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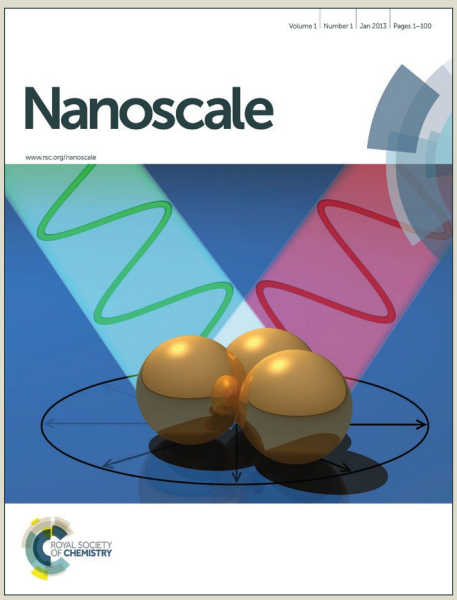





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# Surface coating of siRNA-peptidomimetic nano-self-assemblies with anionic lipid bilayers: Enhanced gene silencing and reduced adverse effects *in vitro*

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Electronic Supplementary Information (ESI) available: non-fusogenic liposomes; cytotoxicity of naked siRNA and the empty vector; immunogenicity; low-magnification images; DOPE/DPPC liposomes. See DOI: 10.1039/x0xx00000x

Cationic vectors have demonstrated the potential to facilitate intracellular delivery of therapeutic oligonucleotides. However, enhanced transfection efficiency is usually associated with adverse effects, which also proves to be a challenge for vectors based on cationic peptides. In this study a series of proteolytically stable palmitoylated α-peptide/β-peptoid peptidomimetics with systematically varied number of repeating lysine and homoarginine residues was shown to self-assemble with small interfering RNA (siRNA). The resulting well-defined nanocomplexes were coated with anionic lipids giving rise to net anionic liposomes. These complexes and the corresponding liposomes were optimized towards efficient gene silencing and low adverse effects. The optimal anionic liposomes mediated high silencing effect, which was comparable to that of the control cationic Lipofectamine 2000, and did not display any noticeable cytotoxicity and immunogenicity *in vitro*. In contrast, the corresponding nanocomplexes mediated a reduced silencing effect with a more narrow safety window. The surface coating with anionic lipid bilayers led to partial decomplexation of the siRNA-peptidomimetic nanocomplex core of the liposomes, which facilitated siRNA release. Additionally, the optimal anionic liposomes showed efficient intracellular uptake and endosomal escape. Therefore, these findings suggest that a more efficacious and safe formulation can be achieved by surface coating of the siRNA-peptidomimetic nano-self-assemblies with anionic lipid bilayers.

## Introduction

In the ongoing search for effective vectors for siRNA delivery, many synthetic cationic carriers including polymers, lipids and peptides show high transfection efficiency, but the implementation of these vectors has been hampered by potential toxicity.[1] Various reports demonstrate that polycations such as polylysine, poly(ethylene imine) and poly(amido amine) display direct cellular toxicity, and they might also trigger complement activation.[2] In addition, interactions between cationic nanoparticles and the *in vivo* environment may potentially induce aggregation, suboptimal biodistribution and immunological reactions at the systemic level.[3] Compared to cationic carriers, anionic siRNA nanoparticles have offered promising evidence of efficacy without significant toxicity.[4] Therefore, it is desirable to engineer and further improve the design of siRNA carriers by exploring promising anionic delivery agents.

We previously synthesized and characterized a library of proteolytically stable α-peptide/β-peptoid peptidomimetics with promising membrane-destabilizing and cell-penetrating properties that we envisage can be exploited for siRNA delivery.[5-8] The molecular structure of these peptidomimetics is based on an alternating hydrophobic/cationic design consisting of N-alkylated β-alanine (β-peptoid) units and α-amino acid units (homoarginine and lysine). This design is believed to benefit from the hydrophobicity of the benzyl side chains of the β-peptoid residues and the cationic charge and hydrogen bond-forming capability of the cationic α-amino acid residues. Such sequences show superior membrane-destabilizing properties and cellular uptake over that of octa-arginine (R8), and the altered peptoid backbone design confers proteolytic stability.[6] Hence, it is hypothesized that these peptidomimetics may facilitate delivery of siRNA across cell membranes. However, their high positive charge density potentially constitutes a toxicity risk for *in vivo* applications, and thus there is a need for formulating efficient siRNA vectors with lower adverse effects. To address this challenge we complexed palmitoylated versions of the peptidomimetics comprising a varied number of cationic charges with siRNA into well-defined nano-self-assemblies. The resulting complexes were subsequently coated with a net anionic binary lipid mixture to mask the cationic charge resulting in the formation of net anionic liposomes. The palmitoylation was introduced to increase the colloidal stability of the complexes in physiologically relevant media via hydrophobic interactions.

Although comprehensive research efforts towards developing cationic lipid formulations for siRNA delivery has resulted in improvement of protein knockdown, an increasing number of reports have demonstrated that many cationic lipid formulations elicit undesired immune responses.[9] Alternative liposomal delivery vectors with reduced immunogenicity and toxicity are therefore needed. In some reports, anionic lipid-based formulations have been utilized for effective DNA and siRNA transfection.[10-14] The group of H. Harashima introduced an anionic lipid envelope structure, and multifunctional nanodevices were engineered based on this delivery approach.[15] However, the application of this lipid vector, in combination with new cationic peptides, and its impact on the efficiency and safety of siRNA delivery remains to be defined. We hypothesize that we might improve the delivery of siRNA and reduce adverse effects via three different means; i) by the use of peptidomimetics with an appropriate cationic charge density for siRNA complexation; ii) by stabilizing these complexes via addition of a hydrophobic acyl moiety to the peptidomimetics; and iii) by subsequent coating of the complexes with anionic lipid membrane bilayers.

In the present work, novel formulations were designed for siRNA delivery using the peptidomimetics as complexing agents in anionic liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS). Thus, siRNA directed against enhanced green fluorescent protein (EGFP) was complexed with a library of palmitoylated α-peptide/β-peptoid oligomers with systematically varied charge density, and subsequently the complexes were coated with anionic membrane bilayers for safe and efficient siRNA delivery. The physicochemical properties of both the complexes and the liposomes were characterized with respect to size, charge and encapsulation efficiency. The gene silencing efficiency, toxicity and immunogenicity were evaluated in vitro. Furthermore, we examined the cellular association, intracellular uptake, subcellular localization and endosomal escape of the EGFP siRNA.

## Materials and methods

**Materials.** Palmitoylated α-peptide/β-peptoid peptidomimetics (Fig. 1A) were synthesized as previously described.[4] DOPE and CHEMS were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 2'-O-Methyl modified dicer substrate asymmetric duplex siRNAs directed against EGFP and negative control firefly luciferase (FLuc) were supplied by Integrated DNA Technologies (IDT, Coralville, IA, USA). The siRNAs had the following sequences and modifications: EGFP, sense 5'-pACCCUGAAGUUCAUCUGCACCACcg-3'; antisense 5'-CGGUGGUGCAGAUGAACUUCAGGGUCA -3'; FLuc sense 5'-

pGGUUCCUGGAACAAUUGCUUUUAca-3' and antisense 5'- UGUAAAAGCAAUUGUUCCAGGAACCAG-3', where lower-case letters represent deoxyribonucleotides, underlined capital letters represent 2´-O-methylribonucleotides, and p represents a phosphate residue.[16] EGFP siRNA labelled with Alexa488 was obtained from IDT. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was provided by Promega (Madison, WI, USA). Phenazine methosulfate (PMS), heparin (MW 14,000 Da) and octyl-β-D-glucopyranoside were purchased from Sigma–Aldrich (St. Louis, MO, USA). Quant-iT™ RiboGreen RNA Reagent and Lipofectamine 2000 (Lip2k) were purchased from Invitrogen (Paisley, UK). Throughout all experiments, RNase-free materials and conditions were carefully applied.

**Formulation of siRNA-loaded nanoparticles and characterization.** Complexes of siRNA and the palmitoylated peptidomimetics (1-4 repeating units, Fig. 1A) were prepared at an N/P ratio of 2: Solutions containing the required amount of the different peptidomimetics were made in 5 mM HEPES buffer, pH = 7.4. A siRNA solution (1.5 µM/27 µg/mL final concentration) was added dropwise to the peptidomimetic solutions while vortexing to facilitate the self-assembly process, and the complexes were then incubated for 30 min at room temperature (rt). The complexes were subsequently coated with lipids by using the lipid film hydration method.[17] In brief, a total amount of 137.5 nmol of DOPE and CHEMS at a molar ratio of 9:2 was dissolved in 250 µL of chloroform, which was then evaporated resulting in the formation of a thin lipid film. A volume of 250 µL of the siRNA-peptidomimetic complex suspension was used to hydrate the film for 10 min while vortexing every min, and the mixture was subsequently sonicated for 1 min using a Branson 2510 bath sonicator (Danbury, CT, USA). The final lipid concentration was 0.55 mM. The liposome mixture was passed through an Econo column (Bio-Rad, Hercules, CA, USA) filled with Sepharose CL-4B matrix (GE Healthcare) to separate non-encapsulated siRNA from the liposomes. The particle size (diameter) and polydispersity index (PDI) of the complexes and the liposomes were determined by dynamic light scattering (DLS), and the surface charge of the particles was measured by analysis of the zeta potential using a Nanosizer NanoZS (Malvern Instruments, Worcestershire, UK) as previously described.[18] A volume of 30 µL siRNA-loaded formulation, diluted 30 times in MilliQ water, was used for the NanoZS measurements per sample, and all samples were measured in triplicate. For measurement of the encapsulation efficiency, complexes and liposomes were dissociated by adding heparin/detergent solution (1 mg/mL heparin and 1 mM octyl-β-D-glucopyranoside in TE buffer). The samples were subsequently diluted and the siRNA was quantified by using the RiboGreen® RNA reagent according to the manufacturer's instructions. The encapsulation efficiency was calculated using the following equation:[18]

$$\text{Encapsulation efficiency} = \frac{\text{actual siRNA loading}}{\text{theorectical siRNA loading}} \times 100\,\%$$

As positive control, Lip2k was complexed with siRNA according to the manufacturer's instructions.

**Cell culture.** The human non-small cell lung carcinoma cell line H1299 (ATCC, Manassas, VA, USA) was maintained in RPMI 1640 medium (Fisher Scientific, Waltham, MA, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all from Sigma-Aldrich), and 10% (v/v) fetal bovine serum (FBS). H1299 cells stably expressing EGFP (H1299 EGFP) were used as reported previously,[16] and the cells were cultured in the same medium supplemented with 0.2 mg/mL geneticin (Invitrogen, Carlsbad, CA, USA). The cells were grown in an atmosphere of 5% CO2/95% O2 at 37 °C changing the growth medium three times a week and sub-cultured twice a week.

**Determination of silencing efficiency *in vitro*.** The H1299 EGFP cells were seeded in 12-well plates at a density of 100,000 cells per well 24 h prior to the experiment. On the following day, the cells were transfected with the nanoparticles loaded with EGFP siRNA or FLuc siRNA, respectively, in serum-free RPMI medium.[16] After 48 h, the cells were washed with phosphate-buffered saline (PBS), trypsinised and resuspended in 300 µL PBS prior to flow cytometric analysis. A total number of 10,000 cells were measured per sample using a Gallios flow cytometer (Beckman Coulter, Fullerton, CA, USA), and the data were analysed using the FlowJo software (Tree Star Inc., Ashland, OR, USA). For each condition, three separate samples were analysed. The percentage of gene silencing was calculated by using the FL-1 mean (mean of the EGFP fluorescence histogram). The cells transfected with nanoparticles loaded with FLuc siRNA were used as negative control.

**Viability assay.** The effect of the peptidomimetics, the complexes and the liposomes, respectively, on the viability of H1299 cells was determined using the MTS/PMS assay as previously described.[18] H1299 cells were seeded at a density of 9,000 cells per well in 96-well plates and cultured for 24 h. The cells were then exposed to solutions of peptidomimetics or dispersions of particles in medium at different concentrations for 24 h at 37 °C. Cells incubated with sodium dodecyl sulfate (SDS) 0.2% (w/v) were used as a positive control, while untreated cells were used as negative control. After incubation, the cells were washed with PBS, and 100 µL of freshly prepared MTS/PMS reagent in HBSS was added to each

well followed by incubation at 37 °C for 1 h. The cell viability was determined by measuring the absorbance of enzymatically formed formazan at 492 nm.

**Toll-like receptor activation assay.** Toll-like receptor (TLR) reporter cell lines (HEK-BlueTM-hTLR2, -hTLR3, -hTLR4, -hTLR7, and -hTLR9 reporter cells) were cultured as instructed by the manufacturer (Invivogen, Toulouse, France). The TLR reporter cell lines were stimulated with the peptidomimetics at concentrations of 0.5 μM, or 5 μM, with the complexes and the liposomes at siRNA concentrations of 50 nM, or with the solvent alone, in a total volume of 100 μL for 20 h at 37 °C. As positive controls, the following agonists were used: PAM3SCK (100 ng/mL) for TLR2, polyinosinic-polycytidylic acid [poly(I:C)] (5 μg/mL) for TLR3, lipopolysaccharide (LPS)-EK (10 ng/mL) for TLR4, CL264 (5 μg/mL) for TLR7 and ODN2006 (10 μg/mL) for TLR9 (all from Invivogen). To detect the reporter protein secreted alkaline phosphatase (SEAP), 20 μL of the supernatant was added to 180 μL of QUANTI-BlueTM substrate (Invivogen) and incubated for 1 h at 37 °C. Levels of SEAP were determined by measuring the absorbance using a microplate reader at 650 nm. Relative SEAP levels were defined as the sample level divided by the solvent control level.

**Dendritic cell maturation assay.** Femurs and tibia of adult CB6F1/CrL mice (6-8 weeks) were flushed with culture medium (Iscove's Modified Dulbecco's Medium) supplemented with 5% (v/v) fetal bovine serum (Lonza, Verviers, Belgium), 50 μM 2-mercaptoethanol (Sigma-Aldrich), penicillin and streptomycin] and the cells were seeded in 12-well plates, at a concentration of 450,000 cells in 1 mL culture medium (adapted from Lutz *et al.* 1999).[20] Dendritic cells were expanded with 20 ng/nL murine recombinant granulocyte macrophage colony stimulating factor (rGM-CSF) (Cytogen, The Netherlands). On day 2, the volume of complete growth medium volume was doubled and on day 5, additional 20 ng/mL rGM-CSF was added. On day 7, bone marrow dendritic cells were stimulated with PBS (1:250), LPS (10 ng/mL) or the peptidomimetics (0.5, or 5 μM) or the complexes and the liposomes at siRNA concentrations of 50 nM (all stimuli buffered to 10 mM HEPES) for 16 h at 37 °C in a humidified $CO_2$ incubator. Staining of surface markers with the indicated antibodies was performed in the presence of Fc block (2.4 G2) for 30 min on ice. Anti-CD11c (N418) and I-Ad/I-Ed (M5/114) were purchased from eBioscience (San Diego, CA, USA), and anti-CD40 (3/23) and -CD86 (GL1) were obtained from BD Biosciences (Breda, the Netherlands). Samples were measured on a FACSCantoII (BD Biosciences, San Jose, CA, USA) and analysed using the FlowJo software. Ethical approval for the mouse experiment was obtained from the Animal Experiment Committee of Utrecht University, The Netherlands.

**Evaluation of morphology.** The morphology of the complexes and the liposomes was determined by transmission electron microscopy (TEM).[18] After negative staining with 2% (w/v) uranyl acetate in water, the particles were visualized by using a Philips CM100 TWIN TEM (Philips, Eindhoven, The Netherlands). Photographs were recorded with a side-mounted Olympus Veleta camera. Further morphological evaluation of the liposomes was carried out by cryo-TEM using a Tecnai G2 20 TWIN instrument (FEI Inc., Hillsboro, OR, USA).[21]

**Heparin decomplexation assay.** To evaluate the binding ability of the siRNA- peptidomimetic in complexes and liposomes, a heparin decomplexation assay was performed as previously described.[22] Complexes and liposomes were freshly prepared as described above. The complexes were equally distributed over the wells (100 μL/well) of a black, flat-bottomed 96-well plate. To each well, 100 μL of Ribogreen solution (in TE buffer, pH 7.4) was added and incubated for 10 min in the dark at rt. A serial dilution of heparin in RNase-free water containing octyl-β-D-glucopyranoside (1 mM) was prepared and 100 μL of each dilution was added followed by incubation for 20 min in the dark at rt. Naked siRNA treated in a similar way as the nanoparticles and nanoparticles without heparin were included as positive and negative controls, respectively. The decomplexed siRNA was quantified by using the RiboGreen® assay as described above. For comparison, the half-maximal effective concentration ($EC_{50}$) of heparin decomplexation was analysed.

**Release of siRNA.** The release of siRNA was measured in TE buffer using complexes and liposomes, respectively, loaded with EGFP siRNA. Briefly, 20 μL of nanoparticle dispersion was suspended in 1 mL TE buffer in RNase-free Eppendorf tubes and shaken in a water bath (50 rpm) at 37 °C. At given times, samples were taken and analysed in triplicates. The released siRNA was quantified by using the Ribogreen assay as described above.[16]

**Cell association of siRNA determined by flow cytometry.** The cellular uptake of the nanoparticles was quantified by using flow cytometry. The H1299 cells were seeded in 12-well plates ($1.5\times10^5$ cells/well) 24 h before the experiment, which was initiated by incubating the cells with nanoparticles at a concentration of 100 nM Alexa 488-labelled siRNA in serum-free medium at 37 °C for 4 h. Cells were rinsed twice with PBS, trypsinised and diluted with medium. After washing and centrifugation, the cell pellet was resuspended in 200 μL PBS and analysed by flow cytometry as described above.

**Intracellular siRNA determined by stem-loop qPCR.** RNA isolation and purification were performed as previously described.[16] The H1299 EGFP cells were washed with PBS and trypsinised at 48 h post-transfection. The sequences of the stem-loop reverse transcription primers and the PCR primers are as described by Colombo *et al.*[16] Briefly, a purified RNA amount of 700 ng was reverse transcribed in a total reaction volume of 20 μL that also included 500 μM deoxynucleotide

mix, 20 U Protector RNase Inhibitor, 10 U Transcriptor Reverse Transcriptase, and 1× Transcriptor Reverse Transcriptase buffer (all from Roche, Basel, Switzerland). The RNA template was first denatured and immediately cooled on ice. The reaction mix and the stem-loop primers (11 nM of each) were then added during the cooling phase. The pulsed RT program consisted of 15 min at 14 °C, 10 min at 42 °C, followed by 25 cycles (15 s at 14 °C, 10 s at 42 °C and 15 s at 65 °C). Subsequently, the mixture was incubated for 5 min at 85 °C for transcriptase inactivation and cooled at 4 °C. The qPCR was performed with a LightCycler 480® (Roche) using the SYBR Green Master mix according to the manufacturer's instructions. An amount of 5 ng DNA was analyzed. The housekeeping gene snoRNA U109 (GenBank ID: AM055742.1) was used for normalization. The PCR program was 95 °C for 5 min, 37 cycles (95 °C for 15 s, 62 °C for 15 s, 72 °C for 1 s) followed by cooling at 4 °C. The PCR data analyses were performed as previously described.[15]

**Intracellular trafficking of siRNA.** The H1299 cells were seeded in 12-well plates ($10^5$ cells/well) containing a cover slip. After 24 h, the cells were incubated for 12 h with the nanoparticles loaded with Alexa488-labelled siRNA (at 100 nM), then rinsed three times with PBS and incubated for 1 h with Lysotracker DND99 (500 nM, Molecular Probes) to stain the late endosomes and lysosomes. After washing with PBS, the cells were fixed with fresh paraformaldehyde (3% in PBS). In order to stain the nuclei, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI, 0.1 µg/mL) for 5 min and rinsed three times with PBS. After washing, the cover slips were placed on a slide using the Vectashield® mounting medium. The slides were imaged using a Zeiss LSM 710 confocal laser scanning microscope equipped with a Zen2010 module (Carl Zeiss, Jena, Germany).

**Statistics.** Values are given as mean values with standard deviations (SD), n = 3. For the statistics and plotting, PRISM (GraphPad, La Jolla, CA, USA) was used. Statistically significant differences were assessed by an analysis of variance (ANOVA) at a 0.05 significance level and followed by Tukey's post test.

## Results and discussion

**Preparation and physicochemical characteristics of complexes and liposomes**

All nanoparticle dispersions prepared in this study are referred to as Pn-C and Pn-L, where Pn, C and L denote *(i)* the number of sequence repeats in the peptidomimetics, *(ii)* the complexes, and *(iii)* the liposomes, respectively. The sequence length of the α-peptide/β-peptoid oligomers was varied from one to four repeats (i.e. P1 to P4, Fig. 1A), and their net positive charge was 2, 4, 6, and 8, respectively. The complexes were generally formed via electrostatic interactions between the polycationic peptidomimetics and the highly anionic siRNA, combined with hydrophobic interactions between the palmitoyl chains and probably also the benzyl groups of the peptides. Complexes were formed by mixing the peptidomimetics with siRNA at a specific N/P ratio, which can be expressed as the charge ratio between the peptide nitrogen and the siRNA phosphate groups. An N/P ratio of 2 was chosen for this study because it resulted in small complexes with a low PDI. All peptidomimetics effectively self-assembled with EGFP siRNA to yield complexes of 90-180 nm in diameter (Fig. 1B). The complexes were further encapsulated with a lipid membrane bilayer composed of neutral DOPE and anionic CHEMS. The Harashima group has successfully applied these lipids to deliver plasmid DNA, and suggested that this packaging mechanism was based on electrostatic interactions between DNA, R8 and lipids.[16] We chose a molar ratio of 9:2 between DOPE and CHEMS, as it has been found that this lipid ratio is required for introducing sufficient negative charge to coat the complexes and shield their net positive charge. Expectedly, the diameter of the liposomes (250-400 nm) was larger than the diameter of the complexes (Fig. 1B), and the zeta potential of all complex particles became negatively charged as a result of the lipid coating (Fig. 1C), indicating that the complexes were in fact coated with a lipid membrane. Also, the zeta potential of the complexes and the liposomes increased with the longer peptidomimetics, and this correlates well with the increased number of cationic charges.

When anionic siRNA was complexed with the cationic peptidomimetics, all complexes showed high encapsulation efficiency (above 98%, Fig. 1C). However, lower encapsulation efficiency (73 %) was observed for P1-L (Fig. 1C), only displaying two cationic charges in the peptidomimetics, which is apparently insufficient for forming a compact particle core. Reduced encapsulation efficiency was also found for P4-L liposomes (63 %). A possible explanation could be that anionic lipid may interact more strongly with the P4 containing the highest number of cationic charges than with P2 and P3 and influence the assembly of the complex. P2-L and P3-L showed higher encapsulation efficiency (above 80%) verifying the necessity of optimizing the charge density for these cationic peptidomimetics-based formulations.

**Silencing effect *in vitro***

The siRNA silencing effect of the complexes and the liposomes were tested in H1299 EGFP cells (Fig. 2). In general, coating with anionic lipid significantly increased the transfection efficiency, as compared to the corresponding complexes at 100 nM siRNA (N/P = 2, Fig. 2A). In addition, P3-L and P4-L (based on the peptidomimetics with the higher charge density) showed higher silencing effect (~ 75%) in H1299 EGFP cells than P1-L and P2-L (~ 35%). There was no significant difference

between the silencing effect of P3-L and P4-L ($p$>0.05). An excess of the cationic component is required to obtain stable particles, whereas a lower cationic charge density and a lower N/P ratio are essential to minimize cellular toxicity.[23] Therefore, P3-L was investigated in further detail and compared to the commercial transfection reagent Lipofectamine 2000 (Lip2k). The gene silencing effect was approximately 77% for the P3-L and 86% for Lip2k, respectively, and P3-L showed almost the same effect as Lip2k at 25 nM siRNA (Fig. 2B). Even at a low N/P ratio of 1, P3-L effectively formed nanoparticles with EGFP siRNA (Supplementary Data, Table 1), and the P3-L liposomes led to decreased EGFP gene expressions in a concentration-dependent manner in a similar way as seen for Lip2k (Fig. 2B). These results suggest that P3-L is an efficient vector for induction of gene silencing, while the complexes exhibit only modest silencing efficiency. A key feature of the lipids used in this mixture is not only their charge, but also their tendency to promote lipid fusion. DOPE is considered a fusogenic lipid,[23] while CHEMS has been used to make liposomes fusogenic as the charge of CHEMS changes at acidic pH.[23] To verify the effects of adding a fusogenic lipid mixture versus adding another lipid mixture, we tested P3-L systems prepared with non-fusogenic lipid mixtures. In contrast, a low silencing effect (<20%) was observed when we replaced DOPE with the zwitterionic phospholipid dipalmitoylphosphatidylcholine (DPPC), as shown in the Supplementary Data, Fig. S1. These results indicate that the coating of the complex surface with a fusogenic lipid mixture plays an essential role in the siRNA silencing process.

**Cell viability**

Toxicity is a major concern when a nanocarrier is designed for therapeutic purposes. The cytotoxicity of the complexes and the liposomes was tested by using the MTS/PMS assay. At concentrations of up to 100 nM siRNA, none of the complexes displayed any toxicity (Fig. 3A), whereas at higher concentrations of siRNA (i.e. 200 and 400 nM), all complexes gave rise to cytotoxicity with up to 70% cell death, indicating that the safe dose window of the complexes is narrow. In contrast, minimal toxicity (<5%) was found for all liposome formulations, even at concentrations corresponding to 400 nM siRNA (Fig. 3A). The calculated half maximal inhibitory concentrations ($IC_{50}$) of the different peptidomimetics infer that the cytotoxicity of the peptidomimetics is increased with oligomer length and thus overall cationic charge (Fig. 3B). Furthermore, neither naked EGFP siRNA nor the empty liposome vector showed any cytotoxicity in H1299 cells (Supplementary Data, Fig. S2). In addition, Lip2k has been reported to be highly cytotoxic as indicated by the $IC_{50}$ (22.9 µg/mL) despite its potent transfection efficiency.[12] Therefore, this clearly demonstrates the importance of charge shielding in achieving safe siRNA transfection.[24]

**Immunogenicity *in vitro***

Antigen-presenting cells, such as dendritic cells, recognize pathogens via pattern-recognition receptors. These receptors can recognize peptidoglycan-, RNA- or DNA-associated patterns, which are characteristic for pathogens but absent in eukaryotic cells. TLRs are widely studied pattern-recognition receptors, and in the present study we measured the potential activation of five different human TLRs (TLR2, TLR3, TLR4, TLR7 and TLR9). At the low and non-toxic concentrations of peptidomimetics (0.5 and 5 µM), P3-C and P3-L (equivalent to 50 nM siRNA), no TLRs activation was observed (Fig. 4A and B, and Supplementary Data, Fig. S3). At the higher, cytotoxic concentrations (Fig. 3A), TLR activation could not be determined (results not shown). Activation of bone marrow-derived dendritic cells was assessed by measuring the expression of the maturation markers CD40 and CD86.[20] None of the four peptidomimetics stimulated dendritic cells at concentrations of 0.5 µM, however, P2, P3 and P4 activated the dendritic cells at a concentration of 5 µM (Fig. 4C), which cannot be explained by the activation of TLRs. P3-C and P3-L did not show any stimulating effects on the dendritic cells at an siRNA concentration of 50 nM. Together, these results indicate that neither the P3-C complexes nor the P3-L liposomes displayed any apparent immunogenicity. Thus incorporation of the peptidomimetics into the complexes and subsequently the liposomes substantially decreased their immune stimulating capacity.

**Morphological evaluation and siRNA release**

The morphology of the complexes and the liposomes was analysed by TEM. The complexes and the liposomes were spherical with a diameter of approximately 90 nm and 250 nm (Fig. 5A and B), respectively, which corresponds to the diameters measured by using DLS (Fig. 1B). The complexes appeared to have a densely packed structure (Fig. 5A). TEM revealed the ultrastructure of the liposomes as being a nuclear core surrounded by one or several concentric lipid membrane bilayers (Fig. 5B). Cryo-TEM further confirmed the formation of membrane bilayer(s) enclosing a more electron-dense core, indicating that the complexes are in fact coated with lipid membrane(s) (Fig. 5C). Corresponding low-magnification images are presented in Supplementary Data, Fig. S4.

When comparing the structure of the complexes before and after membrane coating (Fig. 5A and B), it is evident that the complex cores of the liposomes are much larger in size than the uncoated complex particles as a consequence of the lipid coating. Thus we hypothesize that the lipid coating of the complexes results in a more loose assembly of the complex core in the liposomes. It has been reported that there is a strong positive correlation between the strength of the association in the siRNA-CPP complexes and their resistance to heparin-induced decomplexation.[22] We therefore performed a heparin decomplexation analysis to examine this phenomenon. The electrostatic binding of the EGFP siRNA to the

peptidomimetic in the P3-L liposome core was compared to that of the uncoated P3-C complex. P3-L displayed a heparin decomplexation $EC_{50}$ value of 29 ± 3.1 μg/mL, as compared to 53 ± 4.8 μg/mL for P3-C (Fig. 5D). These measurements indicate that the lipid coating of the complexes affects the binding affinity between the siRNA and the peptidomimetics. This is in line with our estimate that siRNA is tightly packed with the peptidomimetics in the complex, while the complex core is loosened to some extent upon lipid coating. A possible mechanism for the structural transition of the complexes is that anionic lipid may compete with the negatively charged siRNA for positive electrostatic interaction with the peptidomimetic. Another explanation could be that insertion of the palmitoyl chain of the peptide into the anionic membrane bilayer may weaken the association between the peptidomimetic and the siRNA in the complex core.

To further characterize the physicochemical properties, we evaluated the release kinetics for the complexes and the liposomes. A gradual release of siRNA was observed for P3-L, reaching a level of 21% within 12 h. In contrast, siRNA was released more slowly from P3-C: Only 7% of total siRNA was released over 48 h (Fig. 5E). From the release behavior we found that both curves had an initial linear increase followed by saturation, which happened earlier for P3-C than for P3-L. The observed difference in the release profiles is in line with the transition of the complex structure demonstrated above, and we suggest that the more loose structure of the liposome core may contribute to the enhanced siRNA release. Our data is consistent with a previous study, which reported that the most active CPP showed high sensitivity to decomplexation by heparin, indicating the necessity of partial decomplexation for efficient transfection.[22] Thus, it is proposed that a more efficient release of the encapsulated siRNA can explain the enhanced silencing effect of P3-L (Fig. 2A).

**Cellular association and intracellular siRNA quantification**

Flow cytometric analyses were performed to quantify the association of Alexa488-labeled EGFP siRNA with H1299 cells. The fluorescence intensity in cells treated with naked siRNA showed background fluorescence, while considerable differences in the extent of cellular association were detected for the complexes and the liposomes after 4 h of incubation (Fig. 6A). It is well known that positively charged delivery vehicles can facilitate uptake by associating with the negatively charged cellular membranes.[25] As shown in Fig. 6B, cationic LF2k showed a strong association with the cells [mean fluorescence intensity (MFI) 91.0 ± 7.5], and the cellular association of the P3-C complex (MFI 7.0 ± 2.5) was significantly higher ($p$ = 0.0004) than that of naked siRNA (MFI 2.2 ± 0.4). Interestingly, the cellular association of negatively charged P3-L (27.1 ± 4.2) was significantly higher ($p$ = 0.0006) than that of the positively charged P3-C. These observations may be a consequence of different uptake mechanisms for P3-L particles and P3-C particles. Further studies are needed to elucidate this point. It has been reported that the CPP-based complex might deliver siRNA to the cells by receptor-mediated endocytosis.[2] Alternatively, both endocytosis and direct fusion between the liposomes and the plasma membrane might be responsible for the entry of functional siRNA into the cytosol.[26]

To better understand the delivery potential of the carriers, we monitored the relative level of siRNA delivered intracellularly by using stem-loop qPCR (Fig. 6C). Lip2k, P3-C and P3-L exhibited relative intracellular siRNA uptake patterns similar to the membrane association shown in Fig. 5B. In general, the amount of intracellular siRNA delivered by the nanocarriers is directly proportional to their transfection efficiency. Lip2k showed the highest siRNA delivery being 4-fold higher than that of P3-L. However, P3-L showed comparable siRNA silencing efficiency as Lip2k, indicating that the delivery efficiency of P3-L is higher than that of Lip2k. Our data is in agreement with previous studies,[22] suggesting that cellular uptake of siRNA is an important, but not sufficient, prerequisite for a high functional transfection.

**Subcellular distribution of siRNA and endosomal escape**

The intracellular distribution of Alexa488-labeled EGFP siRNA after transfection was visualized by confocal laser scanning microscopy to elucidate the reason for the differential RNAi effects, depending on the particle modification. Considerable differences in the distribution and extent of cellular accumulation were detected between different nanoparticles at the initial 100 nM siRNA after 12 h incubation. Naked siRNA displayed extremely weak fluorescence and in contrast, Lip2k showed a very strong cellular internalization (Fig. 7). For P3-C, noticeable Alexa488-siRNA was seen in the cytoplasm, while P3-L gave rise to a more extensive cytosolic distribution of labeled siRNA. Visualization of the cellular distribution of Alexa488-labeled siRNA internalized via P3-C and P3-L supported the flow cytometry analyses and the cellular siRNA quantification that revealed that P3-L delivered higher amounts of siRNA as compared to P3-C.

It has been reported that nanocarriers can modulate the subcellular drug distribution, eventually resulting in much higher efficacy.[27] An important determinant for siRNA delivery is the efficiency of siRNA escape from the endosomes and the lysosomes into the cytosol.[28] The cationic liposomes Lip2k represents one approach for endosomal escape, as shown in Fig. 7, and despite the fact that a proportion of Alexa488-siRNA (green) co-localizing with the endosomal/lysosomal marker LysoTracker (red) is evident (yellow or orange areas), areas of strong green fluorescence are also present in the cells, suggesting that the Lip2k formulation is capable of inducing considerable endo-lysomal escape. It is proposed that cationic lipids are able to combine with anionic lipids in the endosomal membrane to form ion pairs and thereby destabilizing the endosomal membrane and facilitating the release of siRNA into the cytosol.[29,30] In the case of P3-L

treated cells (Fig. 7), siRNA only partially co-localized with LysoTracker, indicating that the P3-L particles are capable of escaping lysosomal degradation. Interestingly, it is apparent that siRNA is targeted to the nuclei in the cells incubated with P3-L. A possible mechanism could be that the pH-sensitive fusogenic lipid in the P3-L liposomes may facilitate delivery of the payload into the cytosol and nucleus.[31] Because CHEMS is acid-sensitive, P3-L may disassemble as a consequence of a structural change of the stable lamellar CHEMS:DOPE liposomes upon reduction of the pH: At reduced pH CHEMS molecules will change from a cone-like shape to a cylindrical shape due to protonation of the headgroup with a concomitant loss of bilayer-stabilizing properties, and the nonbilayer-forming lipid DOPE will undergo a lamellar-to-hexagonal phase transition, eventually adopting an inverted hexagonal phase that is highly fusogenic, causing membrane destabilization and release of entrapped siRNA.[32] In contrast, reduced cytosolic accumulation of siRNA and lower silencing effect was observed when we replaced CHEMS with the phospholipid DPPC (Supplementary Data, Fig. S5). In addition, cells incubated with P3-C revealed extensively co-localization of siRNA with LysoTracker, suggesting a limited endolysosomal escape of siRNA into the cytosol. Our data is in accordance with the silencing results, which also indicate that the challenge of endosomal escape remains for the development of CPP-mediated siRNA delivery.[2]
These results demonstrate that endosomal/lysosomal escape may be enhanced via modulation of the cellular siRNA distribution as a result of the acid-sensitive anionic lipid-modification on the surface of the nanocomplexes. Therefore, the data suggests that P3-L mediated a higher EGFP silencing primarily by increasing cellular uptake and mediating a more favorable siRNA release in the cytoplasm. P4-L particles also displayed similar results (data not shown). Our findings are consistent with a recent study, which reported that PepFect6/siRNA-PepFect6 liposomes generated a significant RNAi effect *in vitro.*[33] Although there are marked differences in the experimental conditions for P3-L and PepFect6/siRNA-PepFect6 particles (such as CPP type, cell type, target genes and surface modification), the high cellular uptake, efficient endosomal escape, disintegration of complexes, and potent silencing effect of our liposomes and the PepFect6/siRNA-PepFect6 particles may be attributed to the same lipid structure coating the nanocomplexes.

## Conclusions

The optimized anionic liposomes constitute a more effective and robust formulation than the siRNA-peptide complex, and show proficient intracellular uptake and endosomal escape. More importantly, the anionic liposomes elicit negligible cytotoxicity and immunogenicity *in vitro*. Several elements of this study might facilitate the future development of oligonucleotide delivery systems. First, the optimization of the length of the peptidomimetics to display an appropriate positive charge did not only result in small and stable complexes with siRNA at a low N/P ratio, but did also reduce the toxicity caused by this polycationic component. This technology for reducing toxicity may be applied to design other cationic peptides or polymers. Second, these results suggest that P3-L liposomes constitute an efficient and biocompatible vector for siRNA delivery with diminished cytotoxicity and immunogenicity, and that the coating lipid may be extended to other anionic coating agents. Third, although we show that siRNA delivery may be improved in vitro, the P3-L nanocarrier can serve as a template for further surface modification with e.g. targeting ligands and functional molecules especially poly(ethylene glycol) (PEG) capable of prolonging the systemic circulation and minimizing adverse immune responses.[28] In summary, such rational design that address safety concerns and ensure effective delivery will aid the translation of engineered peptidomimetic-based nanoparticles toward the clinic in the future.

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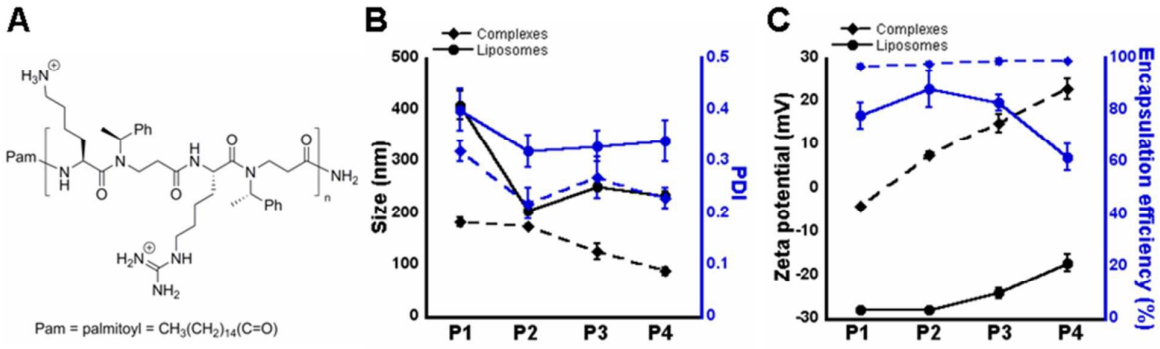


**Fig. 1.** (A) The sequence of α-peptide/β-peptoid oligomers with alternating hydrophobic and lysine and homoarginine motifs containing a variable number (n) of repeats (n = 1, 2, 3 or 4, P1-4), coupled to a palmitoyl lipid chain. (B) Particle size (black lines) and polydispersity index (PDI, blue lines) of complexes (prisms) and liposomes (circles), respectively, prepared using P1-4. (C) Zeta potential (black lines) and encapsulation efficiency (blue lines) of complexes and liposomes, respectively, prepared using P1-4. Values represent mean ± SD (n =3).

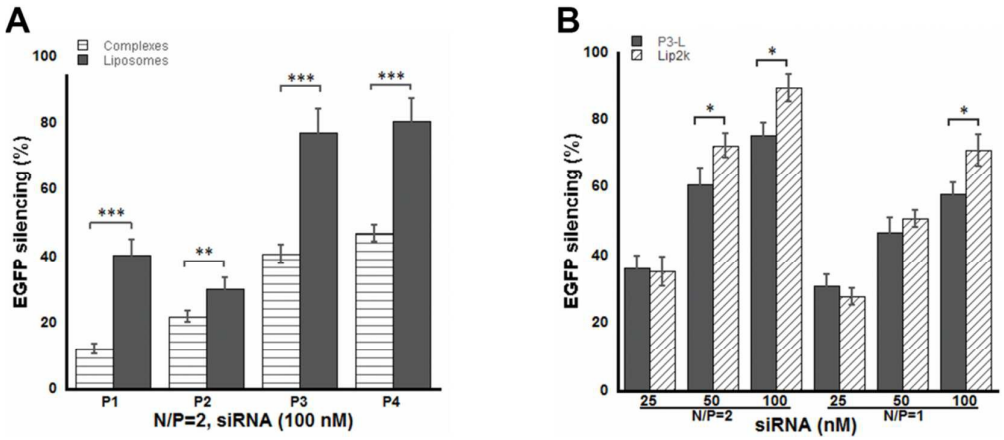


**Fig. 2.** (A) Silencing effect of the four different complexes and the corresponding liposomes at an N/P ratio of 2 in H1299 EGFP cells. (B) Comparison of P3-L with Lipofectamine 2000 (Lip2k) at N/P ratios of 2 and 1. The weight ratio of Lip2K and siRNA was 3:2 at an N/P of 2. Results are expressed as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

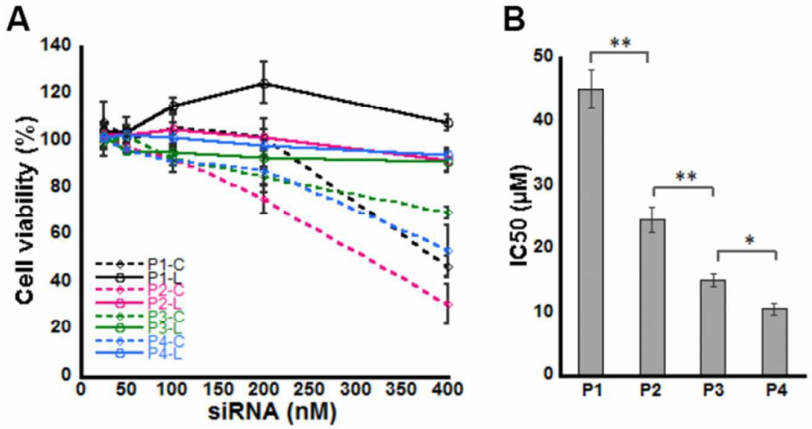


**Fig. 3.** (A) Effect of complexes and liposomes on the viability of H1299 cells. (B) Effect of the four peptidomimetics (P1-P4) on the viability of H1299 cells. The half maximal inhibitory concentrations ($IC_{50}$) values were calculated by using a nonlinear regression analysis. Results are expressed as mean ± SD (n = 3). *$p < 0.05$ and **$p < 0.01$.

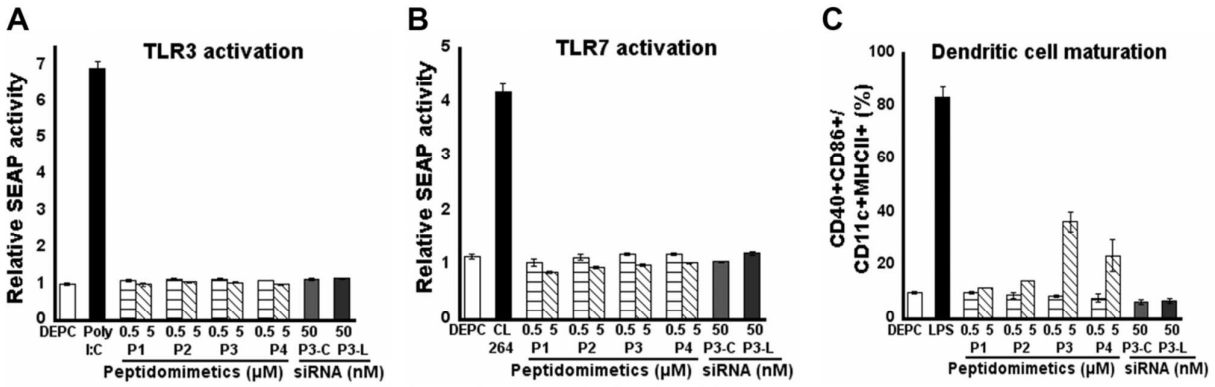


**Fig. 4.** TLR reporter cell lines 3 (A) and 7 (B) were stimulated with P1-P4 at peptidomimetics concentrations of 0.5 μM, or 5 μM, with P3-C and P3-L at siRNA concentrations of 50 nM or with solvent for 20 h at 37°C. The activity of the reporter protein SEAP was determined, and the relative expression levels were defined as the sample level divided by the solvent control level. The agonists of TLR3 and TLR7 are poly I:C and CL264, respecitivly. DEPC water was used as negative control. (C) Bone marrow cells were differentiated into dendritic cells (DC) in 7 days with GM-CSF and were stimulated with P1-P4 at peptidomimetics concentrations of 0.5 μM, or 5 μM, with P3-C and P3-L at siRNA concentrations of 50 nM or with solvent for 16 h at 37°C. Lipopolysaccharide (LPS), a stimulant of DC maturation was used as a positive control, and maturation was determined as the percentage of $CD86^+/CD40^+$ double positive cells within the $CD11c^+/MHCII^+$ double positive population. Results are expressed as means ± SD (n = 3) and data is representative for two independent experiments.

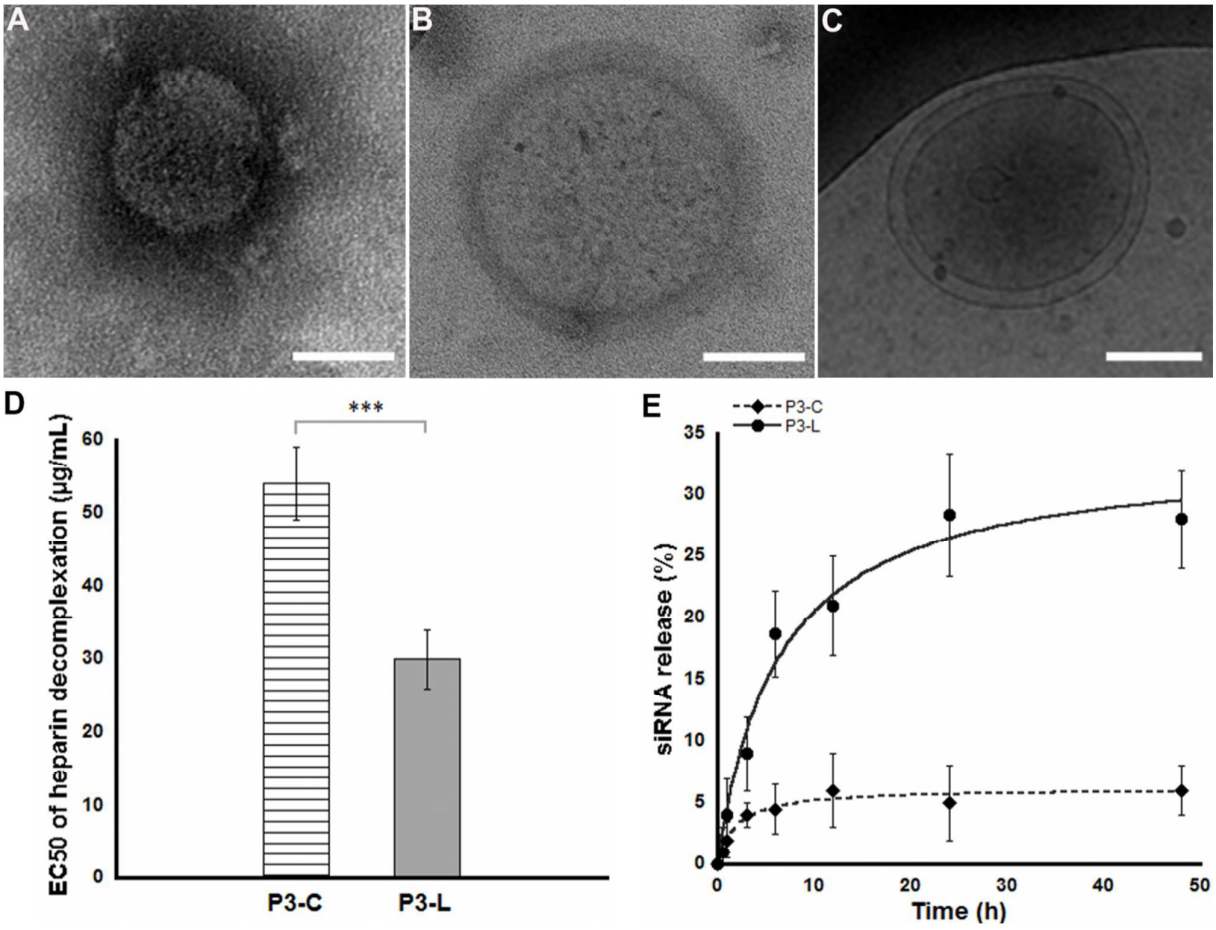


**Fig. 5.** (A) Representative transmission electron micrograph (TEM) image of a P3-C complex. (B) Representative TEM image of a P3-L liposome showing the nuclear nanocomplex within a lipid bilayer envelope. (C) Representative cryo-TEM image of a P3-L liposome showing the bilayer core-structure. Scale bar = 100 nm. (D) The half-maximal effective concentration ($EC_{50}$) of heparin decomplexation. (E) siRNA release from complexes and liposomes in TE buffer at pH 7.4 at 37 °C. Results are expressed as mean ± SD (n=3), *** $p < 0.001$.

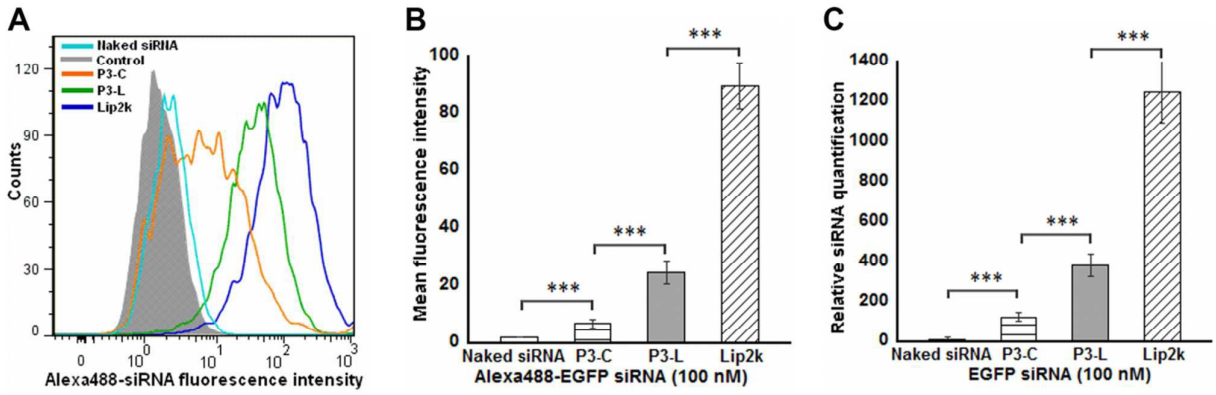


**Fig. 6.** (A) Representative histogram plot of flow cytometric analyses demonstrating the cellular association of siRNA after 4 h incubation of H1299 cells with P3-C, P3-L or Lip2k loaded with Alexa488-EGFP siRNA at a concentration of 100 nM. As controls were used cells incubated with medium alone and non-complexed siRNA, respectively (B) Quantitative comparison of nanocarrier-mediated cellular association of Alexa488-EGFP siRNA by flow cytometry. (C) Relative quantification of EGFP siRNA after 48 h transfection with P3-C, P3-L and Lip2k at 100 nM EGFP siRNA using the stem-loop qPCR. Results are expressed as mean ± SD (n=3), *** $p < 0.001$.

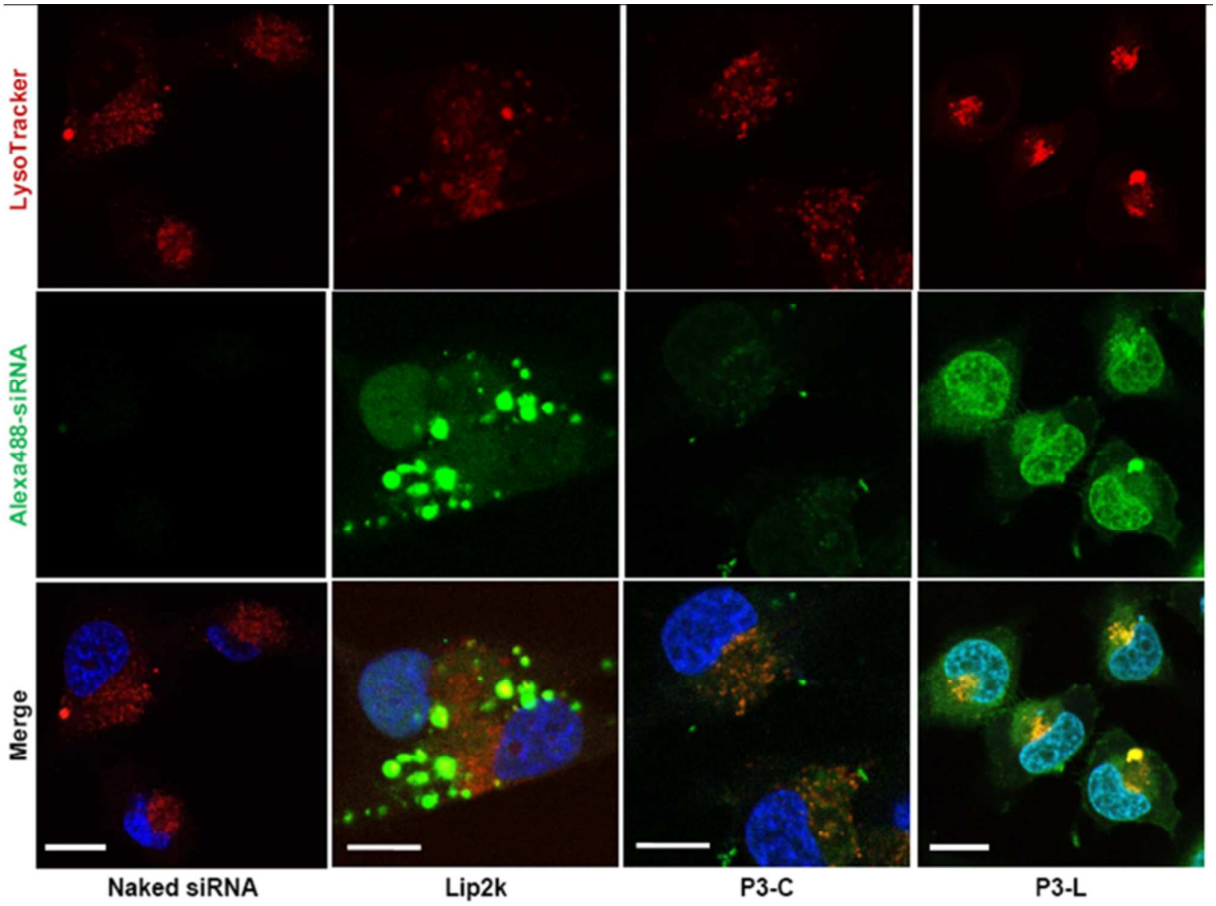


**Fig. 7.** Representative confocal laser scanning microscopy images of H1299 cells (blue: nuclei) transfected with Lip2k, P3-C and P3-L nanoparticles loaded with 100 nM Alexa488-siRNA. The co-localization patterns of siRNA (green) with LysoTracker (late endosome/lysosome marker, red) 12 h after treatment with nanoparticles show different siRNA distributions. Scale bar = 20 μm.