Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 331 (2007) 160–166
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Liposomes for phospholipase A_2 triggered siRNA release: Preparation and *in vitro* test

Camilla Foged *, Hanne M. Nielsen, Sven Frokjaer

Department of Pharmaceutics and Analytical Chemistry, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen O, Denmark

Received 30 July 2006; received in revised form 2 November 2006; accepted 3 November 2006
Available online 9 November 2006

Abstract

Small interfering RNA (siRNA) is potent and highly specific for gene silencing. However, for therapeutic applications, delivery systems are required to protect siRNA from degradation, to enhance cellular uptake and for site-specific delivery. We used a double emulsion technique to encapsulate siRNA into stealth liposomes (SL) to increase entrapment efficiency compared to passive encapsulation. SL are designed for localized, active release of siRNA by secretory phosholipase A_2 ($sPLA_2$). $sPLA_2$ acts as a site-specific enzymatic trigger that actively degrades the liposomal carrier in inflamed tissue releasing entrapped drug. Relatively good encapsulation efficiencies compared to passive encapsulation were demonstrated (7–9%) and SL size was appropriate for i.v. administration (60–90 nm). siRNA targeting enhanced green fluorescent protein (EGFP) entrapped in SL did not silence gene expression of HeLa-cells stably expressing EGFP. However, preliminary flow cytometry and confocal microscopy data showed that the SL siRNA formulation increased uptake of siRNA into vesicular compartments of HeLa-cells in a concentration-dependent manner that could be augmented by exogenuos $sPLA_2$. We hypothesize that the SL can be used to target siRNA to inflammed tissue for silencing of cytokine expression in rheumatoid arthritis.

Keywords: siRNA; RNAi; Delivery; Liposome; PLA_2

1. Introduction

RNA interference (RNAi) has in recent years become a widely used experimental tool and there is increasing enthusiasm for developing therapies based on RNAi (Leung and Whittaker, 2005). RNAi is mediated by small RNA duplexes of 19–23 base pairs (bp) that activate an RNA-induced silencing complex (RISC) and guide sequence-specific degradation of mRNA by RISC in the cytoplasm (Elbashir et al., 2001). RNAi has appeared to be potent and highly specific for gene silencing compared to other antisense strategies (Dykxhoorn and Lieberman, 2005). However, for therapeutic applications, delivery systems are needed to protect siRNA from nuclease degradation, to enhance cellular uptake into the cytoplasm and for site-specific delivery. Both physical and chemical approaches are efficient for introduction of siRNA into mammalian cells *in vitro*, but at present few delivery systems exist for *in vivo* purposes and in particular for systemic administration (Schiffelers et al., 2004; Zimmermann et al., 2006). Therefore, there is a need for development of siRNA delivery systems that efficiently introduce siRNA into the cytoplasm with target cell specificity in order to decrease the siRNA dose administered and to avoid non-specific silencing toxicity for the systemic treatment of multiple diseases.

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by inflammation of joints and destruction of bone and cartilage, and it affects, together with related musculoskeletal disorders, more than 0.5–1% of the world's population (Lawrence et al., 1998). Tumor necrosis factor-α (TNF-α) is a cytokine critically involved in the pathogenesis of RA, and TNF-α-neutralizing drugs are currently used for the treatment of RA (Paulos et al., 2004). TNF-α is a key regulator of the progression of RA and TNF-α is over-expressed by monocytes and macrophages in several RA-diseases in joints. A recent publication has shown that electro-transfer of an siRNA–polyamine complex targeting TNF-α in the articular synovial fluid can ameliorate collagen-induced arthritis in rats (Inoue et al., 2005).

* Corresponding author. Tel.: +45 35 30 64 02; fax: +45 35 30 60 30.
E-mail address: cfo@dfuni.dk (C. Foged).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.11.010

Targeted, siRNA-mediated TNF-α silencing of inflamed joints is thus a promising approach for therapeutic intervention in RA.

Commonly used delivery systems for siRNA are the cationic transfection agents, based on polymers or lipids that form complexes with siRNA via electrostatic interactions and enhance the cellular uptake of siRNA (Medina-Kauwe et al., 2005). However, cationic delivery systems are associated with toxicity, low serum stability and poor biodistribution since cationic complexes aggregate in the lungs upon systemic administration (Mahato et al., 1998). Our research focuses on anionic, lipopolymer-coated SL that have been shown to be promising carriers for other types of drugs and which are planned to enter phase I clinical trials in 2007. SL are used as stable, non-toxic carriers that we hypothesize can increase the stability of siRNA, prolong the drug-carrier circulation time, increase the accumulation of siRNA at sites of inflammation due to the enhanced permeability and retention (EPR) effect and thereby improve the therapeutic index of siRNA (Storm and Crommelin, 1998). SL are furthermore designed for localized, active release of siRNA in inflamed tissue by $sPLA_2$ (Jorgensen et al., 2002; Davidsen et al., 2003).

$sPLA_2$ is a small interfacially active enzyme (14–16 kDa) that catalyses the hydrolysis of phospholipids in the sn-2 position to lysolipids and free fatty acids (Menschikowski et al., 2006). $sPLA_2$ is found at low levels in the blood circulation, but the enzyme is upregulated in cancer tissues and at sites of inflammation, for example in RA (Seilhamer et al., 1989). $sPLA_2$ acts as a site-specific enzymatic trigger that actively degrades the liposomal carrier in inflamed tissue releasing siRNA. Our working hypothesis is that the $sPLA_2$ generated lysolipid and fatty acid hydrolysis products can function locally as membrane permeability promoters facilitating the cellular uptake of siRNA. Previous studies have shown that products of liposomal degradation can act as enhancers for the cellular uptake of small molecule drugs (Andresen et al., 2005), but it is unknown whether this is also the case for larger molecules like nucleic acids.

Commonly used techniques for the entrapment of drugs into anionic and neutral liposomes, *e.g.* passive encapsulation, are not suitable for molecules like siRNA due to low entrapment yield and high manufacturing costs for the synthesis of siRNA. We use a double emulsion technique by which it is possible to increase the entrapment of drug in the aqueous interior of liposomes (Gotfredsen et al., 1983). A water phase containing siRNA is solubilized in an organic phase containing the lipid resulting in the formation of reversed micelles. Liposomes are formed by dispersing the reversed micelles in an aqueous buffer, followed by removal of organic solvent by evaporation. Thereby the localization of siRNA is initially restricted to the compartment that constitutes the internal aqueous phase of the liposomes.

2. Materials and methods

2.1. Materials

If not otherwise stated, compounds were supplied by Sigma–Aldrich (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and dipalmitoylphosphatidylethanolamine-polyethyleneglycol 2000 (DPPE-PEG_{2000}) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Dipalmitoylphosphatidyl[*N*-methyl-^{3}H]choline (^{3}H-DPPC) and Sepharose CL-6B was from Amersham-Pharmacia Biotech, Denmark. siRNAs were purchased from Dharmacon Research (Lafayette, CO) as dried 2′-hydroxyl, Cy3-labelled, annealed, purified and desalted duplexes. Sequences were as follows—EGFP, sense: 5′-GAC GUA AAC GGC CAC AAG UUC-3′ and antisense: 5′-ACU UGU GGC CGU UUA CGU CGC-3′, and, as control, TNF-α, sense: 5′-AGG GAU GAG AAG UUC CCA AUU-3′ and antisense: 5′-UUG GGA ACU UCU CAU CCC UUU-3′.

2.2. Preparation of liposomes

DPPC, DPPG and DPPE-PEG_{2000} were stored at −20 °C as a powder. Dioleoyltrimethylammoniumpropane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) were dissolved in $CHCl_3$ and stored at −20 °C under a nitrogen atmosphere. DPPG:DPPC:DPPE-PEG_{2000} liposomes (77:18:5 and 18:77:5 molar ratios) were prepared essentially as described previously (Gotfredsen et al., 1983), except that the procedure was scaled down to the smallest possible volume. Briefly, Cy3-labelled siRNA directed against EGFP was resuspended in 10 mM Tris buffer (pH 7.40) to a concentration of 18.5 mg/ml and used as internal aqueous phase (40 μl). Reverse micelles were formed in an organic phase of 5:1 dibutylether/chloroform (approximately 2 ml) by sonication of the lipid mixtures suspended in the organic phase and the internal aqueous phase. For determination of lipid concentration, trace amounts of ^{3}H-DPPC were added. The reversed micelles were dispersed in 10 mM Tris buffer upon vigorous agitation in a volume ratio of 1:5, whereby a w/o/w double emulsion was formed. After removal of the organic solvents by evaporation, the phospholipid suspensions were centrifuged for 30 min at 8000 × *g* to remove any lipid aggregates. Non-entrapped siRNA was removed by gelfiltration of the liposome suspension on a Sepharose CL-6B column.

Unilamellar DOTAP:DOPE liposomes for use as control transfection agents were prepared as reported previously (Hope et al., 1985; Davidsen et al., 2001). A 1:1 molar ratio mixture of DOTAP and DOPE (4 μmol total lipid) was dissolved in chloroform in a round bottom flask. For determination of lipid concentration, trace amounts of ^{3}H-DPPC were added to parallel batches prior to solvent evaporation using a rotary evaporator device. The lipid films were then stripped three times with ethanol and dried overnight under low pressure to remove trace amount of organic solvent. Multilamellar vesicles were made by dispersing the dried lipid films in 2 ml HEPES-buffer (10 mM HEPES, 50 mM KCl, 1 mM NaN_3, pH 7.5). The formed liposomes were subsequently extruded 10 times through two stacked 100 nm polycarbonate filters using an extruder from Lipex Biomembranes Inc., BC.

2.3. Characterization of liposomes

The phospholipid concentration was determined by liquid scintillation counting with a Packard TRI-CARB 2100 TR

scintillation counter from Packard Instruments using Ultima Gold scintillation fluid (Perkin-Elmer, Boston, USA). siRNA concentration was measured using the RiboGreen® RNA Quantitation Kit (Invitrogen/Molecular Probes, Leiden, The Netherlands) before and after disruption of the lipid bilayer with octylglucopyranoside (OG). The fraction of encapsulated siRNA was calculated by subtracting non-encapsulated siRNA (measured in the absence of OG) from the total siRNA (measured in the presence of OG). The particle size distribution and the zeta potential were measured in 10 mM Tris buffer (or HEPES-buffer for the DOTAP:DOPE liposomes) using the Zetasizer Nano ZS from Malvern Instruments, Denmark.

2.4. Measurement of $sPLA_2$ concentration

Human tear fluid was collected and stored at −20 °C. The concentration of $sPLA_2$ type IIA was measured in appropriately diluted human tear fluid and in the medium of HeLa-cells following 24 h (time = 0 of transfection) and further 72 h of growth using a commercial ELISA from Cayman Chemical (Tallin, Estonia) according to manufacturer's intructions. The detection limit of the assay was 15 pg/ml.

2.5. Cell culture

HeLa-cells stably transfected with EGFP (HeLa EGFP), kindly donated by Dr. Christian Damgaard and Lars Aagaard (Department of Molecular Biology, Aarhus University, Denmark), were grown in Dulbecco's modified Eagle medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Invitrogen) and 10% fetal bovine serum (FBS) (PAA laboratories GmbH, AT). At confluency, cells were split approximately 1:5. Cells were grown in an atmosphere of 5% CO_2–95% O_2 at 37 °C. Growth media was replaced every other day.

2.6. Transfection

Twenty-four hours before transfection, HeLa EGFP-cells grown to a confluent monolayer were detached from culture flasks by incubating the cells for 5 min at 37 °C with trypsin/EDTA solutions from Invitrogen. The cells were seeded in 12-well tissue culture plates in 1.5 ml of medium, 5×10^4 cells per well, for flow cytometric analysis or onto tissue culture treated 12-well Transwell filters (1.13 cm^2, 0.4 μm pore size; Costar, Cambridge, MA) at a density of 1.5×10^4 cells per filter for confocal microscopy. To prepare lipoplexes for control, DOTAP:DOPE liposomes were mixed with serum free medium (Dulbecco's modified Eagle medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine) and incubated for 15 min at room temperature. siRNA (25 nM final concentration) was added by gentle mixing with a pipette and incubated for 5–20 min at room temperature. The amine (DOTAP) to phosphate (siRNA) ratio (N/P) was 2:1 in the lipoplexes. The amount of media in the wells of the tissue culture plates was adjusted to 500 μl. Human tear fluid was included when appropriate in the medium to a final $sPLA_2$ type IIA concentration of 100 ng/ml, as measured by ELISA. The lipoplexes or the DPPG:DPPC:DPPE-PEG_{2000} liposomes diluted in serum free medium (100 μl total volume) were added drop wise to the cells, and the complexes were distributed evenly by gently rocking of the plates before incubating the cells for 72 h at 37 °C. Cells were harvested by trypsinization, washed and resuspended in ice-cold PBS + 10% FBS for flow cytometry. All transfections were done in triplicate.

2.7. Flow cytometry analysis

The cells were analyzed on a FACScan flow cytometer (Becton Dickinson, NJ, USA) using the CellQuest Software (Becton Dickinson).

2.8. Confocal laser scanning microscopy

Cells grown on filters were washed with PBS, fixed for 15 min in BD CellFIX™ solution (Becton Dickinson, Denmark) and washed again in PBS. Filters were mounted on cover slips, and confocal imaging was performed on a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, GmBH, Germany), using a Zeiss plan apochromat 63× oil immersion objective. Fluorophores were excited using an argon laser line at 458 nm and a HeNe laser line at 543 nm.

2.9. Statistics

Experiments were performed in triplicate, if not otherwise stated. Values are given as means ± S.D. The statistical significance of the results was determined using a Student's *t*-test. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Characterization of liposomes

The composition and characteristics of the liposome formulations are shown in Table 1. DPPG was included in the lipid bilayer in two different molar ratios. DPPG was applied since the human group IIA $sPLA_2$ has an interfacial preference for anionic interface surfaces and requires anionic charges at the interface in order to attain optimal activity (Bezzine et al., 2002). This can be explained by electrostatic interactions between negatively charged phospholipid head groups and the highly cationic i-face of most $sPLA_2$ enzymes. DPPC was added to the formulations to reduce the amount of negative charge, since negatively charged liposomes have been shown to give problems with complement activation upon i.v. administration (Szebeni et al., 2002). Furthermore, 5% of the lipopolymer DPPE-PEG_{2000} was included as a steric barrier in the formulations to avoid the clearance by the reticulo-endothelial system since this concentration of lipopolymer has been shown to give optimal activation of $sPLA_2$ (Vermehren et al., 1998). The size of the liposomes was in

Table 1
Physicochemical properties of stealth liposomes

Liposome composition	Z_{ave} (nm)[a]	PI[a,b]	Yield/lipid[c] (%)	Liposome associated[c] siRNA (%)	Zeta potential[a] (mV)
DPPG:DPPC:DPPE-PEG_{2000} (77:18:5)	92.7 ± 2.5	0.268 ± 0.007	59.9	7.1	−30.3 ± 1.4
DPPG:DPPC:DPPE-PEG_{2000} (18:77:5)	63.1 ± 0.4	0.090 ± 0.013	78.3	8.8	−20.4 ± 0.5

[a] Values represent mean ± S.D., $n = 3$.
[b] Polydispersity index.
[c] $n = 1$.

the range of 60–90 nm which is appropriate for i.v. administration. The polydispersity indexes of the formulations were low (0.09 and 0.27). The zeta potential of the formulations reflected closely the molar ratio of DPPG included in the bilayer (−20 and −30 mV). The lipid loss during the preparation procedure was 22–40% and was mainly caused by aggregates removed during centrifugation and by lipid loss during the gel filtration. The final particle size and the lipid loss were closely related to the degree of removal of organic solvents: incomplete solvent evaporation resulted in higher lipid loss and larger particle size (results not shown). The siRNA encapsulation efficiency was 7.1% for the DPPG:DPPC:DPPE-PEG_{2000} (77:18:5) liposomes and 8.8% for the DPPG:DPPC:DPPE-PEG_{2000} (18:77:5) liposomes. For comparison, control DPPC:DPPE-PEG_{2000} (95:5) liposomes showed an entrapment efficiency of 13.7% (N.B. results not shown in Table 1). This shows that increasing amounts of anionic lipid decreases the encapsulation efficiency of siRNA, probably due to electrostatic repulsion between the siRNA and the negatively charged lipids. Entrapment efficiencies of 7–9% are fare better than those obtainable by passive encapsulation. However, even higher encapsulation efficiencies are though desirable for expensive molecules like siRNA. The double emulsion preparation procedure was initially optimized with DPPC:DPPE-PEG_{2000} (95:5) liposomes without and with siRNA in the internal aqueous phase until consistent results were obtained (results not shown). DPPG:DPPC:DPPE-PEG_{2000} where then prepared using the same preparation procedure. Results in Table 1 are thus representative for only a single preparation of liposomes due to the high cost of siRNA. However, since the formulations presented in Table 1 showed similar size and lipid yield as the DPPC:DPPE-PEG_{2000} liposomes produced by the optimized procedure, the validity of the results is satisfactory.

3.2. *$sPLA_2$-sensitive liposomes do not enhance the transfection efficiency of siRNA to HeLa-cells stably transfected with EGFP*

To study whether the $sPLA_2$ degradable liposomes could enhance the delivery of siRNA targeting EGFP stably expressed in HeLa-cells, various concentrations of liposomes were used in transfection assays. The effect on EGFP expression by HeLa-cells was measured by flow cytometry. There was no statistically significant effect of the liposome formulations on EGFP-expression at any lipid concentration (100–800 μM), relative to cells incubated with medium alone (Fig. 1). The siRNA concentration in the transfection experiments was 12 nM (DPPG:DPPC:DPPE-PEG_{2000}, 77:18:5) and 7.5 nM (DPPG:DPPC:DPPE-PEG_{2000}, 18:77:5) at the lowest lipid concentration tested (100 μM) which is sufficient for EGFP silencing with cationic transfection reagents (results not shown). The lack of effect was not due to the performance of the cell culture system since siRNA complexes with cationic DOTAP:DOPE liposomes were able to reduce the mean fluorescence intensity of EGFP expressing HeLa-cells with approximately 80% (Fig. 1B). HeLa-cells do not secrete detectable levels of $sPLA_2$ in the cell culture medium under the applied conditions, as measured by ELISA (results not shown). It was therefore tested whether addition of exogenous $sPLA_2$ from human tear fluid to the cell culture medium together with the liposome formulations could induce siRNA-mediated gene silencing. Addition of exogenous $sPLA_2$ did not have any effect on EGFP expression (results not shown). However, for the formulation with the highest molar ratio of DPPG at the highest lipid concentration (400 μM), addition of exogenous $sPLA_2$ resulted in complete cell lysis (results not shown). No visually apparent cytotoxicity was associated with the formulation at lower lipid concentrations, with the other formulation, in the absence of $sPLA_2$ or with the control DOTAP:DOPE liposomes at the applied lipid concentrations (results not shown). DPPG:DPPC:DPPE-PEG_{2000} liposomes are thus not able to enhance the delivery of siRNA neither in the presence nor in the absence of $sPLA_2$ to the RNAi pathway in the cytoplasm of HeLa cells *in vitro*.

3.3. *The biological activity of siRNA is not affected by the preparation procedure*

The double emulsion technique applies rather harsh conditions, such as sonication and exposure to organic solvents. To ensure that the lack of effect of the formulations on EGFP expression was not due to loss of biological activity of the siRNA during the preparation procedure, non-encapsulated siRNA from the gel filtration was tested in transfections of HeLa EGFP-cells using the DOTAP:DOPE liposomes as transfection reagent. Non-encapsulated siRNA was able to silence EGFP expression to a level comparable with the silencing effect of control siRNA (Fig. 2). The inhibition of EGFP expression was specific for the applied siRNA since delivery of a control siRNA targeting TNF-α had no effect on EGFP expression (Fig. 2). Neither siRNA nor DOTAP:DOPE liposomes on their own had any silencing effect on EGFP expression (Fig. 2). The lack of transfection efficiency was therefore not due to inactivation or degradation of the siRNA during the double emulsion preparation procedure.

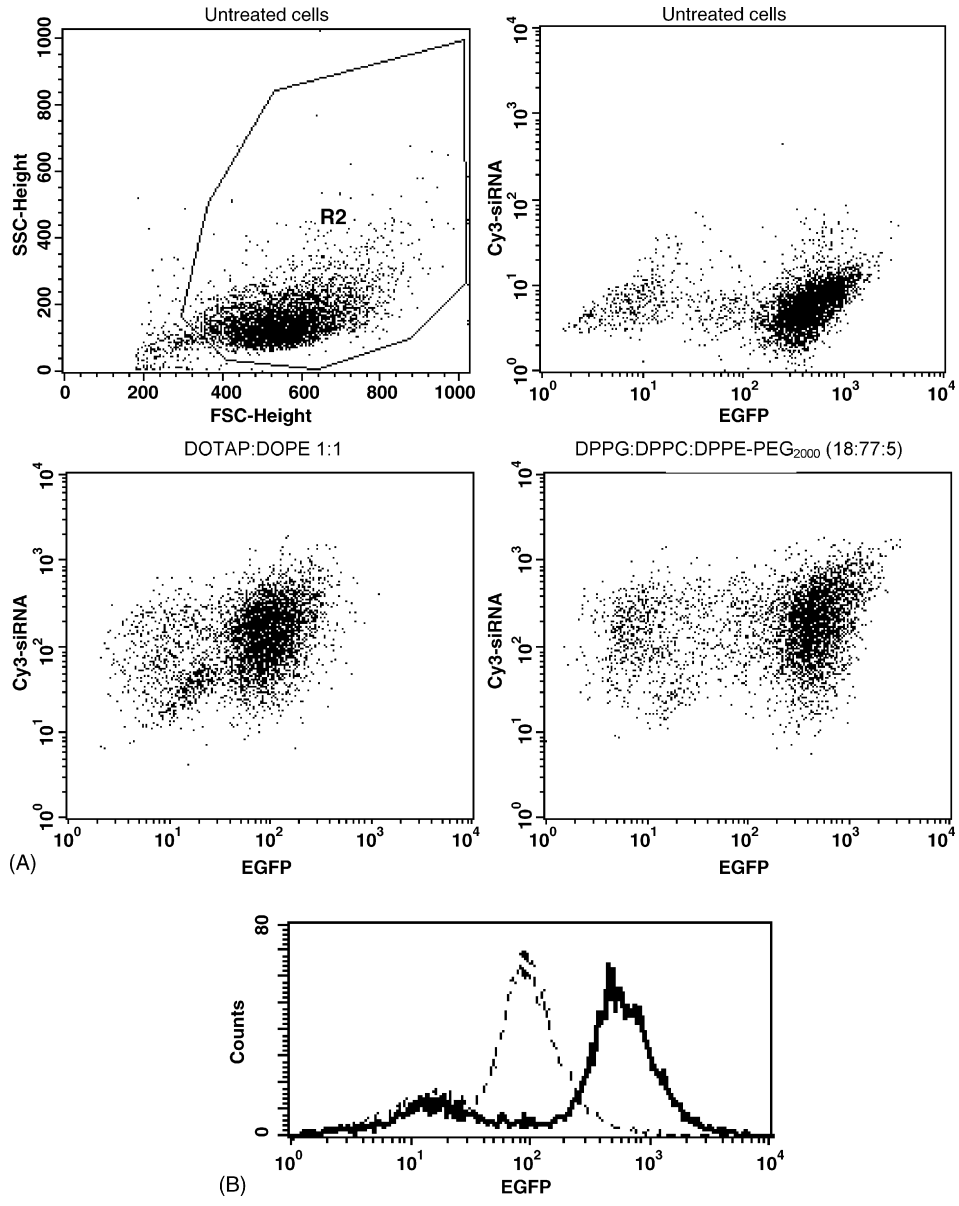

Fig. 1. Representative flow cytometric analysis of HeLa-cells stably expressing EGFP after 72 h of incubation of 5×10^4 cells with DPPG:DPPC:DPPE-PEG_{2000}-Cy3-siRNA liposomes (18:77:5) (400 μM lipid, 31 nM Cy3-siRNA). As positive control was used DOTAP:DOPE liposomes at an amine (DOTAP) to phosphate (siRNA) ratio (N/P) of 2:1. As negative control complexes of DOTAP:DOPE liposomes and Cy3-siRNA targeting TNF-α were used in the same concentrations as for the positive control. (A) Dot plots showing immunofluorescent cells of gated populations of HeLa-cells stably expressing EGFP. Upper left: forward scatter/side scatter plot of untreated cells. The gate used is shown (R1). Upper right: untreated cells. Lower left: incubation with DOTAP:DOPE + Cy3-siRNA (positive control). Lower right: incubation with DPPG:DPPC:DPPE-PEG_{2000} liposomes. (B) Histogram showing down-regulation of EGFP-expression by DOTAP:DOPE-Cy3-siRNA complexes (- - -). Control: incubation with medium (—).

Polyacrylamide gel electrophoresis studies of non-encapsulated siRNA have confirmed this observation as there were no signs of siRNA degradation (results not shown).

3.4. Cy3-siRNA from the liposome formulations are taken up by HeLa EGFP-cells in a concentration dependent manner that can be enhanced by exogenous $sPLA_2$

In order to study whether the lack of silencing effect could be explained by poor cellular uptake of siRNA, HeLa-cells were incubated with various concentrations of liposomes encapsulating Cy3-labelled siRNA in the absence or presence of exogenous $sPLA_2$ and the cell-associated Cy3 fluorescence was measured by flow cytometry. Surprisingly, preliminary results showed that encapsulation of siRNA into DPPG:DPPC:DPPE-PEG_{2000} liposomes increased the uptake of Cy3-fluorescence in HeLa-cells in a concentration-dependent manner that could be enhanced by exogenous $sPLA_2$ (Fig. 3). The effect of $sPLA_2$ addition was most pronounced for the formulation with the highest DPPG concentration (Fig. 3). Other phospholipases produced by HeLa-cells may be involved in the degradation of the liposomes. These results suggest that the lack of silencing effect is not due to lack of cellular uptake of the Cy3-labelled siRNA, although data should be confirmed using shorter incubation periods in order

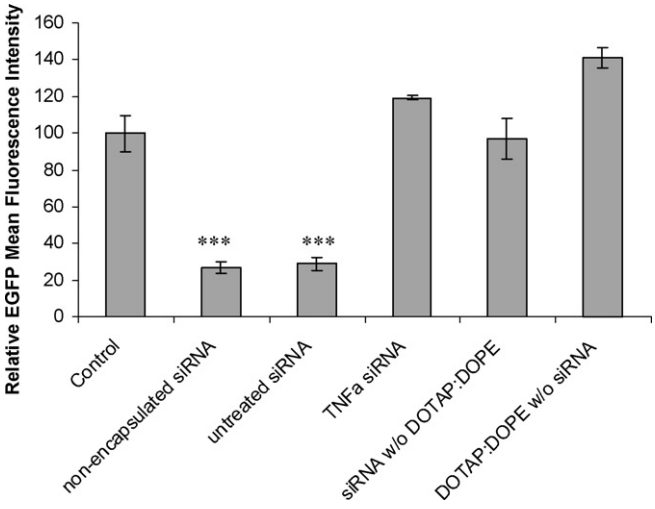

Fig. 2. Relative mean fluorescence intensity (MFI) of HeLa EGFP transfected with lipoplexes of non-encapsulated siRNA from the preparation of DPPG:DPPC:DPPE-PEG_{2000}-siRNA liposomes (77:18:5) and DOTAP:DOPE liposomes (column 2). MFI was normalized to MFI of untreated cells (set to 100%, column 1). For comparison, relative MFI is shown for cells treated with lipoplexes of DOTAP:DOPE and positive control EGFP siRNA (column 3), lipoplexes of DOTAP:DOPE and negative control TNF-α siRNA (column 4), siRNA alone (column 5) and DOTAP:DOPE liposomes alone (column 6) ($n = 3$, average ± S.D.). $^{***}P < 0.001$.

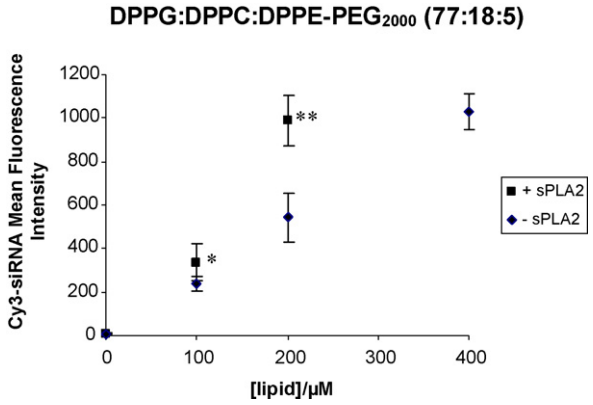

Fig. 3. Cy3-siRNA MFI of HeLa EGFP-cells transfected with various concentrations of DPPG:DPPC:DPPE-PEG_{2000}-siRNA liposomes (77:18:5). The lipid concentration is indicated on the x-axis. Data are represented from cell cultures incubated without the addition of exogenous $sPLA_2$ to the medium and from cell cultures incubated with the addition of 100 ng/ml exogenous $sPLA_2$ from human tear fluid to the medium ($n = 3$, average ± S.D.). Incubation with 400 μM lipid in the presence of exogenous $sPLA_2$ led to complete cell lysis and is thus not reported. $^{*}P < 0.05$ and $^{**}P < 0.01$.

to ensure that Cy3-fluorescence co-localize with siRNA. The $sPLA_2$ degradable, lipopolymer-coated liposomes are thus able to mediate the cellular uptake of siRNA even in the absence of any active targeting moieties coupled to the distal ends of the lipopolymer, such as antibodies or small-molecule ligands. The mechanism by which the $sPLA_2$ degradable liposomes enhance cellular uptake of siRNA is currently unknown, but it is likely that the generated lysolipids and free fatty acids play an important role as membrane permeability enhancers for nucleic acids, as shown for hexadecylphosphocholine (Settelen et al., 2004).

3.5. Cy3 is localized in vesicular compartments

The intracellular localization of the Cy3-labelled siRNA in the EGFP-expressing HeLa-cells was examined by confocal microscopy. A strong Cy3 fluorescent signal was present in vesicular compartments inside the HeLa-cells (Fig. 4). The nature of these vesicular compartments is not known at present, but might be identical to endosomes or lysosomes. These results thus show that siRNA from the liposomal formulations is taken up by the HeLa-cells into vesicular compartments in a way that can be augmented by addition of $sPLA_2$ but that siRNA cannot enter the RNAi pathway in the cytoplasm, probably due to lack of endosomal/lysosomal escape. The enzymatic hydrolysis is expected to occur fast. However, the catalytic capacity of the enzyme could be rate-limiting for hydrolysis of the liposomes since a large molar excess of lipids compared to enzyme is added to the cell cultures. Concomitant with the enzymatic reaction, we believe that the cells take up the intact liposomes. The uptake of fluorescently labeled, empty $sPLA_2$-sensitive liposomes has been examined by confocal microscopy and we found a rapid and substantial uptake into vesicular compartments already after 1 h (results not shown). This indicates that it is likely that intact, siRNA-encapsulated liposomes are taken up by cells shortly after addition of liposomes. We are currently addressing issues,

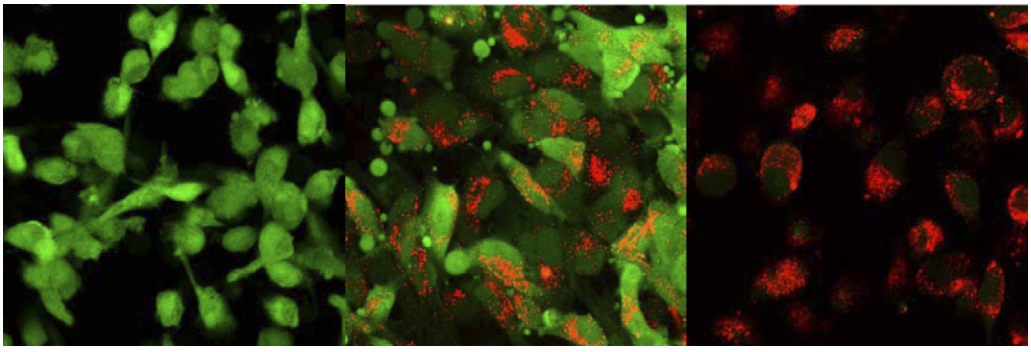

Fig. 4. Two-color immunofluorescence of HeLa EGFP-cells (green) transfected for 72 h with Cy3-EGFP siRNA (red) encapsulated into DPPG:DPPC:DPPE-PEG_{2000} liposomes (77:18:5) (200 μM lipid, 24 nM Cy3-siRNA) with exogenous $sPLA_2$ present in the medium (middle) or complexed with DOTAP:DOPE liposomes (right). Non-transfected cells (left) are shown for comparison. Visualisation was done by CLSM. Background fluorescence levels were adjusted on cells incubated with naked Cy3-EGFP siRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

such as the nature of the vesicular compartments, the detailed kinetics of siRNA/liposome uptake, the stability of the formulations and the siRNA, the release of siRNA from the lipid carrier, the use of various strategies for enhancing the endosomal escape of siRNA and the effect of the formulations in cell types responsible for the over-expression of TNF-α in RA. Future studies will show whether the formulations are useful for the targeted, $sPLA_2$-triggered silencing of TNF-α expression in joints of mice with collagen-induced arthritis.

4. Conclusion

In conclusion, our results showed that siRNA can be encapsulated into $sPLA_2$ degradable liposomes with entrapment efficiencies up to 7–9%. siRNA from the liposomal formulations is taken up by HeLa-cells in a concentration-dependent manner in a way that can be augmented by addition of $sPLA_2$ and siRNA is localized in vesicular compartments. The formulations are not associated with apparent toxicities even at high lipid concentrations and no active targeting moieties are needed for cellular uptake. However, the $sPLA_2$ degradable siRNA formulations do not silence EGFP espression in HeLa-cells. We suggest that siRNA cannot enter the RNAi pathway in the cytoplasm, probably due to lack of endosomal/lysosomal escape.

Acknowledgements

This work was supported by a grant from the Danish Research Council for Technology and Production Sciences, the siRNA Delivery Centre (C.F.). For excellent technical assistance we thank Maria Læssøe Petersen, Bettina Dinitzen and Susanne Nørskov Sørensen.

References

Andresen, T.L., Jensen, S.S., Jorgensen, K., 2005. Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release. Prog. Lipid Res. 44, 68–97.

Bezzine, S., Bollinger, J.G., Singer, A.G., Veatch, S.L., Keller, S.L., Gelb, M.H., 2002. On the binding preference of human groups IIA and X phospholipases A(2) for membranes with anionic phospholipids. J. Biol. Chem. 277, 48523–48534.

Davidsen, J., Vermehren, C., Frokjaer, S., Mouritsen, O.G., Jorgensen, K., 2001. Enzymatic degradation of polymer covered SOPC-liposomes in relation to drug delivery. Adv. Colloid Interface Sci. 89–90, 303–311.

Davidsen, J., Jorgensen, K., Andresen, T.L., Mouritsen, O.G., 2003. Secreted phospholipase A2 as a new enzymatic trigger mechanism for localised liposomal drug release and absorption in diseased tissue. Biochim. Biophys. Acta Biomembr. 1609, 95–101.

Dykxhoorn, D.M., Lieberman, J., 2005. The silent revolution: RNA interference as basic biology, research tool, and therapeutic. Annu. Rev. Med. 56, 401–423.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Gotfredsen, C.F., Frokjaer, S., Hjorth, E.X., Jorgensen, K.D., Debroux-Guisset, M.C., 1983. Disposition of intact liposomes of different compositions and of liposomal degradation products. Biochem. Pharmacol. 32, 3381–3387.

Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R., 1985. Production of large unilamellar vesicles by a rapid extrusion procedure—characterization of size distribution, trapped volume and ability to maintain a membrane-potential. Biochim. Biophys. Acta 812, 55–65.

Inoue, A., Takahashi, K.A., Mazda, O., Terauchi, R., Arai, Y., Kishida, T., Shin-Ya, M., Asada, H., Morihara, T., Tonomura, H., 2005. Electro-transfer of small interfering RNA ameliorated arthritis in rats. Biochem. Biophys. Res. Commun. 336, 903–908.

Jorgensen, K., Davidsen, J., Mouritsen, O.G., 2002. Biophysical mechanisms of phospholipase A2 activation and their use in liposome-based drug delivery. FEBS Lett. 531, 23–27.

Lawrence, R.C., Helmick, C.G., Arnett, F.C., Deyo, R.A., Felson, D.T., Giannini, E.H., Heyse, S.P., Hirsch, R., Hochberg, M.C., Hunder, G.G., Liang, M.H., Pillemer, S.R., Steen, V.D., Wolfe, F., 1998. Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. Arthritis Rheum. 41, 778–799.

Leung, R.K., Whittaker, P.A., 2005. RNA interference: from gene silencing to gene-specific therapeutics. Pharmacol. Ther. 107, 222–239.

Mahato, R.I., Anwer, K., Tagliaferri, F., Meaney, G., Leonard, P., Wadhwa, M.S., Logan, M., French, M., Rolland, A., 1998. Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. Hum. Gene Ther. 9, 2083–2099.

Medina-Kauwe, L.K., Xie, J., Hamm-Alvarez, S., 2005. Intracellular trafficking of nonviral vectors. Gene Ther. 12, 1734–1751.

Menschikowski, M., Hagelgans, A., Siegert, G., 2006. Secretory phospholipase A2 of group IIA: is it an offensive or a defensive player during atherosclerosis and other inflammatory diseases? Prostaglandins Other Lipid Mediat. 79, 1–33.

Paulos, C.M., Turk, M.J., Breur, G.J., Low, P.S., 2004. Folate receptor-mediated targeting of therapeutic and imaging agents to activated macrophages in rheumatoid arthritis. Adv. Drug. Deliv. Rev. 56, 1205–1217.

Schiffelers, R.M., Woodle, M.C., Scaria, P., 2004. Pharmaceutical prospects for RNA interference. Pharm. Res. 21, 1–7.

Seilhamer, J.J., Pruzanski, W., Vadas, P., Plant, S., Miller, J.A., Kloss, J., Johnson, L.K., 1989. Cloning and recombinant expression of phospholipase A2 present in rheumatoid arthritic synovial fluid. J. Biol. Chem. 264, 5335–5338.

Settelen, N., Roch, O., Bock, D., Rooke, R., Braun, S., Meyer, O., 2004. Controlled plasmid gene transfer to murine renal carcinoma by hexadecylphosphocholine. J. Control. Release 94, 237–244.

Storm, G., Crommelin, D.J.A., 1998. Liposomes: quo vadis? Pharm. Sci. Technol. Today 1, 19–31.

Szebeni, J., Baranyi, L., Savay, S., Milosevits, J., Bunger, R., Laverman, P., Metselaar, J.M., Storm, G., Chanan-Khan, A., Liebes, L., Muggia, F.M., Cohen, R., Barenholz, Y., Alving, C.R., 2002. Role of complement activation in hypersensitivity reactions to doxil and HYNICPEG liposomes: experimental and clinical studies. J. Liposome Res. 12, 165–172.

Vermehren, C., Kiebler, T., Hylander, I., Callisen, T.H., Jorgensen, K., 1998. Increase in phospholipase A2 activity towards lipopolymer-containing liposomes. Biochim. Biophys. Acta 1373, 27–36.

Zimmermann, T.S., Lee, A.C.H., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M.N., Harborth, J., Heyes, J.A., Jeffs, L.B., John, M., Judge, A.D., Lam, K., McClintock, K., Nechev, L.V., Palmer, L.R., Racie, T., Rohl, I., Seiffert, S., Shanmugam, S., Sood, V., Soutschek, J., Toudjarska, I., Wheat, A.J., Yaworski, E., Zedalis, W., Koteliansky, V., Manoharan, M., Vornlocher, H.P., MacLachlan, I., 2006. RNAi-mediated gene silencing in non-human primates. Nature 441, 111–114.