ELSEVIER

Biochimica et Biophysica Acta 1509 (2000) 451–466

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.elsevier.com/locate/bba

Novel cationic amphiphilic 1,4-dihydropyridine derivatives for DNA delivery

Zanna Hyvönen [a], Aiva Plotniece [b], Inese Reine [b], Brigita Chekavichus [b], Gunars Duburs [b], Arto Urtti [a,*]

[a] *Department of Pharmaceutics, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland*
[b] *Latvian Institute of Organic Synthesis, Aizkraukles street 21, LV 1006 Riga, Latvia*

Received 27 April 2000; received in revised form 4 September 2000; accepted 5 September 2000

Abstract

In order to find new efficient and safe agents for gene delivery, we have designed and synthesized nine novel single- and double-charged amphiphiles on the base of 1,4-dihydropyridine (1,4-DHP) ring. Some biophysical properties of the amphiphilic dihydropyridines and their complexes with DNA were examined. We investigated the transfer of β-galactosidase gene into fibroblasts (CV1-P) and retinal pigment epithelial (D 4O7) cell lines in vitro. The structure–property relationships of the compounds were investigated in various ways. The net surface charges of 1,4-DHP liposomes were highly positive (25–49 mV). The double-charged compounds condensed DNA more efficiently than single-charged and the condensation increases with the increasing +/− charge ratio between the carrier and DNA. Double-charged compounds showed also buffering properties at endosomal pH and these compounds were more efficient in transfecting the cells, but transfection efficiency of amphiphiles was cell type-dependent. The length of alkyl chains in double-charged compounds affected the transfection efficacy. The most active amphiphile (compound VI) was double-charged and had two C_{12} alkyl chains. At optimal charge ratio (+/− 4), it was 2.5 times more effective than PEI 25 and 10 times better than DOTAP, known efficient polymeric and liposomal transfection agents. Formulation of amphiphiles with DOPE did not change their activities. Our data demonstrate some important effects of amphiphile structure on biophysics and activity. The data also suggest that cationic amphiphilic 1,4-DHP derivatives may find use as DNA delivery system.

Keywords: Gene therapy; Cationic amphiphile; 1,4-Dihydropyridine; Transfection; Gene delivery

1. Introduction

Gene therapy is based on the introduction of specific exogenous sequences of DNA into the target cells for production of the therapeutic gene product [1,2]. The prerequisite for successful gene therapy is efficient and safe delivery of DNA into the cells. Although the viral vectors are currently the most efficient vehicles in gene delivery, they have some disadvantages. Advantages and disadvantages of the vectors were compiled recently by Verma [3]. Disadvantages of the viral vectors include the risk of oncogenicity, immune responses and difficulties in industrial validation and upscaling [2,3]. Problems of viral vectors have prompted the search of efficient and safe non-viral delivery systems.

* Corresponding author. Fax: +358-17-162252;
E-mail: arto.urtti@messi.uku.fi

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00327-8

Positively charged polymers, peptides and lipids have been studied as gene delivery vehicles [4–9]. Cationic liposomes are the most extensively used non-viral systems for gene transfection in vitro and in vivo [3,10]. A large number of cationic lipids have been developed to improve the gene transfer, but toxicity, lack of biodegradability and low transfection efficacy still remain as problems [8,11–14]. Therefore, it is important to develop new transfection agents and to find out the essential structural features for successful gene transfer.

Cationic non-viral vehicles form complexes with DNA. These complexes augment the uptake of DNA by the cells via endocytosis and, furthermore, DNA delivery into the cytoplasm and nucleus [15]. The efficacy of DNA delivery may be affected at several stages: DNA complexation, reaction with extracellular macromolecules, cellular uptake, endosomal escape, nuclear entry and DNA release from the complexes [8,15]. Importance of different stages and the requirements related to the transfection ability are not well- known. Therefore, the design of new effective transfection agents is a difficult task. It is believed that liposomal gene delivery agents must include a cationic component that is attached to a non-polar part. Single and double alkyl chains, saturated and unsaturated, as well as cholesterol have been used as non-polar parts [1,6,9,11,16–20]. The double-chain lipids have shown the greatest efficacies [4]. Most synthetic cationic lipids are either quaternary ammonium salts, or lipoamines, although other moieties have been described [11,17]. For example, Walker et al. [16] showed that bile-acid-based facial amphiphiles and their formulations with fusogenic lipid 1,2-dioleoyl-3-phosphatidyl ethanolamine (DOPE) are effective in gene delivery in vitro. The pyridinium-derived amphiphiles were proposed by Van der Woude et al. [18] as effective and non-toxic gene delivery agents in vitro. Likewise, the cationic fatty acid derivatives of imidazolines [19] or alkyl acyl carnitine ester [20] were shown to complex and transfer the DNA into the cells in culture and in mice. Despite these studies, the structure–activity relationships of the cationic lipids in gene transfer are not well understood and new efficient carriers are needed for gene transfer.

In order to find new efficient and safe agents for gene delivery, we have synthesized and investigated synthetic amphiphilic compounds based on 1,4-dihydropyridine (1,4-DHP) structures. 1,4-DHP derivatives are known mostly as calcium channel blockers in the therapy of cardiovascular diseases [21]. Other physiological features include antioxidant [22,23], radioprotective [24], antibacterial [25] and membranotropic [26] effects.

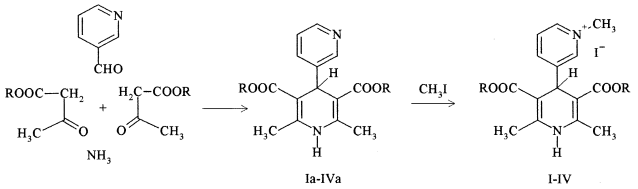
In this study, we introduce novel amphiphilic cationic 1,4-DHP derivatives as transfection agents. After observing that these derivatives form vesicular structures in water, we examined biophysical characteristics of their cationic amphiphile/DNA complexes and transfection efficacies. The results demonstrate that several compounds are able to complex DNA and show very high transfection efficacy in vitro. Furthermore, some critical structural features for activity were revealed.

2. Materials and methods

A series of novel cationic amphiphilic 1,4-DHP derivatives I–IX (Fig. 1) were synthesized in Latvian Institute of Organic Synthesis (Riga, Latvia). *N*-(1-(2,3-Dioleoyloxy)propyl)-*N,N,N*-trimethyl ammonium methylsulfate (DOTAP) and DOPE were obtained from Avanti Polar Lipids (Alabaster, AL, USA), polyethyleneimine of 25 kDa (PEI 25) from Aldrich. Ethidium bromide (EtBr) was purchased from Molecular Probes (Eugene, OR, USA), agarose and *o*-nitrophenylgalactopyranoside (ONPG) from Sigma.

2.1. Synthesis of amphiphiles

2.1.1. General procedure for the preparation of compounds I–IV



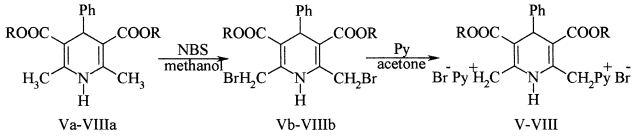
I: R = $C_{12}H_{25}$; II: R = $C_{14}H_{29}$; III: R = $C_{16}H_{33}$; IV: R = $(CH_2)_2OCOC_{15}H_{31}$

Derivatives of 2,6-dimethyl-3,5-dialkoxycarbonyl-

4-(3-pyridyl)-1,4-dihydropyridine (Ia–IVa) were obtained from the respective acetoacetic esters, 3-pyridinecarbaldehyde and ammonia.

To obtain the 1-methyl-3-(2′,6′-dimethyl-3′,5′-dialkoxycarbonyl-1′,4′-dihydropyridyl-4′)-pyridinium iodides (I–IV), the corresponding compounds Ia–IVa (0.003 mol) were dissolved with heating in acetone or a 1:1 mixture of acetone and chloroform (10–20 ml) and methyl iodide (1.3 ml, 2.2 g, 0.015 mol) was added in 2–3 aliquots over 20 min. The product was refluxed for 1–3 h. After cooling, the filtered precipitate was recrystallized from acetone. Yields of products were 68–84%.

2.1.2. General procedure for the preparation of compounds V–VIII

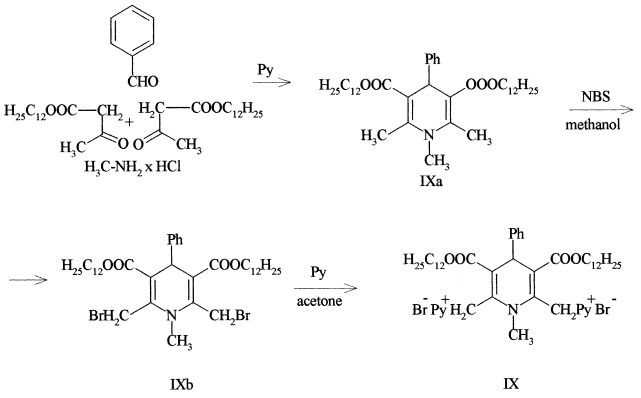


V: $R = C_{10}H_{22}$; VI: $R = C_{12}H_{25}$; VII: $R = C_{14}H_{29}$; VIII: $R = C_{16}H_{33}$

2,6-Dib–romomethyl-3,5-dialkoxycarbonyl-4-phenyl-1,4-dihydropyridines (Vb–VIIIb) were prepared by bromination of corresponding 2,6-dimethyl-1,4-dihydropyridine (Va–VIIIa) (1.6 mmol) with *N*-bromosuccinimide (NBS) (0.6 g, 3.2 mmol) in methanol (100 ml) at 0°C. The mixture was stirred at 0°C for 40 min, diluted with water (40 ml) and kept at 4–6°C until the oil-like residue was obtained. The residue was separated, treated with hexane and filtered.

1,1′-[3,5-Dialkoxycarbonyl-4-phenyl-1,4-dihydropyridine-2,6-diyl)dimethylene]bispyridinium dibromides (V–VIII) were obtained by addition of pyridine (0.42 ml, 5.2 mmol) to a solution of crude product of corresponding compounds (Vb–VIIIb) (0.2 g, 2.6 mmol) in acetone (15 ml). The mixture was stirred at room temperature for 4 h. The precipitate was filtered off and washed with acetone. The precipitate was then crystallized from ethanol, dried and fractionally recrystallized from acetone. Compounds VII and VIII were isolated as dihydrate. Yields of products were 33–40%.

2.1.3. Preparation of compound IX



To obtain 3,5-didodecyloxycarbonyl-4-phenyl-1,2,6-trimethyl-1,4-dihydropyridine (IXa) dodecylacetoacetate (5.40 g, 20 mmol), benzaldehyde (1.06 g, 10 mmol), methylamine hydrochloride (0.67 g, 10 mmol) in pyridine (30 ml) were heated to refluxing for 6 h. The excess of volatile reagents was evaporated under reduced pressure. The residue was triturated with methanol, filtered and recrystallized from ethanol. Yield of the product was 68%.

NBS (0.6 g, 3.2 mmol) in methanol (50 ml) was gradually added to IXa (1.0 g, 1.6 mmol) at room temperature and mixture was stirred for 5 h. The formed oil (derivative IXb) was dissolved in acetone (20 ml) without purification and pyridine (0.24 g, 0.26 ml, 3.2 mmol) was added. The mixture was stirred at room temperature for 5 h, the formed precipitate was filtered off and washed with acetone. Following crystallization from a mixture of chloroform/acetone, 1,1′-[(3,5-didodecyloxycarbonyl-1-methyl-4-phenyl-1,4-dihydropyridine-2,6-diyl)dimethylene]bispyridinium dibromide trihydrate (IX) was obtained. Yield of product was 38%.

All final products were fully characterized by ^1H nuclear magnetic resonance and elemental analysis.

2.2. Plasmid

To assess gene transfer in the cell cultures, we used a pCMV-βGal plasmid containing the β-galactosidase cDNA driven by the CMV promoter. Plasmid was produced in the *Escherichia coli*, extracted by alkaline lysis technique and purified using Qiagen Mega kit (Qiagen GmbH, Germany). The purity of

the plasmid was confirmed by 1% agarose gel electrophoresis followed by EtBr staining and DNA concentration was measured by UV absorption at 260 nm.

2.3. Preparation of liposomes

Cationic liposomes of DOTAP and compounds I–IV were prepared by the thin film hydration method according of Gershon et al. [27]. Shortly, about 10 mg of compound was dissolved in chloroform and the solvent was evaporated under a stream of nitrogen for 45 min in vacuum. The resulting thin films were resuspended in deionized water, vortexed and sonicated in a bath type sonicator for 30 min until solution became clear. The final concentration of liposomes was 1.25 mM. To prepare the liposomes of compounds I–IV and IX, the suspensions were heated to 50–65°C.

To obtain cationic liposomes DOTAP:DOPE (1:1) and compounds I–IV:DOPE (1:1) (molar ratios), 1 ml of 1.25 mM solution of DOPE in chloroform was evaporated to a thin film and, then, 1 ml of cationic liposomes was added to the lipid film. Mixtures were sonicated until translucent.

To prepare the liposomes from 1,4-DHP derivatives V–IX, about 10 mg of the compounds was dissolved in deionized water, vortexed and sonicated 5–7 min in a bath sonicator. The final concentration of liposomes was 2.5 mM.

2.4. pK_a determination

Buffer capacities of the cationic liposomes were estimated according to Budker et al. [28]. Briefly, 3.25 μmol of compound was diluted in 1 ml of chloroform and dried under vacuum with nitrogen stream. Then, the thin film was gently dissolved in 15 ml of 0.5% Triton X-100 at pH 3.5 and titrated with 0.5 M NaOH. pK_a was determined as inflection point of the titration curve. The final concentration of the compound was 0.22 mM.

2.5. Preparation and investigation of complexes

The cationic liposome/plasmid DNA complexes were prepared by adding 0.6 μg of DNA to different concentrations of liposomes to obtain different +/− charge ratios of 0.5–16. After gentle swirling, the mixtures were allowed to stand at room temperature for 25 min prior to analysis or use. The complexes were prepared in MES–HEPES buffer (pH 7.2), water or 5% (w/v) glucose solution.

The zeta-potentials, the sizes of the liposomes and liposome/DNA complexes were determined with a NICOMP 380 submicron particle sizer (NICOMP Particle Sizing Systems Inc., St. Barbara, CA, USA). Size distributions are assessed on the base of NICOMP number-weighted analysis. Zeta-potentials were determined for complexes made in water. The following physical chemical parameters were used in the determination of zeta-potential: medium viscosity 0.933 cPoise, medium refractive index 1.333 and dielectric constant 78.5.

2.6. Gel electrophoresis

The cationic liposome/DNA complexes were prepared at different charge ratios, as described. After 25 min, gel running buffer with bromophenol blue was added to the complexes and they were loaded on 0.9% agarose gel in Tris–borate EDTA buffer, pH 8.0. Voltage of 65 V was applied for 3 h. Following EtBr staining DNA bands were visualized in an UV transilluminator and photographed (Biometra, Bio-Doc II/NT, Video Documentation System, Göttingen, Germany).

2.7. DNA condensation

Ability of the cationic amphiphiles to condense DNA was assessed with an EtBr displacement assay. In 96-well plates, plasmid DNA (0.6 μg DNA per well) was reacted with 0.002% EtBr in 20 mM HEPES–150 mM NaCl buffer, pH 7.4 and fluorescence intensity was measured at 530 nm (excitation) and 590 nm (emission). Intercalation of EtBr molecules between the base pairs of the DNA double helix results in an increased fluorescence signal. Immediately thereafter, the cationic liposomes were added to form complexes at different +/− charge ratios and the quenching of fluorescence intensity was monitored. Condensation of DNA upon complexation with liposomes results in the displacement of EtBr from DNA and in a decreased fluorescence signal. Fluorescence was measured using a FL 500

microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

2.8. Cell cultures

CV1 fibroblast cells derived from African green monkey kidney and D 4O7 retinal pigment epithelial cells [29] were cultured in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively) and 2 mM L-glutamine for the D 4O7 cell line. Cell cultures were maintained at 37°C in a 7% CO_2/air incubator.

2.9. Transfection assay

The cells were collected, counted and seeded in growth medium (100 μl) into a 96-well culture plate, 20 000 cells per well. One day later, the medium was replaced with fresh medium without serum (150 μl). The complexes at different carrier/DNA charge ratios for transfection procedure were prepared just before use in 50 mM MES–50 mM HEPES–75 mM NaCl buffer, pH 7.2 in separate 96-well plates. One hour after changing the serum-free medium, the complexes were added to the cells at 37°C. The dose of DNA per well was 0.6 μg. After 5 h, the complexes were removed, the cells were washed with phosphate-buffered saline (PBS), and the normal growth medium was added. After 45 h of incubation at 37°C, the cells were lysed with 2% Triton X-100, deep frozen and the β-galactosidase activity in each well was determined spectrophotometrically with an ELx 800 automated microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) by monitoring the hydrolysis of ONPG at 405 nm [11]. Purified β-galactosidase from *E. coli* was used to construct a standard curve for calculation of the β-galactosidase activity in the transfected cells.

2.10. Cytotoxicity assay

Cytotoxicity of the compounds was studied using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [30]. Briefly, the cells were plated on the 96-well plate and transfected as described above. After the incubation (37°C in 7% CO_2) for 45 h, cells were washed with PBS, the growth medium was replaced by serum-free medium and 10 μl of MTT (5 mg/ml) was added per well. The plate was allowed to stand for 2 h at 37°C and 100 μl of the solubilizing solution (20% of SDS–50% of DMF, pH 4.7) was added. After incubation for 15 h at 37°C, absorbance at 570 nm was measured using an automatic enzyme-linked immunosorbent assay plate reader (Labsystems Multiscan PLUS, Labsystem, Finland). Results are expressed as a percentage of living cells as compared to the untreated control.

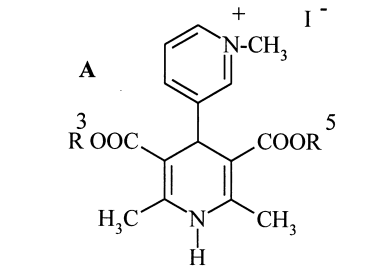
3. Results

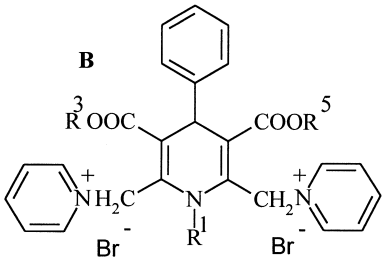
The investigated cationic amphiphiles can be divided into two groups. The single-charged compounds I–IV: 1-methyl-3-(2′,6′-dimethyl-3′,5′-dialkoxycarbonyl-1′,4′-dihydropyridyl-4′)pyridinium iodides (I–III) and 1-methyl-3-(2′,6′-dimethyl-3′,5′-bis(2-palmitoyloxyethyloxycarbonyl)-1′,4′-dihydropyridyl-4′)pyridinium iodide (IV) have quaternized nitrogen in the 4-pyridyl ring and different alkyl chain lengths (C_{12}–C_{18}) at positions 3 and 5 of the 1,4-DHP ring (Fig. 1). The double-charged compounds V–IX: 1,1′-[(3,5-dialkoxycarbonyl-4-phenyl-1-H(methyl)-1,4-dihydropyridine-2,6-diyl)dimethylene]bispyridinium dibromides have two quaternized nitrogens at positions 2 and 6 of the 1,4-DHP ring and different alkyl chains (C_{10}–C_{16}) at positions 3 and 5. Compound IX is 1-N-methylated, otherwise similar to compound VI (Fig. 1).

3.1. Biophysical characteristics of liposomes

All compounds have self-association properties and form liposomes in aqueous media. The vesicles from compounds I–IV, their formulations with DOPE (1:1) and compound IX formed stable translucent suspensions only after heating above the phase transition temperature (50–65°C). Compound I formed initially a clear solution but crystallization was observed within 24 h at room temperature and the clarity was restored upon heating to phase transition temperature. Compounds V–VIII were found to suspend readily in water and to form stable clear solutions upon sonication at room temperature.

Particle sizes of the liposome suspensions in water were determined by quasielastic light scattering. The





A	$R^3 = R^5$	Compound
	$C_{12}H_{25}$	I
	$C_{14}H_{29}$	II
	$C_{16}H_{33}$	III
	$(CH_2)_2OCOC_{15}H_{31}$	IV

B	R^1	$R^3 = R^5$	Compound
	H	$C_{10}H_{21}$	V
	H	$C_{12}H_{25}$	VI
	H	$C_{14}H_{29}$	VII
	H	$C_{16}H_{33}$	VIII
	CH_3	$C_{12}H_{25}$	IX

Fig. 1. Structures of the cationic 1,4-DHP-based amphiphiles.

suspensions of single-charged compounds (I–IV) were heterogeneous, nevertheless the mean diameters of the liposomes were in the range of 15–30 nm (S.D. up to 45–50%). These cationic amphiphiles formed small particles (15–35 nm) also with DOPE. The double-charged amphiphiles (V–IX) formed liposomes with mean diameters in the range of 50–130 nm (S.D. up to 35–45%).

3.2. Sizes of the complexes

Sizes of the freshly prepared amphiphile/plasmid DNA complexes in MES–HEPES buffer (pH 7.2), water and 5% (w/v) glucose were determined and compared with DNA complexes of PEI 25 and DOTAP. The results show that in MES–HEPES buffer, the mean diameters of the complexes at high (≥ 4) +/− ratios are rather small (< 150 nm) for most of the compounds (Fig. 2A). The sizes remarkably increase up to 10–35-fold with decreasing +/− ratio. The mean sizes are at maximum at charge ratios 2:1 and 1:1 (+/−) and in most cases decrease again at negative charge excess (+/− 0.5). At low +/− ratios, the complexes of all examined amphiphiles, DOTAP

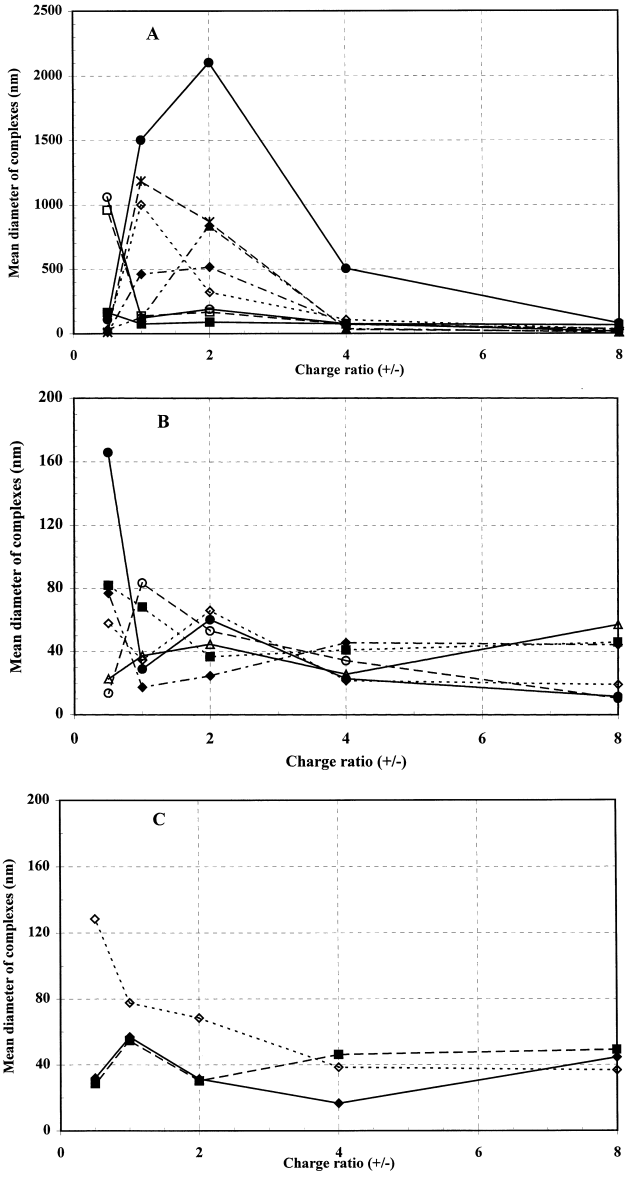


Fig. 2. Mean sizes (nm) of carrier/plasmid DNA complexes at different +/− charge ratio in MES–HEPES (pH 7.2) (A), water (B) and 5% (w/v) glucose (C) for compound II (■), compound II:DOPE (□), compound IV (●), compound IV:DOPE (○), compound V (▲), compound VI (♦), compound VII (△), DOTAP (◇) and PEI 25 (*).

and PEI 25 were stable in buffer only for a short time: already after 3–4 h, or at latest 24 h, visible aggregates were formed. This was not observed at high (≥8) charge ratios. Since DNA is more complexed at the charge close to neutrality, the repulsion between liposomes is reduced. This is leading to fusion and aggregation and results in enhancement of sizes of complexes.

The complexes prepared in water (Fig. 2B) or in 5% (w/v) glucose (Fig. 2C) were smaller (15–120 nm) and their sizes remained at about the same level for 10 days. The sizes were small even at the charges close to neutrality or at the excess of DNA. Similar observations were found for DOTAP/DNA and PEI 25/DNA complexes (Fig. 2A–C), although PEI 25 complexes were too small (< 15 nm) in water and in 5% (w/v) glucose solution for accurate determination with the light scattering method.

3.3. Zeta-potentials of the liposomes and complexes

Table 1 summarizes zeta-potentials of the liposomes and their formulations with DOPE. Zeta-potential measurements indicated that the liposomes had very similar surface characteristics and the net surface charges were positive (25–49 mV). Since surface charge of liposome/DNA complexes may affect the interaction of the complexes with extracellular macromolecules and the cell surface, we examined the zeta-potentials of compound VI/plasmid DNA complexes and DOTAP/plasmid DNA complexes in water. Fig. 3 illustrates that at the excess of cationic liposomes, the zeta-potentials of the complexes were positive, significantly decreased at 1:1 ratio (+/−) and shifted to negative values at charge ratios below 1. This suggests that the amphiphile/DNA ratio defines the surface charge of the complexes. At positive +/− ratios, compound VI/DNA complexes had a

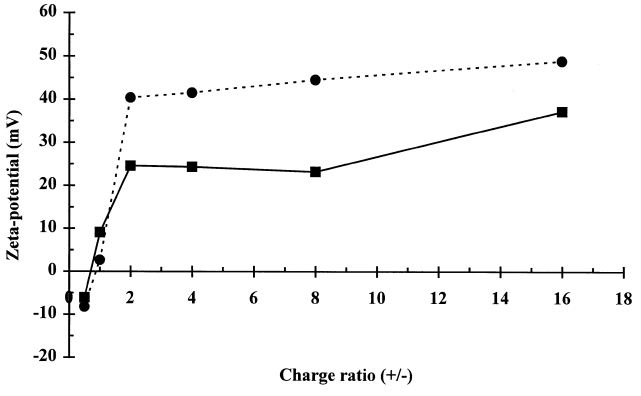


Fig. 3. Zeta-potential of compound VI/DNA (●) and DOTAP/DNA (■) complexes in water at different +/− charge ratios

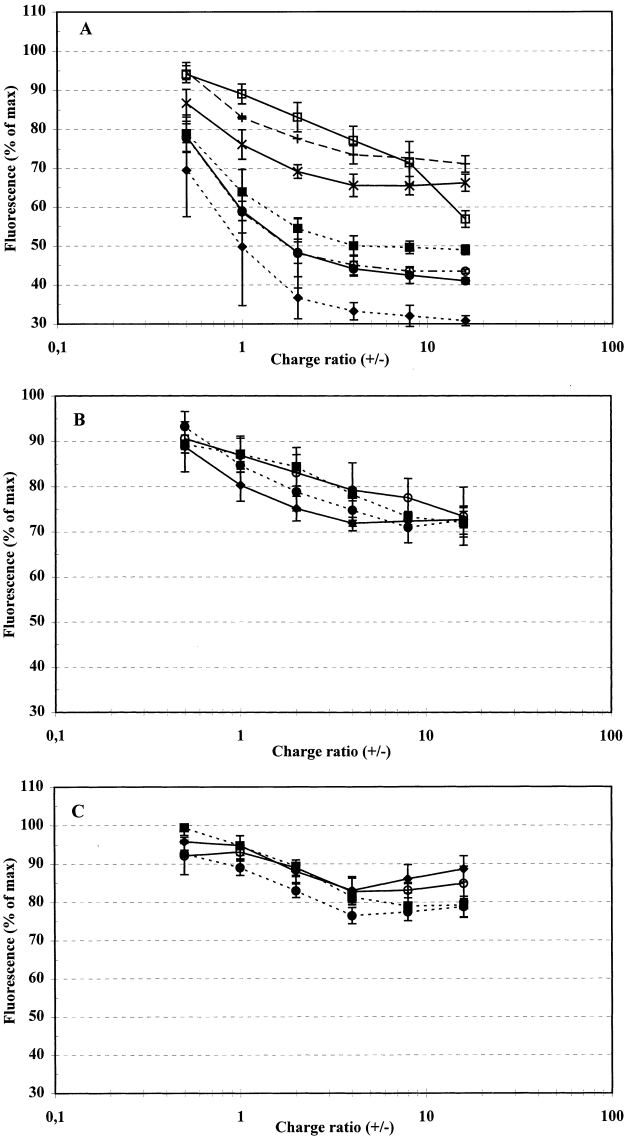


Fig. 4. DNA condensation ability of cationic amphiphiles. (A) Compound V (○), compound VI (●), compound VII (■), compound VIII (□), compound IX (+), DOTAP (*), PEI 25 (♦); (B) compound I (♦), compound II (●), compound III (○), compound IV (■); (C) compound I:DOPE (♦), compound II:DOPE (●), compound III:DOPE (○), compound IV:DOPE (■). The values are expressed as percentage of the maximum fluorescence signal when EtBr is bound to DNA in the absence of an amphiphile. Each data point is from at least triplicate experiments ± S.D.

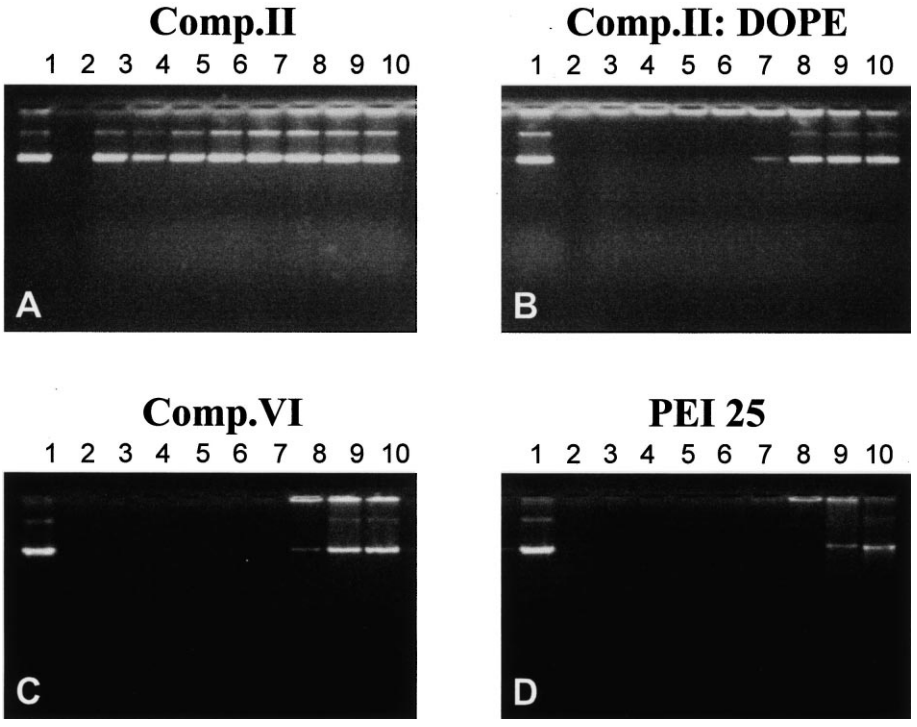


Fig. 5. Gel electrophoresis of the carrier/DNA complexes: (A) compound II, (B) compound II:DOPE, (C) compound VI, (D) PEI 25. In each panel: (lane 1) pCMVβ plasmid DNA (0.6 μg) alone (positive control), (lane 2) carrier (75 μM) alone (negative control), (lanes 3–10) carrier/DNA complexes at charge ratios −/+: 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, respectively.

higher positive zeta-potential than DOTAP/DNA complexes. At higher ionic strengths (e.g. buffer, cell culture medium), the surface charge of the complexes may be shielded by the ions in the solution (data not shown).

3.4. DNA condensation

The ability to form complexes with DNA is necessary for good transfection activity and upon complexation DNA may be condensed. We evaluated the ability of the amphiphiles to condense DNA using EtBr displacement assay. Upon intercalation into DNA, the fluorescence of EtBr is enhanced. Addition of cationic amphiphiles results in an immediate fluorescence quenching due to EtBr displacement from DNA during formation of amphiphile/DNA complexes.

DNA condensation describes the conformation of plasmid DNA, not the mutual interactions between the complexes. The latter is rather described by zeta-potential and fusogenicity of the lipids. Condensation does not reach the maximum at charge neutrality but proceeds further at charge ratios above 1.0 (Fig. 4). The results show that the double-charged compounds (V–VIII) (Fig. 4A) condense DNA more efficiently than single-charged compounds (I–IV) (Fig. 4B,C). Compounds V and VI (C_{10} and C_{12} chains) had a greatest ability to condense DNA, causing a maximal decrease of 56–60% in the fluorescence intensity of EtBr. Compound VII

Table 1
Zeta-potential and electrophoretical mobility of the liposomes in water

Compounds	Zeta-potential (mV)	Electrophoretical mobility (mU)
I	26.4 ± 6.8	2.01 ± 0.55
I:DOPE	26.1 ± 2.5	1.95 ± 0.19
III	33.0 ± 1.3	2.46 ± 0.09
III:DOPE	40.7 ± 2.5	3.03 ± 0.18
IV	35.6 ± 2.5	2.65 ± 0.19
IV:DOPE	44.9 ± 0.9	3.35 ± 0.07
VI	48.9 ± 5.6	3.65 ± 0.41
VII	25.5 ± 2.7	1.91 ± 0.20
VIII	31.6 ± 9.1	2.35 ± 0.68
DOTAP	36.8 ± 1.6	2.74 ± 0.12

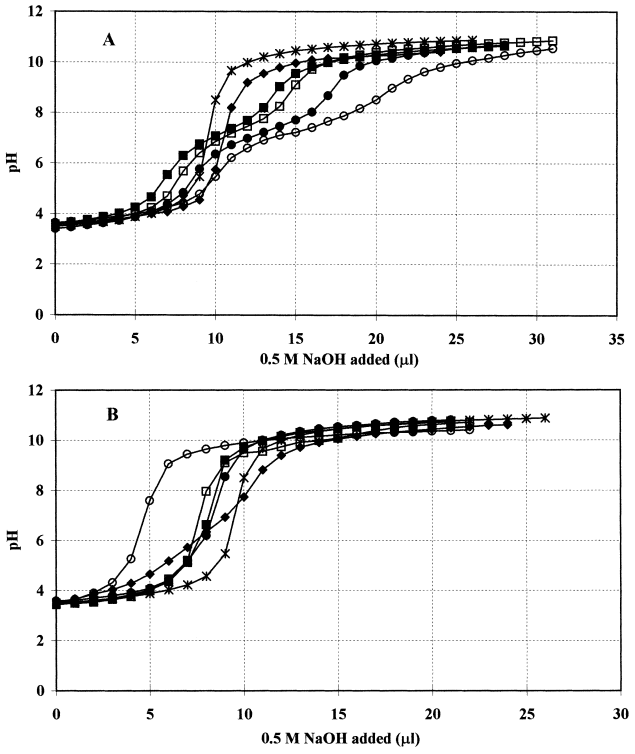


Fig. 6. Estimation of pK_a of compounds by pH titration with 0.5 M NaOH of: (A) compound V (○), compound VI (●), compound VII (□), compound VIII (■), compound IX (♦), control (*); (B) compound I (□), compound III (■), compound IV (○), DOTAP (●), PEI 25 (♦), control (*).

(C_{14}) caused decrease in fluorescence of about 50%, compound VIII (C_{16}) about 30–43% and compound IX only about 20–28% (Fig. 4A). Thus, lengthening of the alkyl chains decreased the ability to condense DNA. Likewise, substitution of the hydrogen at the 1-N position by methyl group led to a reduction in the ability to condense DNA (compound VI vs. IX). PEI 25 was able to decrease the fluorescence by 65–70% and DOTAP condensed DNA maximally by 33–35% (Fig. 4A).

Complexation of plasmid DNA by single-charged compounds (I–IV) led to a decrease of the fluorescence intensity of EtBr by 25–30% (compound I) or less (Fig. 4B). Again, the compounds with longer alkyl chains tended to condense DNA less than the amphiphiles with shorter alkyl chains at positions 3 and 5. Liposomes of compounds I–IV with DOPE condensed DNA less than the liposomes without DOPE (Fig. 4C).

3.5. Gel mobility assay

DNA complexation was confirmed by measuring the changes in DNA migration in agarose gel. Lipid associated with DNA does not migrate into the gel during electrophoresis like free DNA does. The results of the gel retardation assay were consistent with the results of the EtBr displacement experiments. The compounds I–IV condensed DNA slightly in EtBr displacement assay (Fig. 4B) and they did not completely retard DNA even at +/− charge ratios of 16 (Fig. 5A). Hence, the interactions between single-charged compounds I–IV and DNA are weak. Likewise, complexes of monovalent DOTAP with DNA were loose even at +/− 16. Release of DNA from the

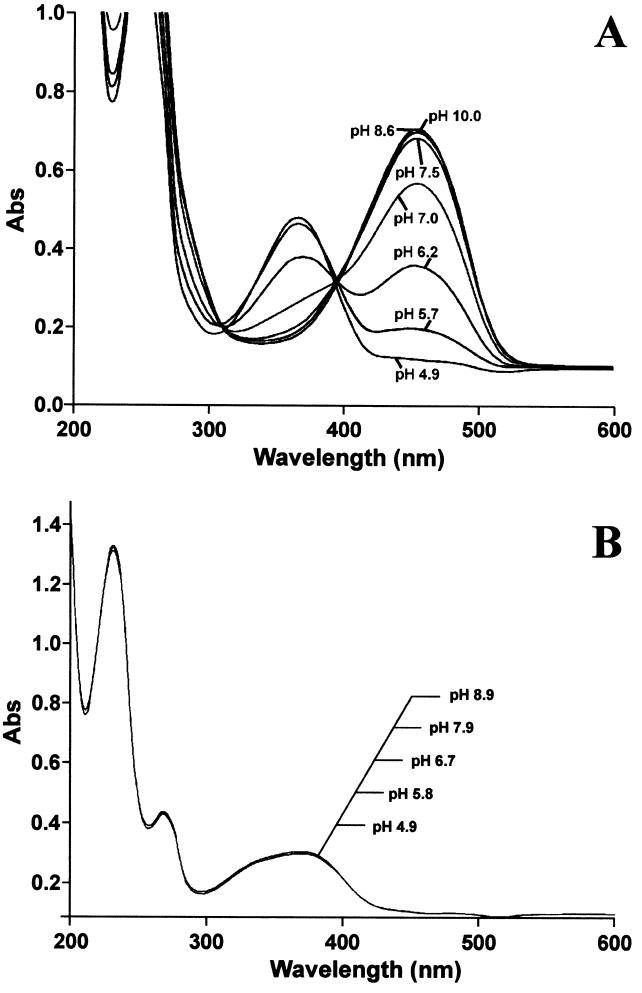


Fig. 7. Effects of pH on the absorption spectra of compounds VI (A) and IV (B).

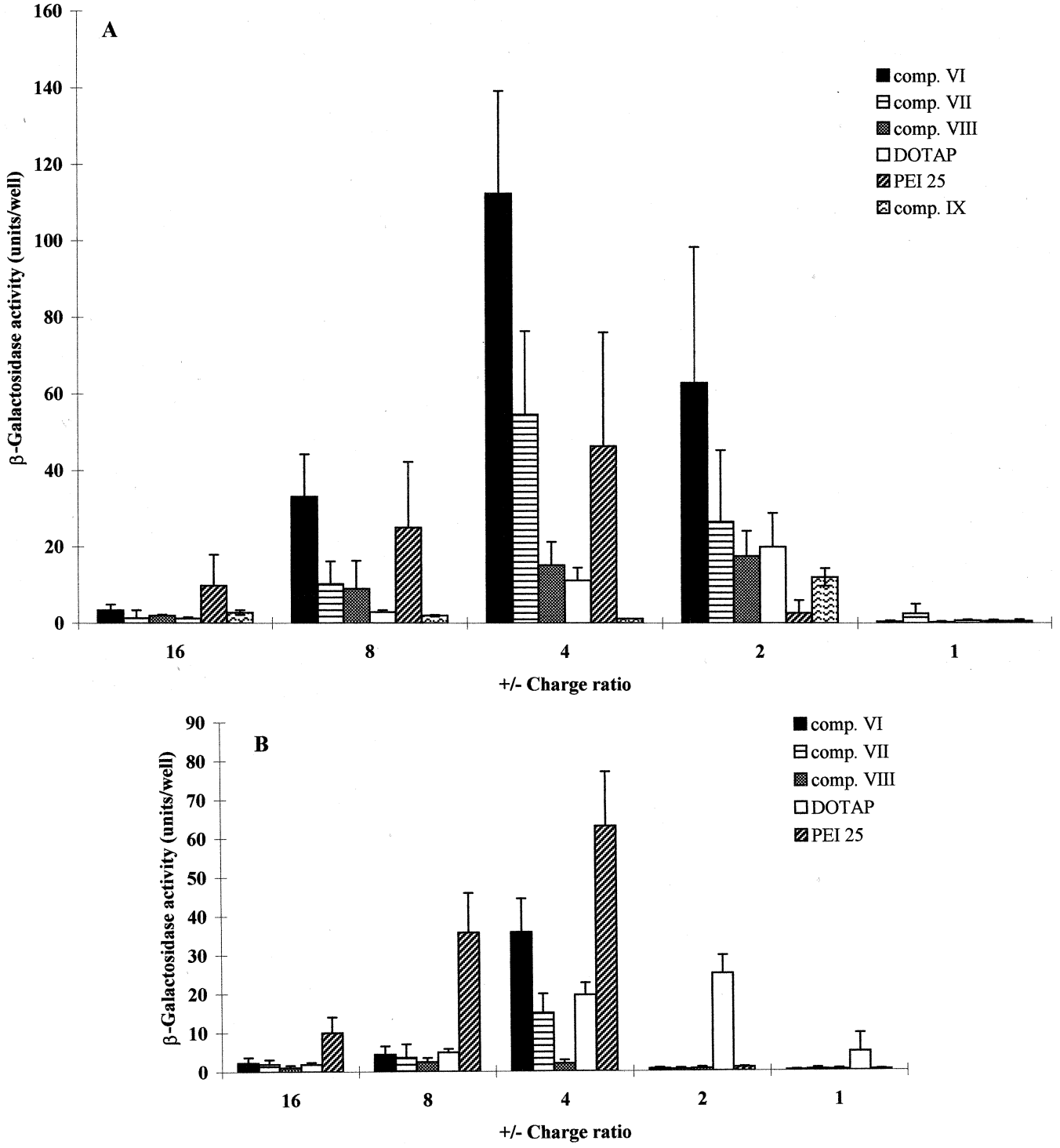


Fig. 8. Transfection efficiencies of compounds VI–IX, DOTAP and PEI 25 in MES–HEPES buffer at different carrier/plasmid DNA charge ratios. Transfections were carried out with fibroblast CV1-P (A) and retinal pigment epithelial D 4O7 (B) cell lines. The cells were transfected for 5 h and incubated for 45 h before β-galactosidase expression assay. Transfection efficiencies are given as β-galactosidase units per well. Each bar represents the average transfection efficiency ± S.D. from at least three experiments. Note that the scale is different for CV1-P and D 4O7 cells.

complexes may be due to the applied electrical field. In contrast, the single-charged compounds with DOPE fully retarded DNA at the +/− charge ratio of 2, suggesting that DOPE somehow helps to stabilize the complexes (Fig. 5B).

Compounds V–IX at +/− 2 fully retarded DNA (Fig. 5C), while for PEI 25 complete retardation was observed at +/− 1 (Fig. 5D).

The gel mobility assay of compound VI/DNA and PEI 25/DNA complexes prepared in water or 5% (w/v) glucose showed that these complexes bind DNA similarly to the complexes prepared in buffer. This binding was retained for at least 10 days.

3.6. Buffering capacity

Cationic liposomes deliver DNA via the endosome compartment [15,31]. It has been proposed that PEIs and dioctadecylglycerospermine (DOGS) are able to buffer the endosomes and, thereby, prevent endosomal acidification and DNA degradation in the cells [32,33]. Therefore, we investigated the buffering capacity of the novel cationic amphiphiles under various pH conditions.

The results showed that double-charged compounds V–VIII have buffering capacity at a pH range of 6–8. The pK_a value for these compounds was approximately 7.0 (Fig. 6A). The compounds with longer alkyl chains tend to have lower buffering capacity. Double-charged compound IX with 1-N-substituted methyl did not show buffering properties. Likewise the single-charged compounds (I–IV) and DOTAP did not display any buffering properties (i.e. no difference from the control) (Fig. 6B). PEI 25 showed buffering capacity over a wide pH range (about 4–9) (Fig. 6B).

The pH-dependent absorption spectra for double- and single-charged compounds were recorded in ion-free water adjusted to various pH values by sodium hydroxide or hydrochloric acid (Fig. 7). Under acidic condition (pH 4.9), the main absorption peak at 365–367 nm was observed. For the double-charged compounds (V–VIII), increased solution pH led to the appearance of a second peak at 454 nm. At pH 7.5, the 365 nm peak disappeared, leaving only absorption maximum at 454 nm (Fig. 7A). For the compounds I–IV and IX, we did not see any pH-dependent spectral features (Fig. 7B).

3.7. In vitro gene transfer activity and toxicity

Cationic amphiphiles were tested for their ability to deliver β-galactosidase plasmid into the monkey fibroblasts (CV1-P) and retinal pigment epithelial cell line (D 4O7), as well as for their cytotoxicity. The transfection efficiencies were compared to PEI 25 and

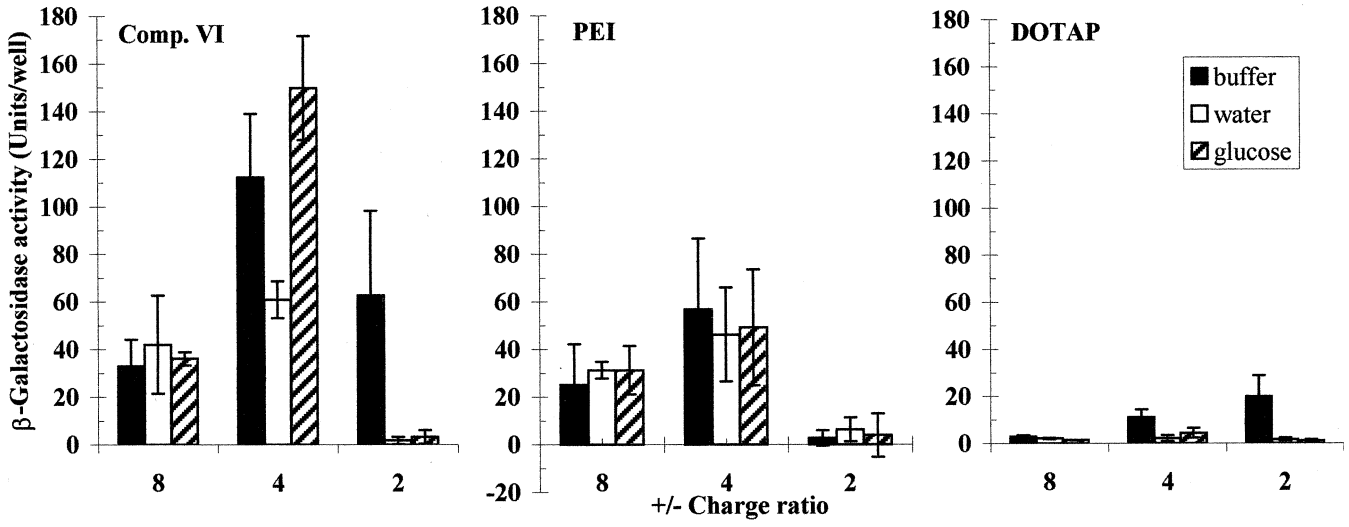


Fig. 9. Transfection efficiencies for complexes of compound VI, PEI 25 and DOTAP in MES–HEPES buffer, water and 5% glucose. Transfection efficiencies are given as β-galactosidase units per well in CV1-P monkey fibroblasts. Each bar represents the average transfection efficiency ± S.D. from at least three experiments.

DOTAP, efficient polymeric and liposomal transfection agents.

CV1-P cells were more susceptible to transfection with double-charged synthetic amphiphiles than retinal D 4O7 cells (Fig. 8). In CV1-P cell culture, the maximal transfection activity of carriers at optimal charge ratios decreased in the order: compound VI > compound VII ≈ PEI 25 > DOTAP ≈ compound VIII > compound IX (Fig. 8A). At +/− charge ratio of 4, compound VI was approximately 2.5 times more effective than PEI 25 and 10 times better than DOTAP. At the same charge ratio, the percentage of viable cells for these carriers was similar (about 90–95%) (Fig. 10A). At high charge ratios (+/− 8–16), the transfection efficacy of the compounds was limited by toxicity (Fig. 10A).

In the retinal pigment epithelial cell line (D 4O7), the transfection level diminishes in the following order: PEI 25 > compound VI > DOTAP ≈ compound VII > compound VIII (Fig. 8B). At +/− charge ratio of 4, the carriers were moderately toxic (70–89% of cell survival) (Fig. 10B), PEI 25 showed more efficient transfection than compound VI, while compound VI was two times better than DOTAP. However, all carriers were more toxic on D 4O7 than on CV1-P cells.

Among the double-charged cationic amphiphiles, compound VI, with two C_{12} alkyl chains, displayed the highest transfection efficiencies in both cell lines. In accordance to the EtBr displacement assay, methylation of 1-N or elongation of alkyl chains to C_{14} and C_{16} reduced the transfection activity. Interestingly, compound with C_{18} chains (oleyl) was completely inactive (data not shown). Likewise, shortening the alkyl chains at positions 3 and 5 to C_{10} resulted in complete loss of transfection activity with no change in toxicity.

It is remarkable that compound VI/DNA complexes prepared in water or 5% (w/v) glucose solution displayed good transfection efficiency (Fig. 9), even though the sizes of the complexes were very small (Fig. 2A,B). Interestingly, activity of DOTAP/DNA complexes in water and 5% (w/v) glucose solution was much smaller than in buffer (Fig. 9). Small complexes are most relevant for in vivo gene therapy and compound VI was about 35 times more active than DOTAP in the case of small complexes. Also, it was three times more active than PEI 25 in this case.

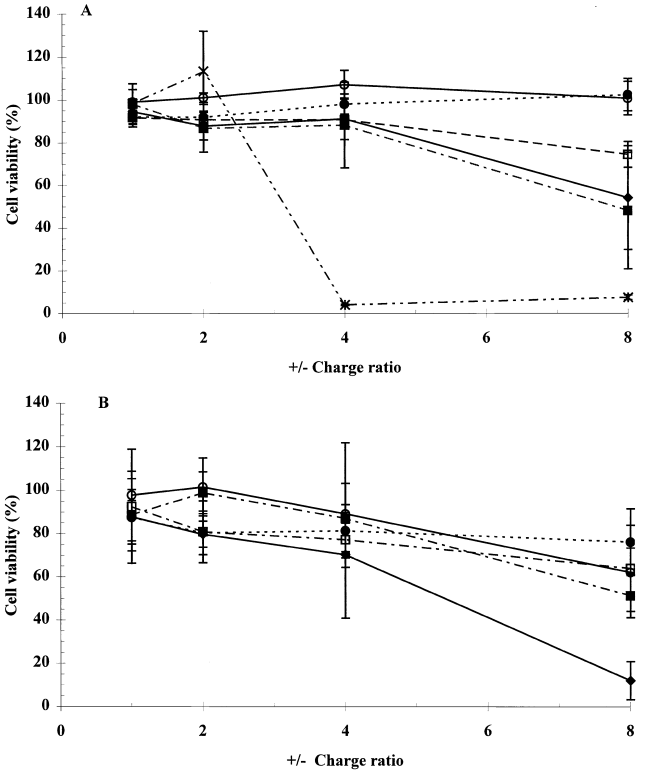


Fig. 10. Cytotoxicity of compound VI (♦), compound VII (■), compound VIII (○), compound IX (*), DOTAP (●) and PEI 25 (□) towards CV1-P (A) and D 4O7 (B) cell lines determined by MTT assay. Cell viability (a percentage of survived cells) displayed as a function of +/− carrier versus DNA charge ratio. Each point represents the average ± S.D. of at least three replicates.

Table 2 summarizes the transfection activity and cytotoxicity of compounds I–IV in both cell lines at optimal charge ratios. In general, the single-charged compounds transfected more effectively D 4O7 than CV1-P cells. In general, they were also more toxic than double-charged compounds towards both cell lines. Alone or formulated with DOPE, single-charged compounds showed moderate transfection activity, although at best their efficiencies are in the same range with DOTAP (Fig. 8A, Table 2).

4. Discussion

In the present paper, we have described some biophysical characteristics of nine novel cationic amphiphilic 1,4-DHP derivatives, their ability to condense DNA and to deliver it into two different cell lines in

Table 2
Transfection efficiency and toxicity of cationic amphiphilic compounds I–IV and their formulations with DOPE (1:1) in CV1-P and D 4O7 cells

Compounds	Carrier/DNA charge ratio (+/−)	CV1-P cells		D 4O7 cells	
		Toxicity (% of surviving cells)[a]	Transfection efficiency (units/well)[b]	Toxicity (% of surviving cells)[a]	Transfection efficiency (units/well)[b]
I	4	40.6	6.0	9.2	0.6
	2	71.8	27.2	55.6	8.8
	1	87.2	0.7	66.1	8.1
II	4	45.6	2.2	12.1	1.2
	2	90.4	4.8	79.1	35.6
	1	89.5	0.3	94.4	16.6
III	2	105.2	2.2	62.6	1.4
IV	2	91.9	2.9	51.1	5.7
	1	94.8	7.7	80.9	20.0
I:DOPE	2	70.0	0.7	29.0	1.1
	1	76.4	0.8	63.6	5.8
II:DOPE	2	77.9	1.5	36.9	3.3
	1	85.7	1.9	68.5	12.8
III:DOPE	2	86.2	8.1	36.4	1.9
	1	90.6	8.4	64.1	9.7
IV:DOPE	2	83.5	8.1	36.1	1.2
	1	85.2	12.0	61.8	3.2
	0.5	91.9	5.5	84.5	1.2

The reported values are the mean of at least three transfection assays.
[a]S.D. ± 15–20%.
[b]S.D. ± 15–25%.

vitro. The investigated compounds have one or two quaternary ammonium groups. The position and structure of the charged groups and the lengths of the alkyl chains at the positions 3 and 5 of the 1,4-DHP ring were modified with the aim to find structural requirements related to the ability of compounds to transfect the cells (Fig. 1). Single-charged compounds I–IV were also formulated with DOPE.

This study suggests that the 1,4-DHP amphiphiles form complexes with DNA like many other cationic lipids [1,8,34–37], but single- and double-charged amphiphiles are clearly different in this respect. We did not find a clear correlation between the size, zeta-potential of complexes and transfection activity, although positive zeta-potential was necessary for good transfection.

Cationic liposomes interact with DNA by electrostatic forces, which results in DNA compaction and complex formation. This complex must then adhere to the cell membrane to be internalized [20,38]. DNA condensation assay revealed that double-charged compounds (V–IX) condense DNA more efficiently than the single-charged compounds (I–IV). In this respect, compounds V–IX resemble PEI 25 and DOGS, and compounds I–IV are like DOTAP [8]. Single-charged amphiphile/DNA or DOTAP/DNA complexes partly disassemble in electric field during electrophoresis, suggesting weak interactions of the carrier with DNA (Fig. 5A). Double-charged compounds and multivalent polymeric PEI 25 clearly form tighter and more compact complexes with DNA than single-charged lipids, suggesting that multiple charges in each cationic molecule might induce a more curved structure in DNA upon complexation. Loosely associated complexes are more likely to be disrupted before they interact with the cell membrane and, therefore, complexation phenomena may influence the transfection efficacy in vitro and in vivo.

Amphiphile/DNA complex is taken up by cells and, then, it is accumulated in the endosomal compartment. The next step is the escape of DNA from endosome; otherwise it will be forwarded to the ly-

sosomes and degraded by nucleases. It has been proposed that cationic lipids cause destabilization of endosomal membrane, lipid mixing and fusion, thereby causing the release of DNA into the cytoplasm of the cells [31,34,38]. For cationic polymers (PEIs) and polyamidoamine dendrimers, a buffering hypothesis has been described [9], suggesting that these carriers prevent acidification of endosomes and, consequently, protect DNA from degradation. In our study, the double-charged amphiphiles (V–VIII) showed buffering capacity at neutral pH range (Fig. 6A). The protonation level increases between pH values from 8 to 6 (Fig. 6B). Since 1-N-methylated compound IX was devoid of buffer capacity, the value of pK_a near 7.0 is related to the 1-NH group of the 1,4-DHP ring. Usually, the 1-NH group of 1,4-DHPs deprotonates at very basic conditions (pK_a near 10–11) and the compounds like I–IV are not expected to deprotonate in water at all. Lowering of the pK_a of 1-NH in compounds V–VIII is probably caused by the proximity of positively charged 2,6-pyridiniomethyl groups. These differences in protonation level of 1-NH group contribute to the buffering capacity. The single-charged compounds (I–IV) did not display buffering properties (Fig. 6B). Likewise, DOTAP with its single quaternary ammonium group did not show any buffering capacity, while PEI 25, a polymer with multiple pK_a values, had a wide buffering range.

The mechanism of DNA delivery is not fully understood yet, but it is clear that the transfection is cell type-dependent. Therefore, it was interesting to examine the new amphiphiles in two different cell lines – CV1 monkey fibroblasts and D 4O7 human retinal pigment epithelial cell line. The gene transfection results showed that in both examined cell lines, the double-charged cationic amphiphiles were more effective and less toxic than the single-charged compounds.

The level of transfection obtained with the compound VI was much higher than DOTAP and at least as high as the level achieved with PEI 25. PEI 25 and DOTAP were selected for comparison, because they are the most active polymeric and liposomal transfection agents currently. The overall rank order of transfection efficiencies was: compound VI $\geq$ PEI 25 > compound VII > compound VIII > DOTAP > others.

It has been proposed that the large size of carrier/DNA complexes can limit the gene transfer in vivo [39]. Particular, small size of the complexes is important for extravasation of the complexes to the target tissues (e.g. tumors). Small size of complexes may also be beneficial for the distribution of the complexes after local application. Interestingly, in contrast to DOTAP, the small-sized DNA complexes of compound VI in 5% (w/v) glucose solution yielded good transfection efficiency. This feature may offer an additional opportunity to use these compounds for gene delivery in vivo.

The length of alkyl chains at positions 3 and 5 of the 1,4-DHP ring were varied and the results show that increased chain lengths from C_{12} in both groups of amphiphiles (single- and double-charged) resulted in declined DNA condensation and transfection activity, so that compound with C_{18} chains was inactive. Effects of alkyl chain length on biophysical properties and transfection have been investigated for some cationic lipids [11,13,38], pyridinium surfactants [18] and alkyl acyl carnitine esters [20]. Elongation of the alkyl chains to C_{16} or C_{18} often reduces transfection efficacy. One of the explanations for our data could be that elongation of alkyl chains increases the phase transition temperature and bilayer stiffness of the vesicles. This might interfere with the ability to fuse with endosomal membrane.

The escape from the endosomes is known to be one of the barriers in gene delivery [15]. High efficacy of double-charged buffering amphiphiles in gene transfection, and also lack of transfection efficacy and buffering capacity of N-methylated compound IX suggest that the buffer capacity at the endosomal pH may contribute to the transfection efficacy. Nevertheless, there are other factors involved. Interestingly, the double-charged compound with two C_{10} chains (compound V) shows self-association properties, high buffering capacity (Fig. 6A) and high ability to condense DNA (Fig. 4A) but does not display a transfection activity in neither cell line. This decrease is not attributed to the toxic effects. Probably shortening of the alkyl chains results in the packing of complexes in a different manner, leading to inactive complexes. The further investigations are needed to define the reasons that result in the different behavior of C_{10} compound.

Often the mixtures of cationic lipids with DOPE

give rise to more efficient transfection than the corresponding cationic lipid alone. DOPE facilitates lipid fusion at different stages of gene delivery [12,40], however, transfection of cationic liposomes is not always enhanced by DOPE [12,38]. Our results showed that for investigated 1,4-DHP derivatives, DOPE did not improve DNA condensation or transfection activity, whereas it increases the toxicity towards both cell lines. Despite the low condensation ability of 1,4-DHP/DOPE combinations, DOPE seems to stabilize the DNA complexes, possibly by changing of complex architecture towards hexagonal phases [41,42].

Finally, we have designed and synthesized novel single-charged and double-charged amphiphiles on the base of 1,4-DHP ring. Our studies demonstrate that the double-charged compounds display high transfection efficiencies in vitro and reveal some important structure–activity relationships. Further studies are needed to prove their usefulness in vivo. Amphiphiles based on the 1,4-DHP ring appear to be a good structural base for the development of DNA delivery systems.

Acknowledgements

We thank M.Sc. Marika Ruponen, M.Sc. Ilpo Jääskeläinen and Prof. Alex Azhayev for their valuable suggestions and advice. Hanna Eskelinen is acknowledged for skilful technical assistance. We wish to thank Dr. Natalia Makarova and Dr. Gunars Tirzitis for their advice in chemical syntheses. This study was supported by Technical Development Centre (TEKES), CIMO and Academy of Finland.

References

[1] D.D. Lasic, N.S. Templeton, Liposomes in gene therapy, Adv. Drug Deliv. Rev. 20 (1996) 221–266.
[2] R.G. Crystal, Transfer of genes to humans: Early lessons and obstacles to success, Science 270 (1995) 404–410.
[3] I.M. Verma, N. Somia, Gene therapy – promises, problems and prospects, Nature 389 (1997) 239–242.
[4] J. Zabner, Cationic lipids used in gene transfer, Adv. Drug Deliv. Rev. 27 (1997) 41–62.
[5] J.-P. Behr, Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy, Bioconjug. Chem. 5 (1995) 382–389.
[6] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielson, Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, Proc. Natl. Acad. Sci. USA 84 (1987) 7413–7417.
[7] E. Wagner, M. Cotton, R. Foisner, M.L. Birnstiel, Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells, Proc. Natl. Acad. Sci. USA 88 (1991) 4255–4259.
[8] M. Ruponen, S. Ylä-Herttuala, A. Urtti, Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies, Biochim. Biophys. Acta 77505 (1998) 1–12.
[9] J.-S. Remy, B. Abdallah, M.A. Zanta, O. Boussif, J.-P. Behr, B. Demeneix, Gene transfer with lipospermines and polyethylenamines, Adv. Drug Deliv. Rev. 30 (1998) 85–95.
[10] S.J. Eastman, C. Siegel, J. Tousignant, A.E. Smith, S.H. Cheng, R.K. Scheule, Biophysical characterisation of cationic lipids:DNA complexes, Biochim. Biophys. Acta 1325 (1997) 41–62.
[11] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, J. Biol. Chem. 269 (1994) 2550–2561.
[12] T. Stegmann, J.-Y. Legendre, Gene transfer mediated by cationic lipids: lack of a correlation between lipid mixing and transfection, Biochem. Biophys. Acta 1325 (1997) 71–79.
[13] C.F. Bennett, D. Mirejovsky, R.M. Crooke, Y.J. Tsai, J. Felgner, C.N. Sridhar, C.J. Wheeler, P.L. Felgner, Structural requirements for cationic lipid mediated phosphotioate oligonucleotides delivery to cells in culture, J. Drug Target. 5 (1997) 149–162.
[14] N. Smyth-Templeton, D.D. Lasic, P.M. Frederik, H.H. Strey, D.D. Roberts, G.N. Pavlakis, Improved DNA:liposome complexes for increased systemic delivery and gene expression, Nat. Biotechnol. 15 (1997) 647–652.
[15] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Poellinger, M.J. Welsh, Cellular and molecular barriers to gene transfer by cationic lipids, J. Biol. Chem. 270 (1995) 18997–19007.
[16] S. Walker, M.J. Sofia, R. Kakarla, N.A. Kogan, L. Wierichs, C.B. Longley, K. Bruker, H.R. Axelrod, S. Midha, S. Babu, D. Kahne, Cationic facial amphiphiles: A promising class of transfection agents, Proc. Natl. Acad. Sci. USA 93 (1996) 1585–1590.
[17] G. Byk, C. Dubertret, V. Escriou, M. Frederic, G. Jaslin, R. Rangara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz, D. Scherman, Synthesis, activity, and structure–activity relationship studies of novel cationic lipids for DNA transfer, J. Med. Chem. 41 (1998) 224–235.
[18] I. Van der Woude, A. Wagenaar, A.A.P. Meekel, M.B.A. terBeest, M.H.J. Ruiters, J.B.F.N. Engberts, D. Hoekstra, Novel pyridinium surfactants for efficient, nontoxic in vitro

gene delivery, Proc. Natl. Acad. Sci. USA 94 (1997) 1160–1165.
[19] I. Solodin, C.S. Brown, M.S. Bruno, C.-Y. Chow, E.-H. Jang, R.J. Debs, T.D. Heath, A novel series of amphiphilic imidazolinium compounds for in vitro and in vivo gene delivery, Biochemistry 34 (1995) 13537–13544.
[20] J. Wang, X. Guo, Y. Xu, L. Barron, F.C. Szoka, Synthesis and characterisation of long chain acyl carnitine esters. Potentially biodegradable cationic lipids for use in gene delivery, J. Med. Chem. 41 (1998) 2207–2215.
[21] N. Baindur, A. Rutledge, D.J. Triggle, A homologous series of permanently charged 1,4-dihydropyridines: novel probes designed to localize drug binding sites on ion channels, J. Med. Chem. 36 (1993) 3743–3745.
[22] G. Tirzitis, I. Kirule, G. Duburs, Antioxidationsaktivitat der 3,5-Dicarbonylderivatives des 2,6-Dimethyl-1,4-dihydropyridins, Fat Sci. Technol. 90 (1988) 411–413.
[23] J. Lehuede, F. Huguet, B. Fauconneau, A. Piriou, J.M. Vierfond, Synthesis, binding affinity and antioxidant activity of new 1,4-dihydropyridines, Eur. J. Med. Chem. 31 (1996) 71–75.
[24] E.V. Ivanov, T.V. Ponomarjova, G.N. Merkushev, G.Y. Dubur, E.A. Bisenieks, A.Z. Dauvarte, E.M. Pilschik, A new skin radioprotective agent Diethon, Radiobiol. Radiother. 31 (1990) 69–78.
[25] L. Fiszer-Maliszevska, J. Wieczorek, M. Mordarski, R. Balicki, L. Kaczmarek, P. Nantka-Namirski, Biological activity of 1,4-dihydropyridine derivatives, Arch. Immunol. Ther. Exp. 33 (1985) 345–352.
[26] D. Tirzite, J. Koronova, A. Plotniece, Influence of some quaternised 1,4-dihydropyridine derivatives on liposomes and erythrocyte membranes, Biochem. Mol. Biol. Int. 45 (1998) 849–856.
[27] H. Gershon, R. Ghirlando, S. Guttman, A. Minsky, Mode of formation and structural features of DNA-cationic liposome complexes used for transfection, Biochemistry 32 (1993) 7143–7151.
[28] V. Budker, V. Gurevich, J.E. Hagstrom, F. Bortzov, J.A. Wolff, pH-sensitive, cationic liposomes: A new synthetic virus-like vector, Nat. Biotechnol. 14 (1996) 760–764.
[29] A.A. Davis, P.S. Bernstein, D. Bok, J. Turner, M. Nachtigal, R.C. Hunt, A human retinal pigment epithelial cell line that retains epithelial characteristics after prolonged culture, Invest. Ophtalmol. Vis. Sci. 36 (1995) 955–964.
[30] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[31] J. Mönkkönen, A. Urtti, Lipid fusion in oligonucleotide and gene delivery with cationic lipids, Adv. Drug Deliv. Rev. 34 (1998) 37–49.
[32] J.P. Behr, B. Demeneix, J.P. Loeffler, J. Perez-Mutul, Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA, Proc. Natl. Acad. Sci. USA 86 (1989) 6982–6986.
[33] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. USA 92 (1995) 7297–7301.
[34] E. Tomlinson, A.P. Rolland, Controllable gene therapy. Pharmaceutics of non-viral gene delivery systems, J. Control. Release 39 (1996) 357–372.
[35] P. Pires, S. Simoes, S. Nir, R. Gaspar, N. Duzgunes, M.C. PedrosodeLima, Interaction of cationic liposomes and their DNA complexes with monocytic leukemia cells, Biochem. Biophys. Acta 1418 (1999) 71–84.
[36] I. Jääskeläinen, J. Mönkkönen, A. Urtti, Oligonucleotide–cationic liposome interaction. A physicochemical study, Biochem. Biophys. Acta 1195 (1994) 115–123.
[37] J.Y. Legendre, F. Szoka Jr., Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes, Pharm. Res. 9 (1992) 1235–1242.
[38] M.J. Hope, B. Mui, S. Ansell, Q.F. Ahkong, Cationic lipids, phosphatidyl-ethanolamine and the intracellular delivery of polymeric, nucleic acid based drugs, Mol. Membr. Biol. 15 (1998) 1–14.
[39] C.W. Pouton, L.W. Seymour, Key issues in non-viral gene delivery, Adv. Drug Deliv. Rev. 34 (1998) 3–19.
[40] H. Schreier, L. Gagne, T. Bock, G.W. Erdos, P. Druzgala, J.T. Conary, B.W. Müller, Physicochemical properties and in vitro toxicity of cationic liposome cDNA complexes, Pharm. Acta Helv. 72 (1997) 215–223.
[41] I. Jääskeläinen, B. Sternberg, J. Mönkkönen, A. Urtti, Physicochemical and morphological properties of complexes made of cationic liposomes and oligonucleotides, Int. J. Pharm. 167 (1998) 191–203.
[42] I. Koltover, T. Salditt, J.O. Rädler, C.R. Safinya, An inverted hexagonal phase of cationic liposome–DNA complexes related to DNA release and delivery, Science 281 (1998) 78–81.