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A hydroxyethylated cholesterol-based cationic lipid for DNA delivery: effect of conditioning

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Abstract

We have synthesised a novel cholesterol-based cationic lipid to promote DNA transfer in cells. This lipid, dimethyl hydroxyethyl aminopropane carbamoyl cholesterol iodide (DMHAPC-Chol) contains a biodegradable carbamoyl linker and a hydroxyethyl group in the polar amino head moiety and is characterised by NMR. Liposomes prepared from this lipid and dioleoyl phosphatidyl ethanolamine (DOPE) in equimolar proportion showed a weak cytotoxicity as revealed by MTT assays and are efficient to deliver plasmids DNA evaluated by the expression of reporter genes in vitro and in vivo. In this paper, we present an original

Abbreviations: AMPGD, [3-4(-methoxyspiro(1,2-dioxetane-3,2′-tricyclo(3.3.1.1)decane-4-yl]phenyl-β-D-galactopyranoside; BSA, bovine serum albumine; CMV, cytomegalo virus; DC-Chol, dimethyl aminoethane carbamoyl cholesterol [3β(*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl)cholesterol]; DDAB, dimethyldioctadecylammonium bromide; DMEM, Dulbeco's modified Eagle medium; DMF, *N*,*N*-dimethyl formamide; DMHAPC-Chol, dimethyl hydroxyethyl aminopropane carbamoyl cholesterol iodide; DMRIE, 1,2-dimyristyloxypropyl-3-*N*,*N*-dimethyl hydroxyammonium bromide; DMSO, dimethylsulfoxide; DOGS, dioctadecylamidoglycyl spermine; DOTAP, *N*-(1-(2,3-dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium methylsulfate; DOSPA, 2,3-dioleyloxy-*N*-(2(spermine carboxamido)ethyl)-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate; DOTMA, *N*-(1-(2,3-dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride; DTT, dithiothreitol; ELSD, evaporative light scattering detector; FCS, fetal calf serum; Lip+, cationic lipid; FTIR, Fourier transform infrared spectroscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; O.D., optical density; ODN, oligodeoxyribonucleotide; PBS, phosphate buffer saline; pCMV-β, plasmid containing β-galactosidase gene, promotor cytomegalovirus; pEGFPN1, plasmid containing green fluorescent protein gene, promotor cytomegalovirus; PFA, paraformaldehyde; pUT650, plasmid containing luciferase gene, promotor cytomegalovirus; QLS, quasi-elastic light scattering; RLU, relative light unit; TBE, tris buffer EDTA; TEAPC-Chol, (3β(*N*-(*N*′,*N*′,*N*′-triethylaminopropane)-carbamoyl)cholesterol iodide); TEAEC-Chol, [3β(*N*-(*N*′,*N*′,*N*′-triethylaminoethane)-carbamoyl)cholesterol iodide]; TMAPC-Chol, [3β(*N*-(*N*′,*N*′,*N*′-trimethylaminopropane)-carbamoyl)cholesterol iodide]; TMAEC-Chol, [3β(*N*-(*N*′,*N*′,*N*′-trimethylaminoethane)-carbamoyl)cholesterol iodide]; TEM, transmission electron microscopy; Transfast, (+)-*N*,*N*-(bis-(2-hydroxyethyl)-*N*-methyl-*N*-[2,3-di (tetradecanoyloxy)propyl] ammonium iodide and DOPE

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method to determine the lipid concentration based on the colorimetric detection of the colipid DOPE and the measure of the molar ratio DOPE/cationic lipid in the liposome by FTIR spectroscopy. The liposomes and lipid/DNA complexes structures were characterized by transmission electron microscopy (TEM) and by quasi-elastic light scattering (QLS). TEM indicated that the complexes correspond to aggregates containing globular substructures with liposomes size. The method of immuno-gold labelling was used to detect plasmid in the complex and reveals the presence of DNA inside the aggregates. Transfection results showed efficient DNA transfer depending on the charge ratio and liposomes conditioning. Gel retardation results indicated that at a molar charge ratio between $X = 1.5$ and $X = 2.5$ (depending on the liposome conditioning), all DNA was taken by liposomes. We showed that conditioning by freeze-drying (lyophilization) facilitates storage and improves transfection efficiency. When the liposomes were lyophilized prior to DNA addition or when the complexes were subjected to freeze-thawing cycles, the obtained complexes showed a transfection with levels enhanced up to four and five-fold respectively for the lyophilized liposomes and freeze-thawed complexes. NMR was used to characterize the modifications under freezing which showed an effect on ^{31}P spectra.

Keywords: Hydroxyethylated cationic lipid; Liposome characterization; Liposome conditioning; DNA delivery

1. Introduction

DNA transfer has been intensively developed using viral or non-viral carriers. Viral carriers are efficient but some risks may exist for the host, so non-viral vectors appear as an alternative. Among the non-viral carriers, cationic polymers and cationic lipids are the most investigated and the latter have been widely studied for DNA delivery. The advantages of lipids over viral vectors include weak toxicity, reduced immunogenecity and thus safety in their use (Lee and Huang, 1997; Zabner, 1997; Rolland, 1998; Schatzlein, 2001).

Since lipofectin was first commercialised, several cationic lipids have been designed to promote DNA transfer (Felgner et al., 1987; Behr et al., 1989; Gao and Huang, 1995). These non-viral carriers constitute a new class of pharmaceutical agents considered as promising. By their positive charges, cationic lipids form complexes with negatively charged DNA, and help DNA to pass through cell barriers. Several assays for DNA delivery in cells, in animal models and even clinical trials used this mean (Nabel et al., 1996). However, their low efficiency represents a significant drawback and still requires constant research of new molecules. In the design of such carriers, the first preoccupation is to have high transfection efficiency. Apart from their extra and intracellular route, the transfection level should depend on the structure of the carrier, on their storage condition and on the structure of lipoplexes, including charge ratio. In such a context, pharmaceutical formulation of liposomes and complexes has been a challenge in the development of non-viral vectors and the knowledge of their characteristics is obviously a crucial step before their use (Lai and van Zanten, 2002; Pedroso de Lima et al., 2001; Dass, 2002).

The stability of the carriers or the complexes is an important factor. Attempts to address the physical instability of these complexes involved variations in solution conditions and methods of preparation. Steric stabilisation and lyophilization to improve physical stability have recently received more attention. There were proofs that mixing with polyethylene glycol (PEG) or lyophilization may reduce the aggregation resulting in maintenance of biological activity (Chern et al., 1999; Allison et al., 2000; Anchordoquy et al., 1997). Up to date, cationic liposomes are generally stored in liquid form at 4 °C as recommended by manufacturers while freezing was required for shipping. The lyophilization was suggested only for phospholipid liposomes (Van Winden et al., 1997) or DNA/cationic lipid complexes (Anchordoquy et al., 1997; Molina et al., 2001). Lyophilization of the complexes implies the pre-determination of their formulation (DNA, charge ratio, etc.) and this parameter cannot be adjusted as requirement. Moreover, even for one cell line, the optimal lipid/DNA ratio is different for whether in vitro or in vivo transfection. Thus, in order to have a flexibility of choice, it may be more useful that stable liposomes and DNA were separately available just before use. In this work, we will investigate the effect of lyophilization of cationic liposomes on the transfection level.

An important challenge is the accurate determination of lipid concentration from which depend the evaluation of the lipid/DNA ratio and the compar-

ative investigation of the transfection efficiency and the cytotoxicity of complexes. However, most lipids have no specific chromophore and fail to be monitored by routine spectrophotometric detection. Up to nowadays, only evaporative light scattering detector (ELSD) has been reported for determining the concentration of DMRIE-C/DOPE liposomes (Felgner, 1997). This method required special equipments and meticulous adjustments to have standard curves. In this work, we propose a two-step method consisting to determine the DOPE/cationic lipid ratio by FTIR and to measure the concentration of DOPE by a colorimetric method (Stewart, 1980), the combination of data will give the concentration of cationic lipid.

For this study, we are interested in cholesterol-based cationic lipids. Indeed, cationic lipids generally belong to two main kinds depending on the hydrophobic part which is whether an acyl tail or a cholesterol derivative (Farhood et al., 1992; Felgner et al., 1994; Okayama et al., 1997). The advantage of the well known dimethyl aminoethane carbamoyl cholesterol (DC-Chol) and other derivatives of this family was demonstrated in several transfections (Gao and Huang, 1991; Kisoon et al., 2002; Sochanik et al., 2000; Hasegawa et al., 2002). Our previous results showed that cholesterol-based cationic lipids with a quaternary ammonium in the polar head and a biodegradable carbamoyl linker are efficient for oligonucleotides and plasmids transfer in cells (Geromel et al., 2001; Reynier et al., 2002; Lesage et al., 2002). The cationic lipid 3-β(*N*-(*N*′,*N*′-dimethyl, *N*′-hydroxyethyl aminopropane) carbamoyl) cholesterol iodide (DMHAPC-Chol) used for this study contains a hydroxyethyl group in the polar head. This cationic lipid forms with dioleoyl phosphatidylethanolamine (DOPE) very stable liposomes with a well defined monomodal size distribution. DMHAPC-Chol and DOPE were mixed in equimolar proportion as this ratio showed high transfection efficiency in vitro and in vivo. We will show that the liposomes obtained are efficient for delivering plasmid constructs incorporating reporter genes encoding the luciferase, the β-galactosidase or the green fluorescent protein. After demonstrating the ability of the lipid to efficiently deliver DNA in melanoma B16-F10 cells, we are particularly interested in the determination of the different characteristics of the carrier, including the structures of the various complexes as a function of the charge ratio and liposomes conditioning (lyophilization). The effect of these parameters on the transfection levels was studied. NMR spectroscopy was used to investigate the freezing effect on the carrier. Finally, for the studied cell line, this cationic lipid was favourably compared to commercial reagents.

2. Materials and methods

2.1. Reagents

DOPE was furnished from Aventi-Polar Lipid (Alabaster, AL) and used without other purification. OPTIMEM, glutamax-containing Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, trypsine-EDTA and phosphate-buffered saline (PBS) were obtained from In Vitrogen (Paisley, UK). Reagents are analytical grade and purchased from Sigma-Aldrich (St. Louis, MI), Carlo Erba (Milano, Italia) and Promega (Madison, WI).

2.2. Synthesis of DMHAPC-Chol

The starting chemicals: cholesteryl chloroformate (>99%, Fluka), 3-(dimethylamino) propylamine (>99%, Aldrich) and anhydrous ether (>99%, Aldrich) were used as received. The treatment of commercial 2-iodoethanol (99%, Aldrich) with sodium thiosulfate, removed free iodine and afforded a product considerably more reactive than the commercial one.

3-β(*N*-(*N*′,*N*′-Dimethyl *N*′-hydroxyethyl aminopropane) carbamoyl) cholesterol iodide or DMHAPC-Chol ($M = 686.81\,\mathrm{g\,mol^{-1}}$) was synthesised as follows. A solution of cholesteryl choloroformate (10^{-2} mol) in 60 ml of anhydrous ether was added dropwise into a solution of 3-(dimethylamino) propylamine (2×10^{-2} mol) in 50 $\mathrm{cm^3}$ of ether maintained at 0 °C (ice-bath), with mechanical agitation. The mixture was then stirred for three hours at room temperature. After removal of the hydrochloride by filtration, the synthesis of the quaternary ammonium iodide salt was carried out by refluxing during 72 h with an excess amount of 2-iodoethanol (3×10^{-2} mol). After removal of the solvent and excess of 2-iodomethanol, DMHAPC-Chol was recrystallized in absolute methanol and purified by chromatography from a silica column

with 10% methanol in $CHCl_3$, 2.3 g of the pure cationic salt where obtained as white or straw-colored powder.

2.3. Liposome preparation

The cationic lipid DMHAPC-Chol and DOPE were dissolved and mixed in chloroform with desired mol/mol ratios. The obtained solution was dried in a rotating evaporator. Solvent trace was removed under vacuum overnight. The obtained film was hydrated with Millipore water. The suspension was sonicated for 1 h in cycles of 15 min to clarity. The suspension was centrifuged for 30 min and the supernatant was recovered. DOPE final concentration was determined by the ammonium ferrothiocyanate assay and the DOPE/DMHAPC-Chol ratio was checked by FTIR. The liposome size was characterised by quasi-elastic light scattering (QLS) using an apparatus constructed by our laboratory. Polystyrene latex spheres of 109 nm in aqueous solution were used for the calibration.

For some experiments, the liposome suspension obtained was lyophilised by freezing at $-80\,^{\circ}C$ and drying under vacuum in a freeze-drier (Virtis) before being re-hydrated at the same concentration and used to prepare DNA/liposome complexes.

2.4. DOPE concentration

The DOPE concentration in the final suspension was determined by a colorimetric method using ammonium ferrothiocyanate (Stewart, 1980). A standard solution of ammonium ferrothiocyanate was prepared by dissolving 27.03 g ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and 30.4 g ammonium thiocyanate (NH_4SCN) in deionized distilled water and making up to 1 liter. For the calibration graph of DOPE, a stock solution of DOPE/DMHAPC-Chol (1/1, mol/mol) in chloroform was prepared. Desired volumes of this solution were pipetted off and completed to 2 ml with chloroform. Two milliliter of the ammonium ferrothiocyanate solution were added to this solution. This biphasic system was then vigorously mixed manually for 1 min. After 45 min of incubation at room temperature, the absorbance of the organic solution was measured at 468–472 nm on a Uvikron (UV-Vis) spectrophotometer 941 (Kronton Instruments).

2.5. Determination of the DOPE/DMHAPC-Chol ratio

The DOPE/DMHAPC-Chol ratio in the obtained liposomes was monitored by FTIR spectroscopy. The DOPE content was calculated from the ratio of the intensity of a band associated with the carbonyl stretching at $1743\,cm^{-1}$ to that of the band at $1712\,cm^{-1}$ corresponding to the carbonyl of DMHAPC-Chol. A calibration curve was established from spectra of DOPE/DMHAPC-Chol mixtures with various ratios. For these FTIR experiments, DOPE and DMHAPC-Chol were mixed in chloroform at desired ratios and the solution was placed directly on a zinc selenide trough and evaporated. Infrared spectra were acquired on a Perkin-Elmer 2000 spectrometer equipped with a DTGS detector at ambient temperature. For each spectrum, three scans were collected with a final resolution of $1\,cm^{-1}$. A linear function was used to fit the baseline in this region prior to the determination of the absorbance. To characterize samples with unknown DOPE/DMHAPC-Chol ratios, the aqueous lipid suspension was lyophilised and dissolved in chloroform.

2.6. Plasmids DNA: reporter genes pCMV-βgal, pEGFP-N1 and pUT650

pCMV-βgal plasmid used for this work was a plasmid of 7.164 kb containing the β-galactosidase reporter gene sequence under the control of the cytomegalovirus promoter (pCMV) (Clontech, Palo Alto, CA). The plasmid coding for enhanced green fluorescent protein (pEGFP-N1) was purchased from Clontech (Palo Alto, CA). DNA plasmid was amplified in JM109 stain of *Escherichia coli*, and purified using a Qiagen Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). The concentration of plasmid DNA was measured by UV absorption at 260 nm and plasmid DNA purity was controlled using A260/A280 ratio and agarose gel electrophoresis. pUT650 was a plasmid of 5.149 kb containing the firefly luciferase reporter gene sequence under the control of the promoter CMV (Cayla, Toulouse, France). Plasmids were conditioned in Tris–EDTA buffer (pH 8) except pUT650 which was conditioned in sterile water.

2.7. Formation of DNA/liposome complex

For the formation of DNA/liposome complexes, DNA and liposomes (lyophilized or not) were separately diluted in equal volumes of sterile water. For in vitro experiments, DNA and liposomes were mixed to a final volume of 20 μl with desired charge ratios (*X*). *X* corresponds to the ratio of positive charges (one per molecule of DMHAPC-Chol) to negative charges (3 nmol/μg of plasmid). The DNA solution was added over the liposome suspension. The complexes were incubated at 20 °C for 15 min prior to use.

For specific applications, the prepared complexes were subjected to three cycles of rapid freezing in liquid nitrogen and thawing to the ambient temperature prior to use (FT lipid/DNA complexes).

2.8. Gel retardation electrophoresis assay

Formation of complexes between plasmid DNA and cationic liposomes results in a retardation of DNA electrophoretic migration which permits to estimate the charge ratio corresponding to the complete DNA complexation. For this purpose, the complexes were prepared as previously described: 1 μg of plasmid was mixed with DMHAPC-Chol/DOPE (1/1, mol/mol) liposomes at the indicated cationic lipid/DNA ratios. After 15 min of incubation at room temperature, aliquots of complexes were mixed with loading buffer (Blue 6X loading dye, Promega) and analysed electrophoretically on 1.3% agarose gel containing ethidium bromide (0.5 μg/ml) in 1× TBE. DNA was visualised by UV illumination. The measure was done with lyophilised liposome and the untreated liposome suspension.

2.9. Transmission electron microscopy: characterization of liposomes and DNA/liposome complexes by negative staining and immuno-gold labelling

TEM was used to observe the ultrastructure of liposomes and liposome–plasmid complexes and to estimate the plasmid location. The liposomes or complexes were spread on Formwar/C copper grids treated with a solution of bacitracin (1 mg/ml), then stained by a solution of uranyl acetate (0.75%).

For the observation of the plasmid in the complex, we used immuno-gold labelling. We first prepared digoxigenin-labelled plasmids as indicated by the provider (DIG-Chem-Link from Roche Diagnostics, Meylan, France). In a sterile vial, 3 μl of reactive digoxigenin was added to 15 μg of pCMV-β plasmid in a final volume of 150 μl. After incubation for 30 min at 50 °C in a waterbath, the reaction was stopped by adding 37.5 μl of the stop solution. The digoxigenin-labelled plasmid was used to prepare liposome–plasmid complexes, and the complexes were spread on Formwar nickel grids as previously described. The treated grids were dried at the open air for at least 2 h. The grids were then incubated for 30 min at room temperature with diluted ultrasmall gold-labelled anti-digoxigenin antibody Fab fragments (Aurion, Wageningen, The Netherlands) in 0.2% acetylated BSA. After three washings in water (for 5 min), the samples were treated with the silver enhancement reagent of Aurion and rinsed again. Finally, the grids were stained by a solution of uranyl acetate (0.75%).

TEM was performed using a Philips CM10 microscope operating under a voltage of 60 kV.

2.10. Cell lines and growth condition

Murine melanoma cells B16-F10 were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were grown on plastic ware in monolayers at 37 °C in a humid atmosphere containing 5% CO_2 in air. The glutamax-containing culture medium DMEM was supplemented with fetal calf serum (FCS, 10%) and penicillin/streptomycin (50 U/ml).

2.11. Assay of cell viability by MTT test

Subconfluent cells were trypsinized, harvested and resuspended in 96-well plates. B16-F10 cells were seeded at 10^4 per well. The following day, culture medium was replaced with 100 μl of different concentrations of cationic lipids or 100 μl of complexes at various charge ratios (with constant liposome concentration) in serum free OPTIMEM medium. After 6 h, OPTIMEM was removed, replaced with culture medium containing serum. After 24 h, the medium was removed and 100 μl of 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyl tetrazolium bromide (MTT) at 1 mg/ml (Sigma, St. Louis) was added to the cells for further 3 h. The formazan crystals were dissolved in 100 μl of dimethylsulfoxide (DMSO, Sigma). Absorbance was measured at 570 nm on a P450 microplate reader (Bio-Rad, Hercules, CA) and used to calculate the percentage of viable cells compared to untreated cells.

2.12. Transfection protocol and transfection level measurements

In order to determine the transfection levels, cells were seeded in a 24-well Falcon plate (5×10^4 cells per well) on the day preceding transfection. Just before transfection, culture medium was replaced with 0.5 ml OPTIMEM without serum, and 20 μl liposome–plasmid complexes at desired charge ratio *X* (containing 1.5 nmol of plasmid) were added. After 6 h of incubation, OPTIMEM was removed, replaced with culture medium containing serum. After 24 h, cells were twice washed with PBS and the transfection level was measured using the chemiluminescence of β-galactosidase in the presence of AMPGD (3-(4-methoxyspiro(1,2-dioxetane-3,2′-tricyclo(3.3.1.1)decan)-4-yl)phenyl-β-D-galactopyranoside) substrate with a Tropix Galacto-light Plus kit (Applied Biosystems, Bredford, MA). Following the procedure of the supplier, after lysing the transfected cells with 100 μl of a lysis solution containing 0.5 mM of dithiothreitol (DTT) freshly added, 25 μl of the cell extract was incubated with 100 μl of the chemiluminescent substrate reagent (diluted to 1%) before 150 μl of the light emission accelerator reagent was added. Immediately after the mix, luminometric measurement was made using a BCL luminometer (Gouteyron Technologies, Vals le Puy, France) operating at integration mode for 10 s. Protein was titrated by using the Bio-Rad DC Protein assay kit (Hercules, CA), in order to normalize results expressed in relative light unit per miligram of protein (RLU/mg).

To compare the transfection levels of DMHAPC-Chol/DOPE liposomes with well-known cationic liposomes, the transfection was performed with optimised lipid/plasmid ratios. For DMHAPC-Chol/DOPE liposomes, DMHAPC-Chol/DOPE freeze-thawed complexes, DC-Chol/DOPE and Transfast (Promega) liposomes, the optimised lipid/plasmid ratio was fixed to 1.5. In the case of DMHAPC-Chol/DOPE liposomes, lyophilised and rehydrated, a ratio of 2 was used. Lipofectamine and DMRIE-C (Life Technologies) were used as suggested by the provider.

2.13. Tumor transfection

Pathogen-free female four-week-old nude mice (NMRI) were purchased from Harlan (Harlan, Belgium). The animals were housed in a laminar flow cabinet under specific pathogen-free conditions and fed ad libitum. Cultured B16-F10 cells were quickly trypsinized with 0.25% trypsin and 0.02% EDTA. The harvested cells were washed twice with free serum glutamax-containing DMEM medium and were resuspended in PBS. Three tumours were induced in each animal by subcutaneous injection of 100 μl of PBS containing 2×10^6 cells.

When convenient tumour size was obtained (about 10 mm), animals were anaesthetised using a cocktail of tribromoethanol and *tert*-amylalcohol (Aldrich). Tumours were injected with a volume of 100 μl of lipid/DNA complexes. The cationic lipid/DNA complexes (15 μg of pUT650) were administrated by central bolus. The same amount of naked DNA was diluted in sterile water and injected for control. After 24 h post-injection, mice were sacrificed by cervical dislocation and tumours were removed for analysis. Each tumour was weighed and lysed in 200 μl of lysis solution containing 1 mM of DTT freshly added, 5 μg/ml of leupeptin and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) freshly added. They were crushed and centrifuged at 18,000 × *g* then supernatants were collected for measurement. The luciferase activity was measured as described in Reynier et al. (2002). The results were evaluated for statistical significance by a distribution-free test of Mann–Whitney *U*-test.

2.14. Fluorescence microscopy: GFP expression

The day before transfection, B16-F10 cells (2×10^4 per well) were seeded onto a four-well permanox slide Lab-Tek (Nunc, Napperville, IL). Plasmids pEGFP-N1 (0.5 and 1 μg) complexed with liposomes (charge ratio of 1.5) were added to the cells following the transfection protocol described above. After 6, 24 or 48 h of incubation, the media was removed, and cells were

rinsed twice with PBS followed by a fixation step with 4% paraformaldehyde (PFA) in PBS and mounted in Mowiol. Fluorescence expression was observed with a Zeiss Axiophot fluorescence microscope. Untransfected cells or cells treated with the plasmid alone were used as control.

2.15. NMR spectroscopy

^{1}H (500 MHz), ^{13}C (125 MHz) and ^{31}P (202 MHz) NMR spectra were recorded on a Varian Unity INOVA spectrometer operating with a 5-mm gradient indirect detection probe at 25 °C. Chemical shifts were referenced to internal signal of water (4.78 ppm) or to internal signal of $CDCl_3$ (7.24 ppm) for proton and to external phosphoric acid (85%) (0 ppm) for phosphorus.

^{1}H spectra (32 or 64 transients) were obtained with 8.3 μs 90° pulse length, 1 s of relaxation delay, 6000 Hz spectral width and 16 K data size. For the hydrated samples, the water signal was suppressed by a presaturation pulse during the relaxation delay. A 0.5 Hz line broadening was applied to the free induction decay (FID) prior to Fourier transform. COSY spectra were performed with 256 t1 increments of 16 transients each. FIDs were acquired using 2 K data points and data were processed with a shifted sine bell function in both dimensions.

For ^{31}P, FIDs were accumulated for up to 1500 transients in $CDCl_3$, 30 μs at 55 dB 90° pulses length, 2 s of recycle delay, 2 kHz spectral width and 2 K data size was employed. The FID was processed with an exponential multiplication corresponding to 5 Hz line broadening prior to Fourier transform.

One-dimensional ^{13}C experiments were recorded in $CDCl_3$. The spectral width was 30 kHz, the data size was 32 K, the 90° pulse length was 15.5 μs for 53 dB. (^{13}C–^{1}H) HSQC experiments were performed using a 15.5 μs 90° non-selective pulses length (53 dB) for carbon and 8.3 μs (58 dB) for proton. The $1/4J_{CH}$ delay correspond to a coupling constant J_{CH} of 140 Hz. A total of 256 experiments of 64 transients each were collected.

2.16. Statistical analysis

The results were evaluated for statistical significance by analysis of variance and *t*-test.

3. Results

3.1. Characterization of DMHAPC-Chol by NMR and FTIR

^{1}H spectral assignments were mostly based on COSY and (^{13}C-^{1}H)-HSQC experiments. Fig. 1A shows ^{1}H chemical shift assignments of each compound, DMHAPC-Chol and DOPE in $CDCl_3$. Severe signal superimpositions between 0.9 and 3 ppm made us unable to complete the assignment of the large number CH_2 chemical shifts in DMHAPC-Chol and DOPE. These results can be used to analyse an equimolar mixture (Fig. 1B). Practically, no differences exist between recorded spectra of lipids in the mixture and those taken separately for each compound.

The comparison of dry cholesterol and DMHAPC-Chol FTIR spectra revealed the disappearance of a band at 1052 cm^{-1} corresponding to the C–O stretching of the alcohol group of the cholesterol and the apparition of two bands at 1250 and 1712 cm^{-1} corresponding respectively to the C–O stretching of the ester group and to the carbonyl stretching of DMHAPC-Chol.

3.2. Determination of cationic lipid concentration

The concentration of cationic lipid DMHAPC-Chol was measured by a two-step protocol: measurement by colorimetric method of DOPE concentration and determination by IR absorption of the DOPE/cationic lipid ratio. Two calibration plots were established, one corresponding to the DOPE quantification (Fig. 2A) and the other representing the variation of the ratio between the IR absorption peak heights at 1712 and 1743 cm^{-1} of respectively the carbonyl groups in DMHAPC-Chol and in DOPE as a function of the DOPE/cationic lipid ratio (Fig. 2B). For samples used in this work, the concentration of DOPE was typically 2.1 mM as determined by the colorimetric method of Stewart so that the concentration of cationic lipid DMHAPC-Chol was 2.5 mM with an accuracy about 10%.

3.3. Characterization of liposomes and complexes formed with pCMV-β

Liposomes and complexes at various ratios with plasmid DNA were characterised by dynamic light

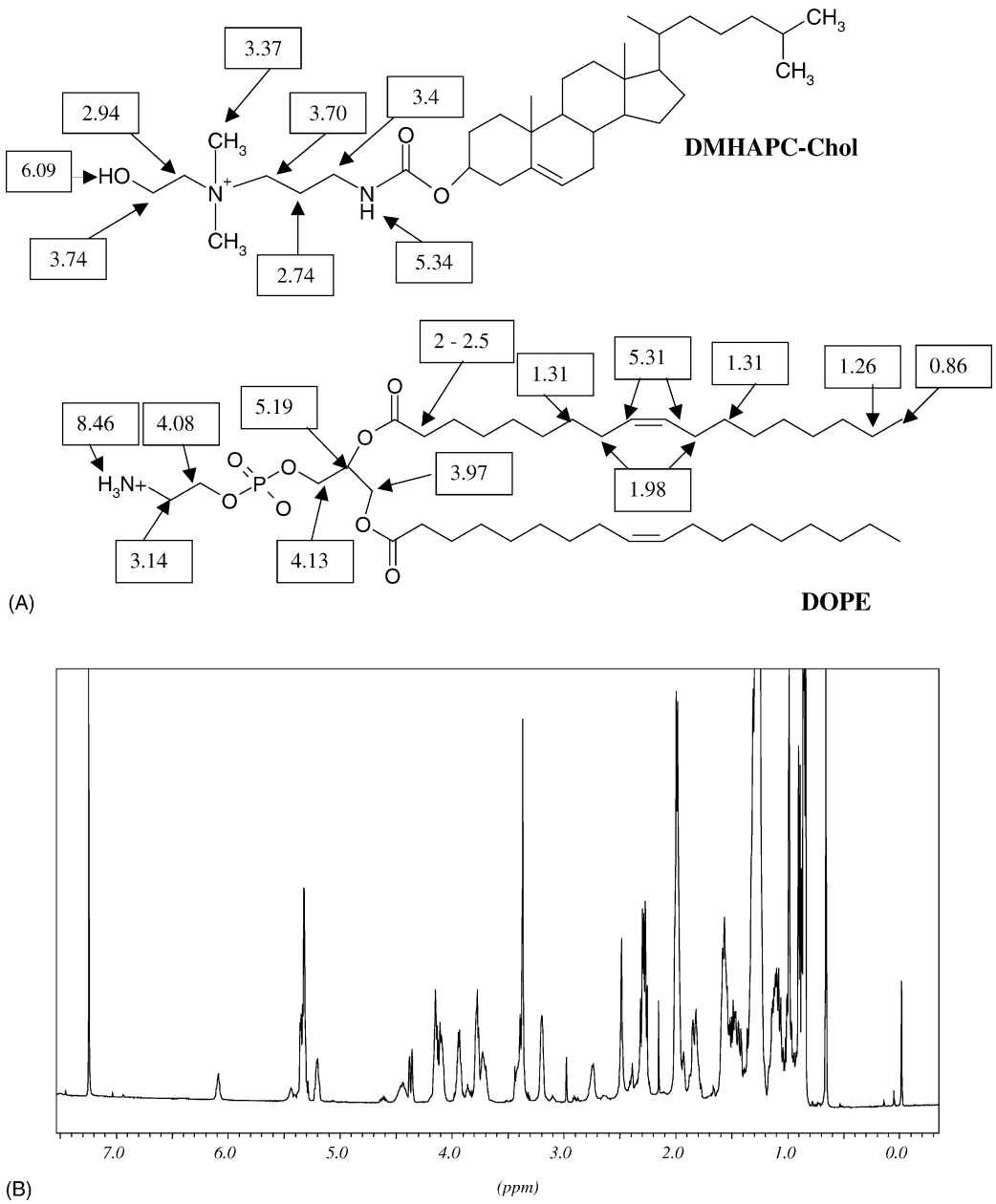

Fig. 1. Chemical characterization: (A) the schemes and ^{1}H NMR chemical shifts (in ppm) of DMHAPC-Chol and DOPE dissolved in $CDCl_3$ and (B) the ^{1}H NMR spectrum of DOPE/DMHAPC-Chol (1/1) in $CDCl_3$ at 25 °C. SW = 6000 Hz, 32 scans, lb = 0.5 Hz.

scattering and transmission electron microscopy after negative staining using uranyl acetate.

Light scattering results are shown in Fig. 3 where the average hydrodynamic diameters, and the scattered intensity of sonicated liposomes were represented as a function of the charge ratio X. The plots indicate sizes varying from 80 nm for uncomplexed liposomes to 180 nm for the complexes with a maximum value of size and scattered intensity for $X = 1.5$. For the lyophilised liposomes, simple observation of samples

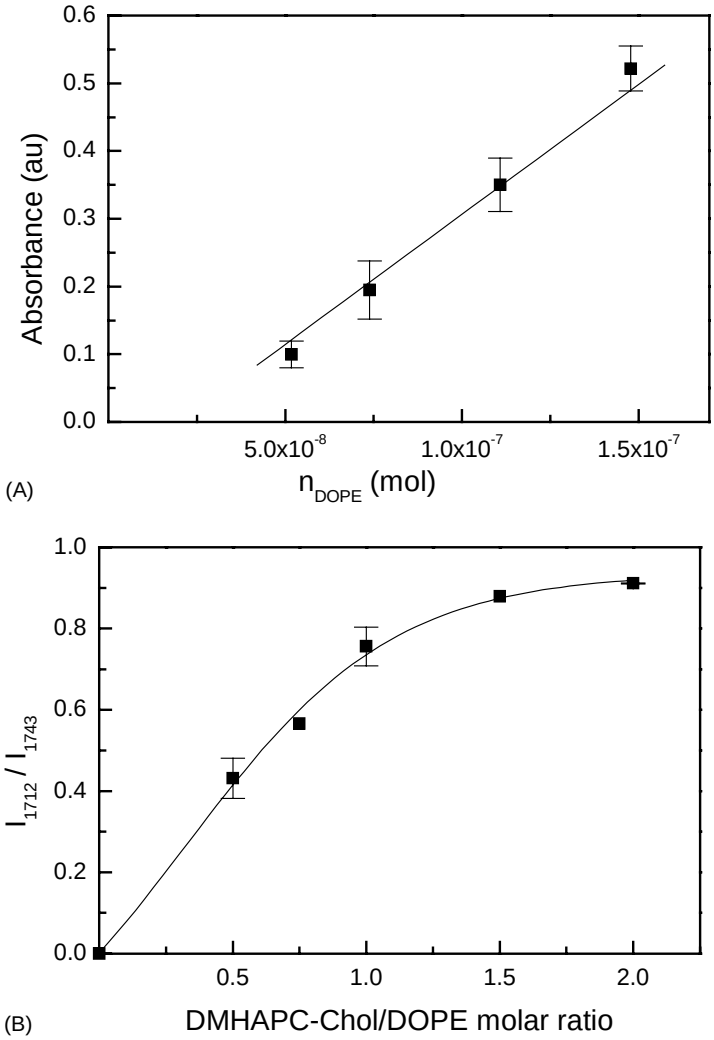

Fig. 2. Determination of the cationic lipid concentration: (A) the calibration curve used to estimate the DOPE content in $CHCl_3$ solutions following the colorimetric method of Stewart. The absorbance was measured in the organic solution at 468–472 nm for binary DMHAPC-Chol/DOPE (1/1) mixtures with known DOPE concentrations; (B) the calibration curve plotted to determine the DOPE content in the DMHAPC-Chol/DOPE mixtures. I_{1712}/I_{1743} represents the height ratio of the carbonyl band attributed to DMHAPC-Chol over the carbonyl band attributed to DOPE in the FTIR spectra of anhydrous DMHAPC-Chol/DOPE mixtures. Each result represents the mean ± S.E. of three spectra.

reveals a turbid suspension. The particles are too large and heterogenous to be accurately characterised by light scattering. QLS results roughly indicated a mean diameter of about 860 nm with a polydispersity of 1, confirming the macroscopic observation.

Results of electron microscopy are given in Fig. 4. Liposomes uncomplexed with DNA are shown in Fig. 4A (unlyophilized liposomes) and 4B (lyophilised liposomes). On Fig. 4A, a close examination of the TEM pictures showed a mean size of 90–120 nm for the unlyophilized liposomes. The sizes observed by TEM were consistent with the hydrodynamic diameters measured by QLS. The liposomes conserved their spherical shape with their bilayer structure. While the unlyophilized liposomes are relatively homogenous in size, lyophilised liposomes present at least four

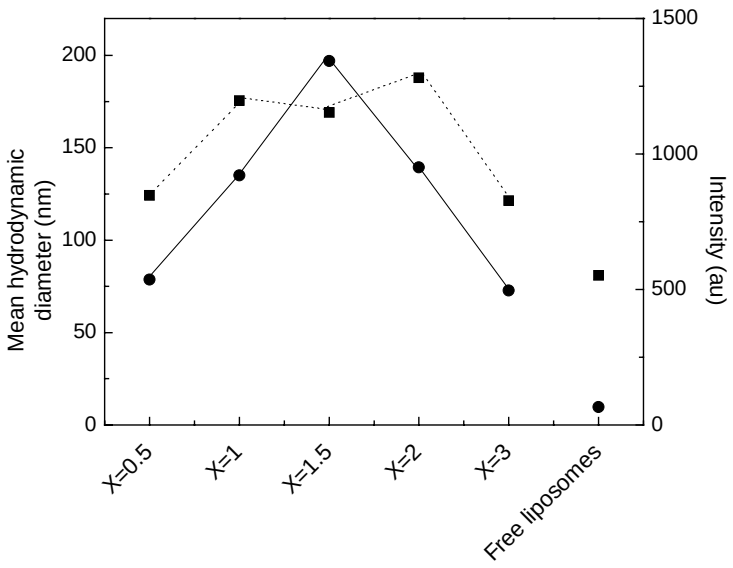

Fig. 3. Light scattering measurements. Effect of the lipid/plasmid ratio on lipoplex hydrodynamic diameter (■) and intensity (●) monitored by light scattering. The measurements were performed with DMHAPC-Chol/DOPE untreated liposomes.

kinds of shape going from globular structures, with the presence of spherical units inside to chain structures with liposomes fusionning on the periphery. Fig. 4C–H are depicted for complexes with DNA at three charge ratios $X = 0.5$; $X = 1.5$ and $X = 3$.

The micrographs show a general feature of lipoplexes resulting from aggregation of several independent liposomes, the global shape varying with the DNA ratio.

When plasmid DNA was added to unlyophilized liposomes, long aggregates were obtained at molar ratio $X = 3$ (excess of cationic lipids with regard to DNA) (Fig. 4G). These aggregates were 1 to 2 μm long and 90–120 nm wide, presenting a rod-shape with some short lateral expansions. Liposomes can be detected inside these aggregates. If more DNA was added ($X = 1.5$), the aggregates became shorter and thicker rods (400 nm × 800 nm) (Fig. 4E). Then at $X = 0.5$, the aggregates were more developed in width and became pulpy till a globular shape (Fig. 4C). In these large aggregates, substructure with a size ranging from 200 to 500 nm can be observed, with globular structures inside (about 100 nm). However, for the three studied molar ratios, globular particles (90–120 nm) were discernible inside the large aggregates and may correspond to liposomes.

Addition of DNA to lyophilised and rehydrated liposomes ($X = 3$) give rise to approximately spherical large aggregates ranging from 1 to 2 μm (Fig. 4H). Inside these structures, we distinguished small particles of liposomal size (90–120 nm). If more DNA was added ($X = 1.5$ and $X = 0.5$), the spherical shape and the size of the aggregates were preserved (Fig. 4D and F). Substructures of 120–400 nm were observed.

With immuno-gold labelling, we were able to observe the plasmid location in the complexes. Amplified gold particles correspond to the black spots in the observed field. In this case, the magnification used was 72,500 in order to see the structure details. In both cases, unlyophilized and lyophilised and rehydrated samples (Fig. 4J and K), gold particles are located in the border regions, at the surface of the substructures present in the large aggregates. On Fig. 4K, in the low right corner, a series of gold particles is perfectly aligned.

3.4. Complex formation efficiency

Liposome–plasmid complexes (lipoplexes) were quickly formed after the addition of an aqueous solution of plasmid to DMHAPC-Chol/DOPE liposomes. Association of plasmid and DMHAPC-Chol/DOPE

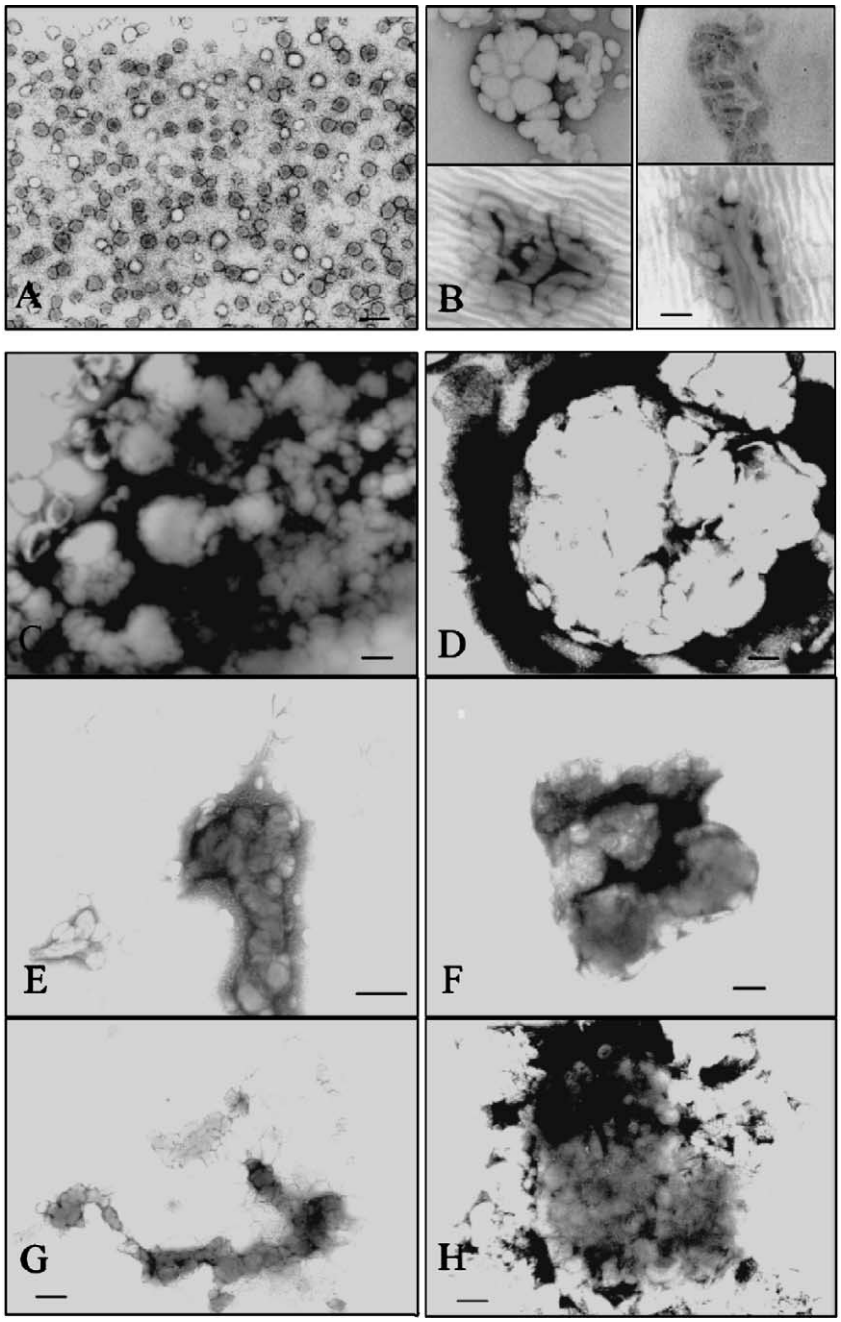

Fig. 4. Ultrastructures observed by TEM of DMHAPC-Chol/DOPE (1/1) liposomes and complexes for unlyophilized (A, C, E, G, and J) and lyophilised and rehydrated samples (B, D, F, H and K). (A) and (B) correspond to the free liposomes, (C) and (D) to a lipid/plasmid molar charge ratio of 0.5, (E) and (F) to a ratio of 1.5 and (G) and (H) to a ratio of 3. For (J) and (K), gold-labelling was used to observe plasmids in the complexes. The plasmids were labelled with digoxigenin detected by antibodies conjugated to gold particles enhanced by colloidal silver and appear as black points on the pictures. (J) and (K) correspond to DMHAPC-Chol/DOPE (1/1) complexes with labelled plasmids at molar charge ratio of 1.5 made respectively with unlyophilized and lyophilised and rehydrated liposomes. Bars represent 200 nm.

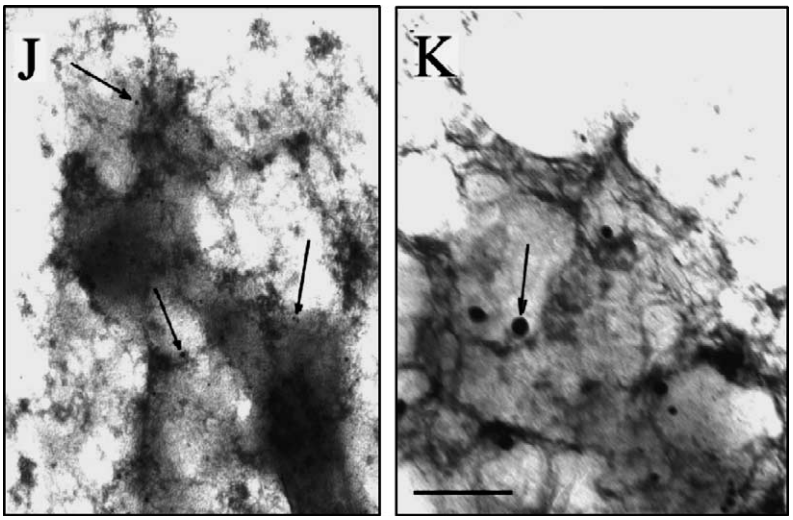

Fig. 4. (*Continued*).

liposomes was monitored by gel retardation electrophoresis. Fig. 5A was depicted to unlyophilized liposomes and Fig. 5B indicated complexation with lyophilised and rehydrated ones.

Fig. 5A indicated changes in the pattern of plasmid DNA electrophoretic migration when pCMV-βgal plasmid DNA was mixed with unlyophilized liposomes at different cationic lipid/DNA (Lip+/DNA) molar charge ratios X ranging from 0.5 to 3. Migration of pCMV-βgal is gradually slowed down when the (Lip+/DNA) charge ratio X is increased from 0.5 to 1.5 (Fig 5A, lanes 2–4). Beyond the value of 1.5 no migration was observed with an increase of the charge ratio (Fig. 5A, lanes 5–7). DNA was thus entirely bound to liposomes at a molar charge ratio in the range of $X = 1.5$ and $X = 2$ (between lanes 4 and 5). A similar result was obtained with lyophilised liposomes (Fig. 5B). However, the cutoff value is between $X = 2$ (lane 5) and $X = 2.5$ (lane 6). In both cases, these ratios are roughly equal to 2 and could correspond to the neutral form of the complex if we consider that the unilamellar vesicle structure is preserved when the complex is formed. With this configuration, only half of cationic lipid molecules located in the outer leaflet of the liposomes was exposed to DNA.

In any case, the yield of the liposome–plasmid complexes formation can achieve 100% when the molar charge ratio is appropriate.

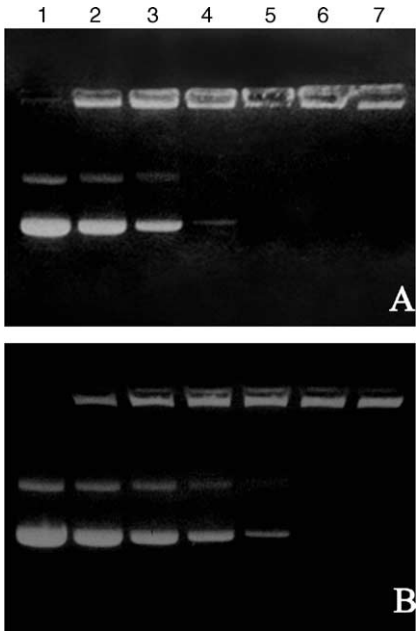

Fig. 5. Gel retardation electrophoresis assay of DNA complexed with DMHAPC-Chol/DOPE liposomes for unlyophilized (A) and lyophilised and rehydrated (B) liposomes. Lane 1: uncomplexed pCMV-βgal plasmid DNA. Lanes 2–7, pCMV-βgal plasmid DNA complexed with DMHAPC-Chol/DOPE liposomes at various lipid/DNA molar charge ratios: $X = 0.5$, $X = 1$, $X = 1.5$, $X = 2$, $X = 2.5$, and $X = 3$. Electrophoresis conditions: 1% agarose gel, 80 V.

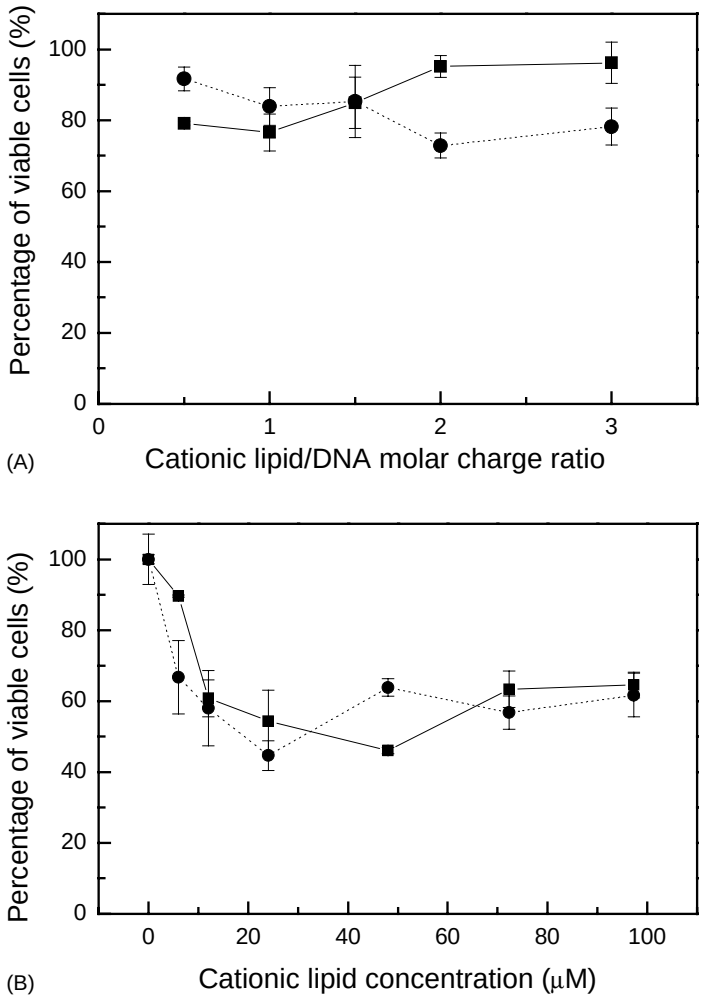

Fig. 6. The cytotoxicity of DMHAPC-Chol/DOPE liposomes was estimated by measuring the cellular viability by MTT test as described in the text. Assays were performed in triplicate 24 h after B16-F10 cells were exposed to liposomes. Data were done for liposomes (■) and lyophilised and rehydrated liposomes (●): (A) the viability for complexes at various charge ratios with a constant lipid concentration of 6 μM; (B) the viability of uncomplexed liposomes with various lipid concentrations.

3.5. Cytotoxicity study

The cytotoxicity of DMHAPC-Chol/DOPE liposomes was studied using the MTT test. Results are indicated in Fig. 6. One can clearly see that with a given cationic lipid concentration, plasmid DNA at different doses has no effect on the cell viability (Fig. 6A). Fig. 6B showed that the percentage of viable cells decreases rapidly from 100% in the range of 0–20 μM in cationic lipid but this percentage was larger than 50% for cationic lipid concentration ranging up to 100 μM. No significant difference was observed between untreated and lyophilised and rehydrated liposomes.

3.6. Transfection efficiency of DMHAPC-Chol/DOPE liposomes in vitro and in vivo

First, we measured the transfection efficiency of DMHAPC-Chol/DOPE liposomes in vitro in B16-F10 cells as a function of the molar cationic lipid/DNA charge ratio. The results of β-galactosidase expres-

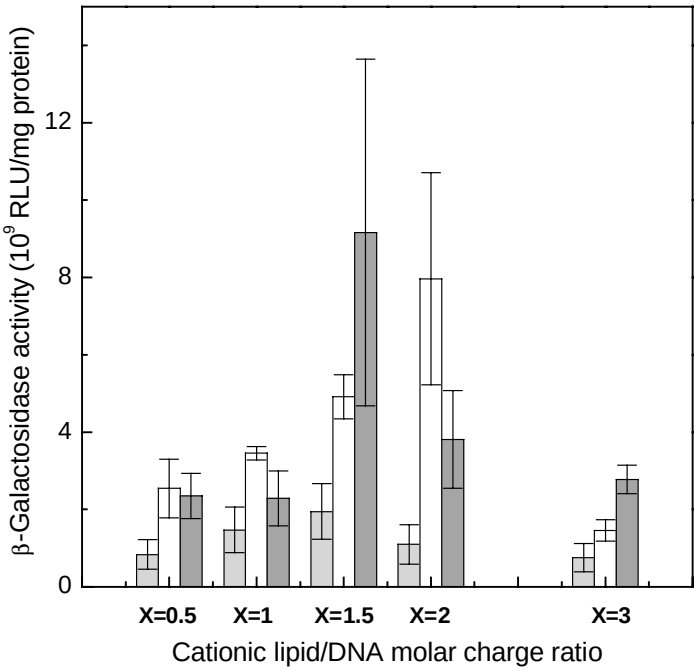

Fig. 7. Transfection level in B16-F10 cells of β-galactosidase plasmid delivered by cationic liposomes DMHAPC-Chol/DOPE. 0.5 μg of pCMV-βgal plasmid DNA containing the β-galactosidase gene was complexed with liposomes (light grey), lyophilised and rehydrated liposomes (white) and frozen and thawed complexes (dark grey). The charge ratios were ranging from $X = 0.5$ to $X = 3$. Each result represents the mean ± S.E. of three experiments.

sion 24 h after transfer in cells by cationic liposomes DMHAPC-Chol/DOPE are indicated in Fig. 7. When the cationic lipid/DNA molar charge ratio was changed from 0.5 to 3, DMHAPC-Chol/DOPE liposomes showed a maximum transfection level at $X = 1.5$. This maximum corresponds to the total complexation of DNA as measured by gel electrophoresis.

Second, the transfection level was estimated in vivo in B16 tumours. In a previous work, we have shown the importance of the molar charge ratio, the effect of injection volume and the influence of DOPE on the lipofection of B16 tumour in order to maximize intra tumoral gene delivery (Reynier et al., 2002). With the optimal conditions established, we have investigated the efficiency of the new cationic liposome DMHAPC-Chol/DOPE to carry DNA into tumours induced by B16-F10 melanoma cells in nude mice (Fig. 8) and statistically analysed by *t*-test. Control levels were obtained with 15 μg of unvectorized pUT650 diluted in sterile water and injected in tumours. After 24 h of incubation, compared to unvectorized plasmid DNA, the levels for DMHAPC-Chol/DOPE liposome observed at a charge ratio $X = 0.5$ were significantly enhanced by a factor of 26-fold ($P < 0.01$). For comparison, Fig. 8 showed also the levels obtained with DC-Chol and four cationic liposomes in the same series with either trimethylated polar head, trimethylaminoethane-carbamoyl cholesterol iodide (TMAEC-Chol) and trimethylaminopropane-carbamoyl cholesterol iodide (TMAPC-Chol) or triethylated polar head, triethylaminoethane-carbamoyl cholesterol iodide (TEAEC-Chol) and triethylaminopropane-carbamoyl cholesterol iodide (TEAPC-Chol). The spacer of these lipids was either 2C or 3C. All the formulations contain DOPE in equimolar proportion. While DC-Chol and the short lipids TMAEC-Chol and TEAEC-Chol with 2C spacer give low transfection levels, TEAPC-Chol and TMAPC-Chol with 3C spacer increased respectively 10- and 30-fold the transfection level with respect to the control. The results clearly showed the superiority of the cationic lipids constituted by three carbons in the spacer, i.e., TEAPC-Chol, DMHAPC-

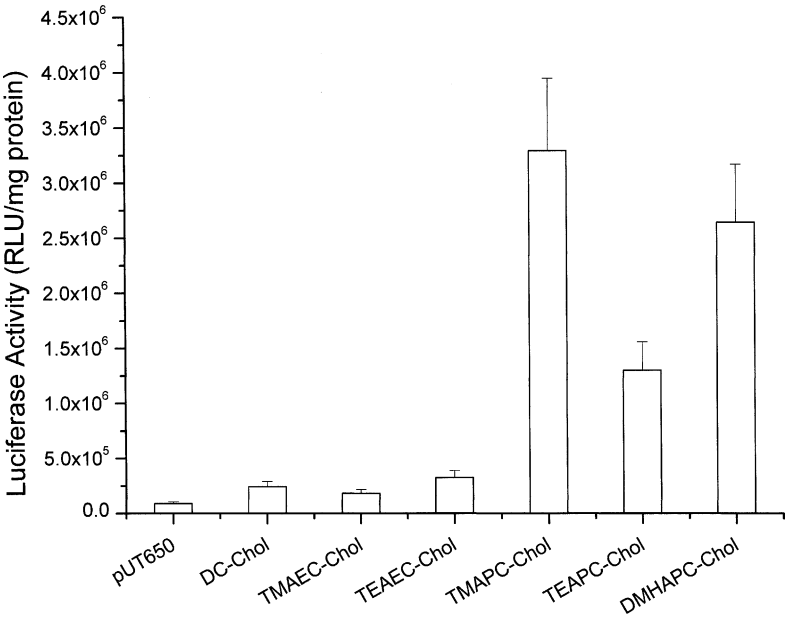

Fig. 8. Transfection level after intratumoral administration in B16-F10 tumors by liposomes prepared from DMHAPC-Chol/DOPE and other lipids in the same series derived from cholesterol. All the formulations were prepared with DOPE in equimolar proportion. Fifteen micrograms of plasmids pUT650 was complexed with cationic lipid/DOPE (1/1) liposomes at lipid/DNA molar charge ratios $X = 0.5$ in water (75 μl final) and injected directly into melanoma tumours. Tumours were removed 24 h post-injection and luciferase was evaluated as described in materials and methods. Error bars correspond to the mean ± S.E. of six tumours. Statistical analysis was used for comparison of results.

Chol and TMAPC-Chol and the potential influence of the hydration of the polar head.

3.7. Effect of conditioning on DMHAPC-Chol/DOPE liposomes efficiency in vitro

DMHAPC-Chol/DOPE liposomes were lyophilised and rehydrated and lipid/DNA complexes were subjected to three cycles of freeze-thawing prior to transfection in order to estimate the conditioning effect on DNA delivery. The results of β-galactosidase expression 24 h after transfer in B16F10 cells are indicated in Fig. 7 as a function of the molar cationic lipid/DNA charge ratio. Depending on the conditioning, DMHAPC-Chol/DOPE liposomes showed a maximum transfection level at $X = 1.5$ for untreated liposomes, $X = 2$ for lyophilised and rehydrated liposomes and $X = 1.5$ for frozen and thawed complexes.

The transfection level obtained with frozen liposomes is 4–5-fold higher in comparison with unlyophilized liposomes ($P < 0.01$). The lyophilization of the liposomes and the freeze-drying of the complexes substantially influence the transfection efficiency of the lipidic carrier. A comparable enhancement of transfection level was obtained by freeze-thawing of complexes DNA/lipofectamine and DNA/DOTAP:DOPE without any addition of cryoprotectant (Anchordoquy et al., 1998).

Transfection levels in B16-F10 cells of DMHAPC-Chol/DOPE liposomes using pCMV-β plasmids encoding β-galactosidase were analysed statistically and compared to those of liposomes in the same chemical series and to commonly known commercial reagents (Fig. 9). For the latter, the transfection protocols were as recommended by the manufacturers. As previously observed, the transfection level obtained with lyophilised and rehydrated liposomes is four-fold higher in comparison with unlyophilized liposomes. At the molar charge ratio $X = 1.5$, complexes subjected to three cycles of freeze-thawing gave a transfection level up to five-fold greater than unfreeze-thawed ones and this is the same level obtained with

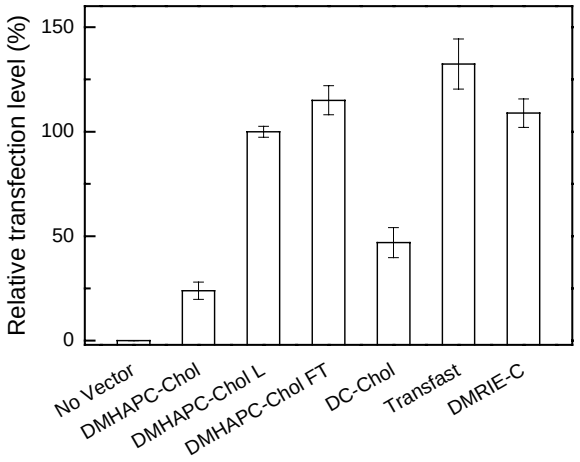

Fig. 9. Comparison of transfection levels in B16-F10 cells of β-galactosidase plasmid delivered by DMHAPC-Chol/DOPE liposomes with various formulations (liposomes, lyophilized and rehydrated liposomes (L) and frozen and thawed lipid/plasmid complexes (FT)) and well-known cationic liposomes. Values presented are relative to the transfection level by lyophilized and rehydrated DMHAPC-Chol/DOPE liposomes (100%). Each result represents the mean ± S.E. of four or more experiments. Statistical analysis was used for comparison of results.

DMRIE-C. Results in Fig. 9 indicated that in comparison to Transfast (Promega) and DMRIE-C (Invitrogen), unlyophilized DMHAPC-Chol/liposomes present a significantly lower level but the lyophilised and rehydrated liposomes gave a comparable level.

The expression in cells of GFP genes delivered by DMHAPC-Chol/DOPE liposomes was used to monitor the effect of lyophilization on the number of transfected cells. The functionality of the plasmid delivered by DMHAPC-Chol/DOPE liposome was checked inside B16-F10 cells (Fig. 10). Observations of the cells 6 h after transfection showed practically no expression of GFP. But at 24 h (Fig. 10C and D) and more especially at 48 h of transfection (Fig. 10E and F), the fluorescence in cell cytoplasm was high, with the same intensity using 0.5 or 1 μg of plasmid. However, the fluorescence was more intense and there were more transfected cells after transfection by complexes formed with lyophilised liposomes than with unlyophilized liposomes. Control with untranfected cells (Fig. 10A) or cells treated with the plasmid alone (Fig. 10B) did not present any fluorescence.

3.8. Effect of lyophilization on ^{31}P NMR spectra

In order to understand the enhancement of transfection level observed with lyophilised liposomes, ^{31}P NMR spectroscopy was employed. Liposomes were satisfactory analysed in D_2O, but the aggregate size of samples rehydrated after lyophilization did not give suitable spectra. Also, as a first investigation, we recorded spectra of samples dissolved in $CDCl_3$ instead of D_2O.

The phosphorous spectrum in Fig. 11A of pure dry DOPE powder in $CDCl_3$ showed one signal at 0.9 ppm, corresponding to the phosphate group belonging to DOPE polar head. No change was observed when the sample was lyophilised and redissolved in the same solvent. The ^{31}P spectrum of the equimolar mixture DMHAPC-Chol/DOPE powder directly dissolved in $CDCl_3$ presented only one peak at 0.62 ppm (Fig. 11B).

When the liposomes DMHAPC-Chol/DOPE 1:1 prepared in H_2O were lyophilised and then dissolved in $CDCl_3$, two signals appeared (Fig. 11C): one at 0.64 ppm, which corresponds to the signal already found in $CDCl_3$ and a new resonance at −1.07 ppm. When DOPE lipid was first dispersed in H_2O, lyophilised and then dissolved in $CDCl_3$, two large signals were also present in the ^{31}P spectrum at 0.88 ppm and −0.32 ppm (Fig. 11D). These signals correspond to two different environments for DOPE phosphate groups in these samples, first dispersed in H_2O and then lyophilised.

4. Discussion

The results obtained with assays in vitro and in vivo showed that DNA transfer can be efficiently carried out with the new cationic lipid DMHAPC-Chol, derived from cholesterol and containing a hydroxyethyl group $–C_2H_4OH$ in the polar head. In some specific conditions of use, the observed transfection level is the same or even better as compared with commercialised reagents. This lipid was designed following other members TMAEC-Chol and TEAPC-Chol, all containing a biodegradable carbamoyl linker –OCONH–, demonstrated to be efficient in the transport of DNA (Cao et al., 2000; Lesage et al., 2002) but the polar head has been modified to contain a

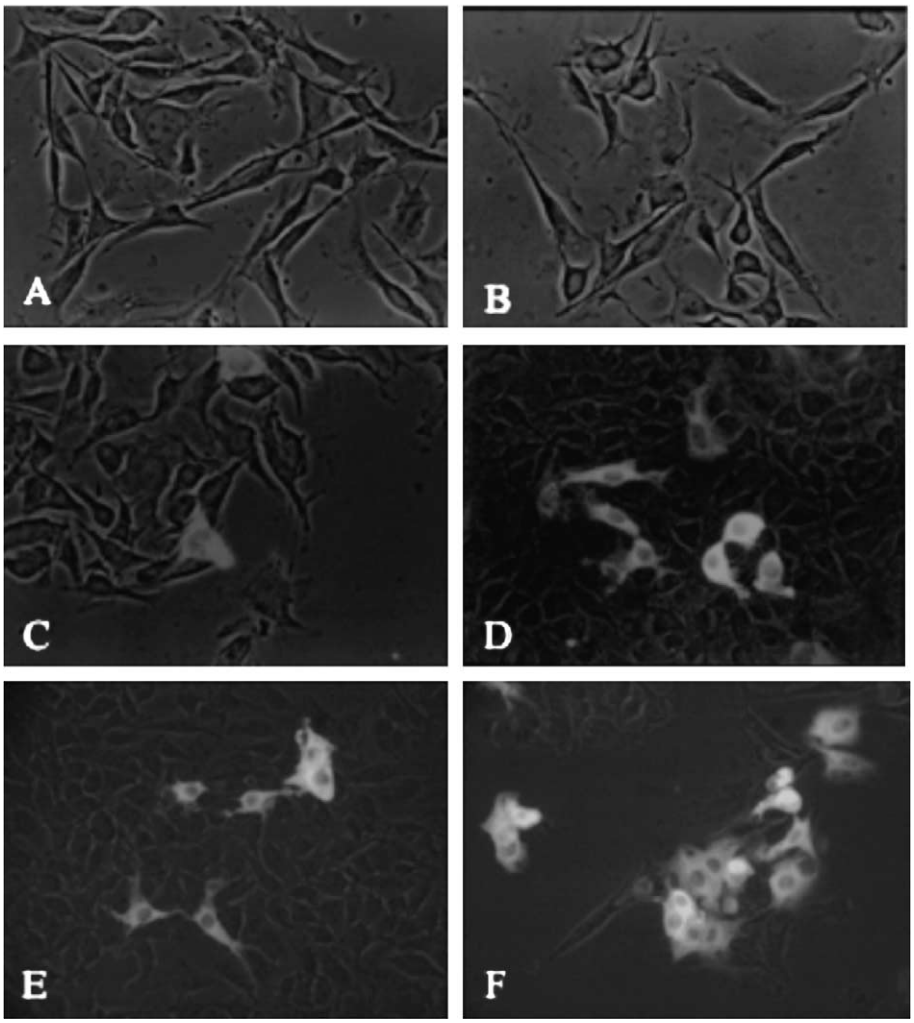

Fig. 10. Expression of gene encoding for GFP transferred into B16-F10 cells by DMHAPC-Chol/DOPE liposomes. The cells are observed in epifluorescence combined with phase contrast: (A) cells alone (B) cells transfected with plasmid pEGFP-N1 alone; (C and E) and (D and F) are transfected with DMHAPC-Chol/DOPE (1/1) complexes made respectively with unlyophilized and lyophilized and rehydrated liposomes with a lipid to plasmid ratio of 1.5; (C) and (D) pictures were obtained 24 h after transfection; (A), (B), (E) and (F) were obtained 48 h after transfection.

hydroxyethyl group. In comparison with the lipid TEAPC-Chol with three ethyl groups, the $–C_2H_4OH$ group should render the polar head more hydrophilic than TEAPC-Chol. The contribution of this hydroxyl moiety on the quaternary ammonium in the enhancement of transfection has been demonstrated with the reagents DMRIE-C (Invitrogen) (Felgner et al., 1994) and Transfast (Promega) (Bennett et al., 1997; Wang et al., 2002) whose cationic lipids molecules contain respectively one and two hydroxyethyl groups.

First, it is interesting to discuss about results observed from in vivo transfection. In comparing with two other cationic lipids in the series, TEAPC-Chol and TMAPC-Chol, different by the spacer length (2C or 3C) or by the ramified groups in the polar head, the results presented in Fig. 8 clearly showed (i) the superiority of the cationic lipids TEAPC-Chol and TMAPC-Chol constituted by three carbons in the spacer over cationic lipids DC-Chol, TMAEC-Chol and TEAEC-Chol (ii) the probable influence of the

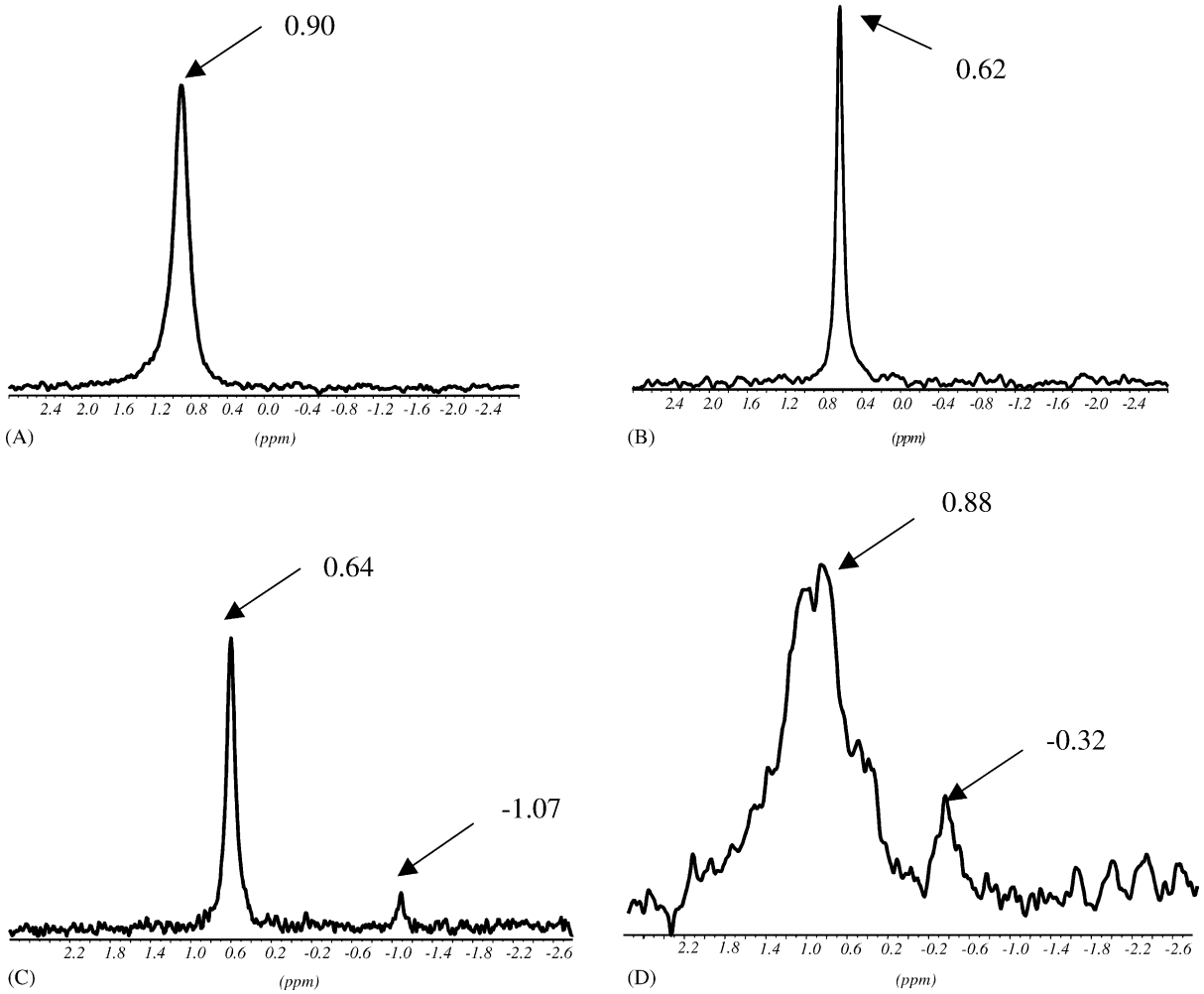

Fig. 11. ^{31}P NMR spectra of dry (A and B) and hydrated and lyophilized (C and D) samples dissolved in $CDCl_3$: (A) DOPE; (B) DMHAPC-Chol/DOPE (1/1) dry mixture (SW = 2000 Hz, 1500 scans, lb = 5 Hz); (C) DMHAPC-Chol/DOPE liposomes (SW = 2000 Hz, 1500 scans, lb = 5 Hz); (D) DOPE dispersion (SW = 2000 Hz, 25,000 scans, lb = 10 Hz), hydrated and lyophilized prior to analysis in $CDCl_3$.

hydration of the polar head. Between the lipids having the same spacer length 3C, transfection results with hydroxyethyled DMHAPC-Chol gave one of the highest transfection level. It is also important to note that the molar charge ratio used was $X = 0.5$. This value was suggested by our previous results indicating that the optimal level in vivo is $X = 0.5$ while that in vitro is $X = 1.5$–2. This discordance between optimal charge ratios for in vitro and in vivo conditions was already mentioned (Son et al., 2000; Reynier et al., 2002).

For this study, a simple original method to determine the concentration of cationic lipid has been used. Indeed, an accurate knowledge of the concentration is necessary for an accurate preparation of complexes with DNA at a desired molar charge ratio. Because of a lack of absorption in the UV region, the measurement of cationic lipid concentration became a severe problem. To resolve this difficulty, we proposed an indirect determination by the measurement of DOPE concentration by a colorimetric method combined with the determination of the cationic lipid/DOPE molar

ratio by FTIR using a calibration curve established prior to measurements. Once the calibration plot of the peak height ratio corresponding respectively to the carbonyl groups of cationic lipid and DOPE as a function of known molar ratios determined, this two-step method become a relatively simple routine handling, more practical than the evaporative light scattering detector, one of the rare published methods used to detect cationic lipid (Felgner, 1997).

Another important finding in this study is the enhancement of the transfection level observed with lyophilised DMHAPC-Chol/DOPE liposomes and freeze-thawed complexes. Reproducible results showed that the β-galactosidase activity was increased up to four fold when plasmids was delivered in cells by lyophilised in comparison with unlyophilized cationic liposomes. In the same range, transfection was increased five-fold by freeze-thawing of the complexes.

Transfection using GFP confirmed the effect of lyophilization on the enhancement of DNA delivery in cells and showed that more cells are transfected in this latter case.

Up to date, the effect of lyophilization on liposomes was only investigated from a structural point of view (Gregoriadis et al., 1996; Van Winden et al., 1997). This surprising enhancement observed in transfection may open a new way for pharmaceutical uses of cationic liposomes. The lyophilization presents several advantages. First, the lyophilization allows to avoid the storage of liposomes under the liquid state which is generally the cause of instability. Second, it facilitates the liposome shipping. Third, the storage of cationic liposomes separately with respect to the storage of DNA will allow the complexation with any DNA in choice and at any desired ratio for use in a precise purpose.

Lyophilization and freeze-thawing promote changes in the complexes structure which modify the available positive charges of liposomes and the lipid/DNA ratio for which DNA is fully taken as indicated by electrophoresis results. In this work, we characterised the untreated and lyophilised and rehydrated liposomes by QLS and microscopy to follow the size changes.

Both techniques showed that complexes are aggregates whose size depends on the molar charge ratio X between lipid and DNA. However, if the increase in size of aggregates as well in polydispersity factor (data not shown) were maximum around $X = 1.5$ as given by QLS results, the observation of their substructures by TEM revealed that these particles were formed by the aggregation of liposomes with DNA. In any case, with complexes formed either with unlyophilized or lyophilised liposomes, spaghetti shapes were not detected, in contrary to that observed with other carriers (Sternberg et al., 1994). It may be that these vesicles were responsible for the observed highest transfection level obtained with a molar charge ratio X around 1.5–2.

It is worth noting that DNA in complexes was observed by the immuno-gold labelling technique. With this technique, although the entire shape of DNA was not observable, simultaneous observations of the presence of DNA and liposomes were possible in the same field scale, taking in mind the difference of two orders in their sizes, liposomes with 100 nm in diameter and DNA with 2 nm in section. We observed that in both cases (untreated and lyophilised liposomes), plasmid was detected on the surface of the substructures. No significant difference could be noticed between both complexes but these results support the hypothesis of an aggregation between liposomes with DNA as a linker.

For untreated liposomes, the size and charge changes of the complexes can be associated to an increase of the transfection efficiency. In the case of lyophilised liposome, as discussed above, a change in the structure of the complexes may result in a modification of charge ratio giving the optimal transfection with respect to the untreated system. In parallel, we detected a significant transfection efficiency increase. One has to notice that in this case, no stabilising cryoprotectant additive as sucrose were added during the freezing step, as previously mentioned in the literature (Anchordoquy et al., 1997). Transfection would not be directly correlated to the maintenance of particle size, as generally admitted (Molina et al., 2001).

Both lyophilization and freeze-thawing are associated with liposomes aggregation. In this latter case, the transfection is largely increased by this pre-treatment of the complexes. During freezing of the complexes a rearrangement of DNA and cationic lipids in complexes can be responsible for the transfection improvement.

To elucidate the changes induced by lyophilization from a molecular point of view, we used NMR results. ^{31}P NMR showed that in the lyophilised

DMHAPC-Chol/DOPE liposomes, even after dissolution in $CDCl_3$, two signals were observed at 0.64 and −1.07 ppm, apparently belonging to two conformations of the phosphate group of DOPE. Two signals at 0.88 and −0.32 ppm were also observed when DOPE was first dispersed in D_2O, lyophilised and dissolved in $CDCl_3$. When comparing the latter spectrum with that of pure unhydrated DOPE presenting an unique band at 0.90 ppm the second signal at −0.32 ppm may be due to a modification of the PO_2^- group environment of DOPE in the bilayer during lyophilization. These data indicated that there are two PO_2^- populations with different behaviours with respect to lyophilization.

Regarding these results, it is important to note that most cationic lipids do not form liposomes alone but need the presence of DOPE. The ability of this lipid to form bilayers in L_α state or to exist in inverted hexagonal phase was known. The role of this colipid has been discussed elsewhere (Farhood et al., 1992; Fasbender et al., 1997; Hope et al., 1998). It was suggested that DOPE has a double role, to help cationic lipids to form bilayers and also, once the complex liposome/DNA internalised in cellular endosomes, it destabilises the latter to release DNA in the cytosol (Zelphati and Szoka, 1996; Zhigaltsev et al., 2002). This should be directly related to the transfection level. In our case, it is likely that the mutual effect of DOPE and cationic lipid DMHAPC-Chol with a cholesterol base was that the latter stabilises DOPE in L_α phase (Fenske and Cullis, 1992) and DOPE helped DMHAPC-Chol to exist in bilayer state. During the freezing phase of lyophilization, probably ice was locally formed but heterogenously so that the environment of phosphate groups of DOPE was affected with more or less hydration states (Sanderson et al., 1993) which conferred to the liposomes a structure favouring a better transfection. After lyophilization, the rehydration may be incomplete for a fraction of PO_2^- sites, favouring the existence of DOPE in the hexagonal inverted phase, especially when the latter are internalised in the endosomes at low pH. This should be favourable for the destabilisation of endosomal membrane and the release of DNA in cytosol which in turn enhance the transfection level.

In conclusion, we showed that DMHAPC-Chol associated with DOPE can be successfully used for DNA delivery in tumours. We also pointed out the effect of conditioning on these DOPE/hydroxyethyl cholesterol-based cationic liposomes efficiency. Lyophilization and freeze-thawing of DNA/liposome complexes without use of cryoprotectants substantially enhanced their ability to deliver DNA in cells.

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