European Journal of Pharmaceutics and Biopharmaceutics 90 (2015) 63–69

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Fine tuning of mixed ionic and hydrogen bond interactions for plasmid delivery using lipoplexes

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ARTICLE INFO

Article history:
Received 6 August 2014
Accepted in revised form 3 November 2014
Available online 21 November 2014

Keywords:
Cationic lipids
Thiourea lipids
Lipoplexes
DNA delivery
Transfection
Nanomedicine

ABSTRACT

Non viral gene transfection has been mostly reached via cationic polymer and lipid, required for DNA complexation and cell internalisation. However, cationic charges often induce cytotoxicity and limit the efficacy of the lipoplexes *in vivo* due to their fast elimination from the blood stream. Few years ago, we had developed noncationic lipid interacting with DNA via hydrogen bond interactions. To take advantage of both the internalisation efficacy of cationic complexes and the higher DNA release efficacy of non cationic lipids, we chose to mix both ionic and hydrogen bond interactions within one lipoplex. The idea behind this strategy would be to reduce the overall charge while maintaining a high level of transfection. Four mixed formulations of cationic lipid and thiourea lipid were prepared. We found that decreasing ionic interactions and increasing hydrogen bond interactions improved cationic lipoplexes properties. Indeed, we showed that replacement of net positive charges by hydrogen bond interactions with DNA phosphates led to efficient lipoplexes for *in vitro* DNA transfection at lower cationic charge content, which consequently reduced lipoplex cytotoxicity.

1. Introduction

Nonviral gene transfection could be of high interest to combine safe and efficient nanotechnologies. However, this is not fully achieved yet, mainly because of their lower efficiency as compared to viral vectors. Nevertheless, improvement is still achievable, and few nonviral gene therapy entered clinical trial recently [1]. Few years ago, we had hypothesised that the cationic charge used for DNA interaction beared by either lipids or polymers was the tree which hid bits. Indeed, *in vitro* transfections were highly efficient for all cationic complexes performed without serum, but led to inefficient *in vivo* transfections. The reason for this was the extremely fast elimination of complexes from the blood stream. In this context, lipofectamine, PEI, DOGS [2] and our own lipids amongst which DMAPAP were developed [3]. Various strategies were then proposed to reduce the overall charges, such as PEG shielding [4], PEG shielding with anionic PEG [5] and postgrafting [6]. Moreover, interest of tunable lipids neutral at physiological pH and becoming charged at lower pH fulfilled this hypothesis that complexes should present a lower cationic overall charge [7,8]. Our choice was to develop noncationic lipids able to interact with DNA phosphates via hydrogen bonds [9]. The shape of the so-called lipothiourea lipids is strictly copied from the shape of cationic lipids, in which amines were replaced by thiourea functions [10]. This family of lipids evolved towards the years to yield a lipid quite efficient to transfect *in vitro* despite its low internalisation into the cells [11]. The ability of lipothiourea to transfect cells efficiently was attributed to the fact that DNA was released very efficiently into the cells thanks to the low binding forces between thiourea and phosphates functions. Based on the very high efficiency of cationic lipids to transfect cells and the capacity of lipothiourea to interact less tightly with DNA, we thought that a mixture of cationic lipids and lipothiourea could be of great interest to reduce the lipoplexes overall charge while providing a nanovector able to transfect efficiently. Noteworthy, combining cationic and hydrogen

Abbreviations: PEI, polyethylene imine; DOGS, dioctadecylamidoglycylspermine; DMAPAP, dimyristoylaminopropylaminopropyl; EtOH, ethanol; DMEM, dubelcco minimum essential medium; FBS, foetal bovine serum; NaCl, sodium chloride; PBS, phosphate buffer saline; PFA, paraformaldéhyde; EDTA, ethylene diamine tetraacetic acid; RLU, relative light unit; GFP, green fluorescent protein; DAPI, DiAmidinophenylindol.

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http://dx.doi.org/10.1016/j.ejpb.2014.11.001
0939-6411/

bonds for DNA interaction via thiourea function have been proposed on two other nanosystems recently reported in the literature. First, lipidic cyclodextrins combining on its hydrophilic face both cationic and thiourea functions have shown higher transfection efficiency [12–14]. More recently, polyethylene imine whose charges have been masked by methylthiourea have been proposed [15].

The aim of this work was to compare various combinations of cationic and lipothiourea lipids, starting from the more cationic to the less cationic, to evaluate the interest of this combination in terms of nanoparticle surface charge, DNA interaction, *in vitro* gene transfection and cytotoxicity.

2. Material and methods

2.1. Lipids used in the study

DMAPAP and DDSTU lipids (Fig. 1) were synthesised as previously described [6,16]. DOPE-Rhodamine was purchased from Avanti Polar Lipids.

The *Dimyristoyl-glycine-cyanine 5* was obtained as followed. The dimyristoyl amine reacted with 1.2 eq Boc-Glycine in the presence of NEt_3 and BOP during 30 min at room temperature. After successive washing steps with KHSO4, NHCO3 and saturated NaCl, the product was deprotected in DCM/TFA 9/1 during 1 h. The aminolipid obtained was reacted with 1.2 eq. cyanin-5-NHS in THF during 1 h, and then purified by silica gel chromatography.

2.2. Preparation of the liposomes

Liposomes were prepared by ethanolic injection as described [17]. Briefly, appropriate amounts of DMAPAP and DDSTU were diluted in ethanol, mixed according to the desired ratio (Table 1), and dropped at 1 mL/min on a solution of stirred water filtered under 0.2 μm.

2.3. Preparation of the plasmid

Plasmid pVax2 was used for all experiments. pVax2 is a derivative of the commercial plasmid pVax1 (Gibco, Invitrogen, France), which was digested with the restriction enzymes HincII and BamHI to excise the promoter. The plasmid was then blunted with the Klenow fragment, dephosphorylated with alkaline phosphatase, pCMVbeta (Clontech, Palo Alto, CA, USA), and was digested with EcoR1 and BamHI to excise the cytomegalovirus (CMV) promoter. The CMV promoter was blunted with Klenow enzyme and ligated into the blunted pVax1 to give pVax2. The plasmid pXL3031 was digested with EcoRI and BamHI and then treated with the Klenow fragment to produce a blunted fragment containing the luciferase cDNA. This fragment was ligated into pVax2 after EcoRV digestion and phosphatase alkaline dephosphorylation to give the pVax2-Luc [18].

2.4. Preparation of the DNA/lipid complexes

Plasmid (200 μL, 0.01 g/l in H_2O) was added dropwise with constant vortexing to various amounts of liposomes (in 200 μL of H_2O). N/P indicates the ratio in nanomoles of cationic charge for 1 μg of DNA phosphates for the physico-chemical characterisation and 0.5 μg for the cell transfection (1 nmol lipid/μg Phosphate is equivalent to 1 nmol lipid /3 nmol Phosphate), counting 3 cationic charges per cationic lipid. Total lipid ratio indicates the ratio in nanomoles of total lipid for 0.5 μg of DNA phosphates.

2.5. Physicochemical characterisations

The hydrodynamic diameter of the lipoplexes was measured by dynamic light scattering (Zeta Sizer Nanoseries, Malvern Instruments. The time-dependent variation of the light intensity was linked to their radius with the Stokes–Einstein equation $D = kT/6\pi R\eta$ where D is the diffusion coefficient, T the temperature, k the Boltzmann constant, R the particle radius, and η the solvent viscosity. Zeta potential measurements were performed on the same instrument using the Smoluchowsky theory. Lipoplexes were diluted in 20 mM NaCl to maintain the conductivity constant between the samples to be compared.

2.6. DNA accessibility

Picogreen (In vitrogen USA) is an intercalating agent which fluoresces when intercalating between the base pairs. Samples corresponding to 50 ng of DNA (10 μL) were loaded in 96 black well plates. A solution of picogreen (90 μL, diluted 200 times in Tris EDTA) was added to the wells. Measurements were performed with a Multilabel Counter Perkin Elmer (Wallac Victor^2 1420) with excitation and emission wavelengths of 460 and 490 nm. Percentage of fluorescence was calculated as follows: % fluorescence = Intensity (Lipoplex sample − Background)/Intensity (Free DNA-Background). Background corresponded to the value of picogreen added in the suspension of liposomes which did not contain DNA.

2.7. In vitro transfection

Murine melanoma B16 cells and CT26 colon carcinoma cells were cultured in Dubelcco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, L-glutamine (29 mg/mL), penicillin (50 U/mL) and streptomycin (50 U/mL) (GibcoBRL, Life Technology) with 5% CO_2 at 37 °C. Cells were seeded in 96 well plates 24 h prior transfection (12,000 cells/well). Complexes containing 0.5 μg DNA were loaded on each well. After 7 h, medium was changed and the cells left for 17 h. Cells were then washed twice with PBS, and then lysed with Luciferase Cell Culture Lysis Reagent (Promega) Transfection efficacy was evaluated with a luciferase kit (Luciferase Assay System, Promega). To lysed cells supernatant (10 μL) was added luciferin and ATP (50 μL), then luminescence was measured with an automator multilabel counter (Wallac Victor^2 1420, Perkin

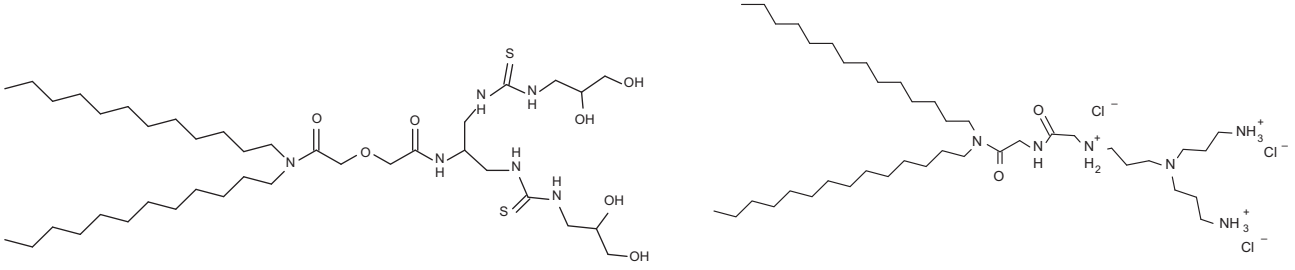

Fig. 1. Lipids DDSTU (left) and DMAPAP (right) used in the present study.