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Asymmetric liposome particles with highly efficient encapsulation of siRNA and without nonspecific cell penetration suitable for target-specific delivery

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ABSTRACT

The discovery of siRNA has been an important step in gene therapy, but the problem of delivering siRNA to a target organ limits its use as a therapeutic drug. Liposomes can be used as a nonviral vector to deliver siRNA to target cells. In this study we developed a novel method of producing asymmetric liposome particles (ALPs) with highly efficient siRNA encapsulation. Two kinds of lipid inverted micelles were prepared for the purpose of obtaining ALPs. The inner one is composed of ionizable cationic 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), which entrap siRNA, and the outer one is composed of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), DOPE, polyethylene glycol-1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (PEG-PE), and cholesterol. After mixing the inverted micelles, ALPs encapsulating siRNA were obtained by solvent evaporation and dialysis. This process allowed more than 90% siRNA encapsulation as well as the negatively charged surface. The ALPs protected siRNA from ribonuclease A degradation. ALPs without any surface modification elicited almost no uptake into cells, while the surface-modified ALPs with a polyarginine peptide (R12) induced nonspecific cell penetration. The conjugation of the anti-human epidermal growth factor receptor antibody (anti-EGFR) to ALPs induces an EGFR-mediated uptake into the non-small cell lung cancer cell lines but not into NIH-3T3 cells without the receptor. The siRNA encapsulated in ALPs showed the R12- or anti-EGFR-dependent target gene silencing in NCI-H322 cells. These properties of ALPs are useful for target-specific delivery of siRNA after modification of ALPs with a target-specific ligand.

1. Introduction

The discovery of RNA interference (RNAi) opened a new window for the treatment of gene-related diseases such as cancer [1–5]. Small interfering RNAs (siRNAs) induce sequence-specific cleavage of their target mRNA through the endogenous RNA-induced silencing complex [6,7]. Therefore, siRNAs have great potential in the treatment of diseases caused by genetic disorders [8]. However, negatively charged siRNAs cannot penetrate the cell membrane, and siRNAs are rapidly eliminated by nucleases in the blood and body fluids [9,10]. Appropriate delivery systems involving the use of vectors such as siRNA carriers have been developed to enhance the target delivery of siRNA to specific cells and to improve the cellular uptake by means of systemic administration.

Viral systems such as retroviruses, adenoviruses, adeno-associated viruses are powerful tools for efficient gene delivery, but they have major disadvantages such as inflammation and oncogenic potential [9,11]. Moreover, they cannot be used for the delivery of synthetic siRNAs. Because of these disadvantages, the research has focused on non-viral vectors for siRNA delivery.

Many cationic polymers can spontaneously produce particles called polyplexes by polymer mixing with nucleic acid. Positively charged polyplexes electrically interact with the negatively charged siRNAs, and they carry the siRNA, effectively protect it from enzyme degradation, and promote the uptake of siRNA into cells [12,13]. The disadvantages of cationic polyplexes are removal by means of the reticuloendothelial system (RES) and accumulation at the primary organs as a result of nonspecific interactions with blood components [14–17].

Lipoplexes, which are siRNA-cationic lipid complexes, were developed to deliver siRNAs into cells [18,19]. The lipoplex encapsulation depends on the charge interaction between siRNA and cationic lipids. Although lipoplexes efficiently encapsulate and protect siRNA from nuclease degradation, the rapid removal and clearance of lipoplexes from blood by RES clearance and the accumulation in the primary organ during systemic delivery are major problems for their usage as a carrier [17,18,20]. This problem is overcome by using polyethylene

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glycol (PEG) to prevent the positively charged lipids from having direct contact with the blood components. The PEGylation decreases the RES clearance of the lipoplex [21], and the PEGylated liposomes also have some advantages, such as accumulation at the tumor site [22] and prolonged circulation of the liposome in mice [23]. In addition, the encapsulation of siRNA inside the liposome is considered the best way of protecting siRNA from nuclease degradation. Several methods have reportedly achieved good encapsulation of siRNA in PEGylated liposomes [10,24,25]. These formulations are insufficient to protect the nonspecific delivery that is needed for target-specific delivery of siRNA. Recently, a spontaneous vesicle formation method was used to successfully encapsulate siRNA [10,26], and clinical trials are currently in progress. However, the main *in vivo* delivery mechanism is dependent on the enhanced permeability and retention effect. In contrast, neutral or negatively charged liposomes can minimize the nonspecific absorption of siRNA into cells but their lower efficiency in terms of encapsulating siRNA is a major disadvantage [27].

In this paper, we describe a novel method of producing asymmetric liposome particles (ALPs) which have a high efficiency in encapsulating siRNA and a negatively charged surface. The cationic lipids that encapsulate siRNA are localized at the inner layer, whereas the neutral and PEGylated lipids face the outer environment. The ALPs were unable to internalize into cells, but their surface modification by a polyarginine peptide or a target-specific antibody (anti-EGFR) induced the cellular uptake of ALPs and siRNA-dependent gene-silencing effects. We believe that ALPs may be useful for target-specific systemic delivery of siRNA after conjugation with proper target-specific ligands.

2. Materials and methods

2.1. Material

The lipids for this study, which were purchased from Avanti Polar Lipids, Inc. (USA), were 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (miPEG-PE), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and cholesterol (CH). TRIzol reagent and lipofectamine 2000 were obtained from Invitrogen (USA), and an RT-PCR kit was obtained from New England Biolabs (USA). A solid-phase method involving Fmoc chemistry (REF) was used to synthesize the cell-penetrating peptide R12 (RRRRRRRRRRRRGGC). All other chemicals were purchased as analytical and molecular biology grades. Double-strand siRNAs were purchased from Samchully Pharm Co., Ltd (Korea), and fluorescein isothiocyanate (FITC)-labeled siRNA (FITC-siRNA) was obtained from the Bioneer Corporation (Korea). Samples of dialysis membrane with molecular weight cut-off values of 10 K, 50 K, and 300 K were purchased from Spectrum Laboratories (USA). The non-small cell lung carcinoma (NSCLC) cells A549, NCI-H322, NCI-H460, and NIH-3T3 were obtained from ATCC (USA). 2-Iminothiolane·HCl was supplied by Thermo Scientific (USA). Anti-EGFR (Erbitux) was obtained from Merck & Co. Inc. (USA), and anti-human IgG antibody-cy3 and streptavidin-Cy3 were obtained from Sigma-Aldrich (USA), respectively. All solutions were made up in DEPC water.

2.2. ALP preparation

As shown in Fig. 1, the ALPs were prepared by means of a modified reverse phase evaporation method [28]. The inner and outer inverted micelles were prepared separately in two different test tubes. The lipid components of the outer layer were DSPC, DOPE, PEG-PE (which is a mixture of mPEG-PE and miPEG-PE), and CH with a molar ratio of 3:3:2:4 or 4:3:1:4 (1.7 μmol total). The lipid components of the inner layer were DODAP and DOPE with a molar ratio of 9:1 (1.5 μmol total). After making the outer and inner thin films, we hydrated the outer lipid film in a mixture of 200 μl HBS (20 mM HEPES and 150 mM sodium chloride; pH 7.5) and 120 μl ethanol; we then hydrated the inner lipid film in 150 μl of 150 mM sodium citrate (pH 4) containing 100 μg of siRNA. To make the outer and inner inverted micelles, we added 600 μl and 400 μl samples of diethyl ether to the outer and inner layer solutions, respectively, and sonicated the mixture with a cup-horn probe of a Sonics ultrasonic processor (Sonics & Materials, USA). The inverted micelles were mixed, and the diethyl ether was evaporated under nitrogen blowing. The ALPs that encapsulated siRNA (siRNA/ALPs) were dialyzed with a 10 K membrane in HBS at 4 °C to remove the remaining organic solvents, and the final volume was adjusted to 500 μl by adding HBS. The same method was used to

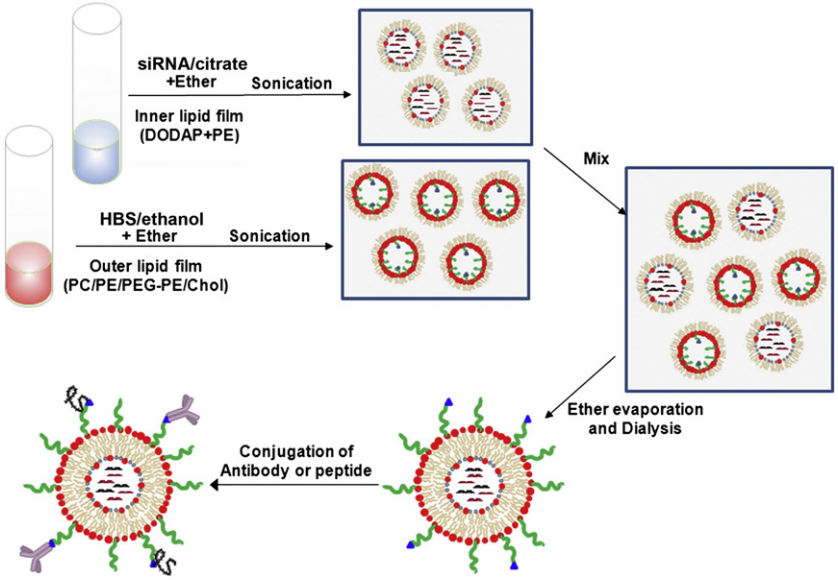

Fig. 1. Schematic of the ALP preparation. The inner and outer lipid films were hydrated with sodium citrate (pH 4.0) containing siRNA and HBS/ethanol, respectively. The inner and outer inverted micelles were prepared by sonication after the addition of diethylether. The inverted micelles were then mixed and evaporated. The resulting liposomes were equilibrated with HBS.

prepare the ALPs that encapsulate the FITC-siRNA, though 100 μg of FITC-siRNA was used instead of the siRNA. To modify the ALP surface, we added 55 nmol miPEG-PE to the outer layer lipids. We also made liposomes by changing the inner pH from 4 to 5 and by using DOTAP instead of DODAP. The particle sizes and zeta potentials of the ALPs were analyzed with an ELS-Z zeta-potential and particle size analyzer (Photal Otsuka Electronics, Japan).

2.3. Evaluation of siRNA encapsulation in ALPs

The efficiency with which ALPs encapsulate siRNA was monitored by using a 4% agarose gel electrophoresis method with a Tris-borate-EDTA (TBE) buffer system. To analyze the encapsulation of siRNA, we loaded the ALPs on the 4% agarose gel in the TBE buffer with 0.5 μg/ml ethidium bromide in the absence and presence of 1% nonidet-P40 (NP40). After the electrophoresis, siRNA bands were visualized with a gel documentation system using an ultraviolet transilluminator (Bio-Rad, USA). The siRNA band intensities were determined by means of densitometric analyses using the Bio2D program (Vilber Lourmat, France).

2.4. Nuclease protection assay of siRNA in ALPs

To evaluate the nuclease protection of the siRNA encapsulated in ALPs, we mixed 4 μl of 20 μg/ml ribonuclease A (RNase A) or 8 μl of fresh mouse serum with 8 μl of siRNA/ALPs containing 1.6 μg siRNA. The mixture was then incubated at 37 °C for up to 24 h. Agarose gel electrophoresis was conducted in the presence of 1% NP40 to monitor the remaining siRNA. An equivalent amount of free siRNA was used as a positive control to check the RNase A activity.

2.5. Serum stability and toxicity of siRNA/ALPs

Serum stability of siRNA/ALPs was studied by turbidity change in 70% fetal bovine serum (FBS). Thirty microliters of siRNA/ALP solution was mixed with 70 μl of HBS or FBS in the 96 well culture plate, and then the mixtures were and incubated at various times up to 24 h at room temperature. One hundred microliters of HBS or a mixture of 70% FBS and 30% HBS, respectively, was used as a control. The turbidity of the mixtures was measured by the absorbance at 550 nm. The relative absorbance values (%) were calculated according to $(As-Ac)_t/(As-Ac)_0\times 100$, where As and Ac denote the mixtures of siRNA/ALP and FBS or HBS and the corresponding control, respectively, and $(As-Ac)_t$ and $(As-Ac)_0$ denote the absorbance differences at given time (t) and initial time (0), respectively.

The cell toxicity of siRNA/ALPs was measured by cell viability. The siRNA/ALP solution (6.4 mM lipid concentration) was serially diluted in DMEM containing 10% fetal bovine serum (DMEM-FBS) up to 1:4 (1.2 mM lipid concentration). One hundred microliters of the diluents was treated to 40% to 50% confluent cells in 96 wells, and the cells incubate for 24 h in CO_2 incubator. The cell viability was measured according to the manufacturer's protocol by using WST-1 kit (Takara, Japan).

2.6. Preparation of a Fab′ fragment

A Fab′ fragment of the anti-EGFR was obtained in a subsequent treatment of the antibody with pepsin and 2-mercaptoethylamine-HCl as shown in Supplementary Fig. 1A. Briefly, a 4 mg/ml to 5 mg/ml sample of the antibody was equilibrated with 0.1 M of sodium acetate; the pH level was 4.0. The sample was digested with 0.1 mg/ml pepsin (Sigma, USA). After 2 h to 3 h of incubation, the reaction was stopped by adding a tenth of the volume of 1.5 M Tris–HCl, pH 8.8. The resulting $F(ab')_2$ was dialyzed against PBS–EDTA (PBS solution containing 5 mM EDTA) and then reduced in a 90 min treatment with a tenth of the volume of 6 mg/ml 2-mercaptoethylamine–HCl. The resulting Fab′ was dialyzed against PBS–EDTA. All the products were analyzed by means of a reducing and non-reducing process of SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Surface modification of ALPs

To conjugate R12 to ALPs, we added R12 with 5 times molar excess than miPEG-PE to the ALP solution. The mixture was incubated for 3 h at room temperature. The unbound peptide was removed by dialysis with a 50 K membrane in HBS. To conjugate the anti-EGFR and Fab′ to ALPs, they were initially thiolated for 20 min to 30 min with a ten-fold to twenty-fold molar excess 2-iminothiolane. The thiolated proteins were then dialyzed in PBS–EDTA, and then the thiolations were estimated by an Ellman assay using 5,5′-dithiobis-(2-nitrobenzoic acid. Fifty micrograms of thiolated antibody (tAb) or Fab′ (tFab′) and 100 μg of unmodified Fab′ were incubated for 16 h to 20 h at 16°C with 100 μl of an miPEG-PE formulation that contained ALPs. The unreacted maleimide groups of ALPs were neutralized by the addition of a hundredth volume of 200 mM 2-mercaptoethanol. The resulting ALPs were mixed with the equal volume of 74% sucrose, and then were purified by a buoyant sucrose density gradient centrifugation with 9%, 25%, 30%, and 37% sucrose concentrations. A 9% fraction containing ALPs was collected and dialyzed against HBS. Agarose gel electrophoresis was used to analyze the encapsulation of siRNA, and SDS-PAGE was used to analyze the antibody conjugation to the ALPs.

2.8. Immunocytochemistry

The expression of the EGFR on the cell membrane was monitored by means of immunocytochemistry. The NSCLC cells, namely A549, NCI-H322, and NCI-H460, and the NIH-3T3 cells were cultured in micro-slide eight-well microscopy chambers (ibidi, Germany) in DMEM-FBS. The cells were fixed with 4% formaldehyde in PBS, washed with PBS, and subsequently incubated for 1 h with the anti-EGFR and anti-human IgG antibody-cy3. The cells were incubated for a further 5 min with 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in PBS, and the cell bound antibody was monitored with a confocal microscope (Carl Zeiss LSM 510 META, Germany).

2.9. Effect of antibody modification on the cellular uptake

The EGFR antibody was conjugated with FITC (Sigma, USA) or biotin-maleimide to test the effect of the modification on the receptor-mediated antibody uptake. For the FITC modification, the antibody was mixed with FITC (with a fivefold molar excess) in PBS, and the mixture was incubated for 1 h. For the biotin-maleimide modification, tAb was mixed with a tenfold molar excess sample of biotin-maleimide (Sigma, USA) in PBS–EDTA, and the mixture was incubated for 1 h. All the modified antibodies were dialyzed in PBS and applied to the NSCLC cells (A549, NCI-H322, and NCI-H460) and the NIH-3T3 cells; they were cultured in micro-slide eight-well microscopy chambers (ibidi, Germany) in DMEM-FBS. The cells treated with the biotin-modified antibody were fixed with 4% formaldehyde, permeabilized with 0.1% Triton-X100, and then treated with streptavidin-cy3. The antibody localizations were monitored with a confocal microscope.

2.10. Cellular uptake assay of ALPs

The NSCLC cells (A549, NCI-H322 and NCI-H460) and the NIH-3T3 cells were cultured in micro-slide eight-well microscopy chambers (ibidi, Germany) in DMEM-FBS. The cells were then treated with FITC-siRNA/ALPs modified by R12, tAb or tFab′ in DMEM-FBS. After 1 day of incubation, the cells were rinsed twice in PBS and fixed with 4% formaldehyde in PBS at room temperature for 15 min. After the subsequent DAPI treatment, the cellular uptake of FITC-siRNA was monitored with a confocal microscope.

2.11. Gene-silencing efficiency of siRNA/ALPs

To test the gene-silencing efficiency of the ALPs that encapsulate siGL3 (siRNA for GL3-luciferase; antisense: 5′-UCGAAGUACUCAGCGUAAGdTdT-3′) [29], we cultured NCI-H322 cells until the confluency level in the DMEM-FBS reached 80% to 90%. The cells were transfected with a pGL3 control vector (Promega, USA) by means of lipofectamine 2000 (Invitrogen, USA). They were then cultured for 16 h to 24 h. The transfected cells were subcultured overnight in a 24-well plate and treated for two more days with samples of the siGL3-Lipofectamine 2000 complex and siGL3/ALPs with or without modification with tAb. The luciferase activity was analyzed with a Promega luciferase assay system (Promega, USA), and the luminescent lights were monitored with a multilabel plate reader (PerkinElmer VICTOR 3 1420, USA).

To prevent the expression of TMPRSS4 expression, we applied siTMPR4 (siRNA for TMPRSS4: antisense: 5′-AAGUUGUCGAAACAGGCAGAGAACC-3′) to NCI-H322 cells as described previously [30]. Briefly, the siRNA/ALPs (with and without ligand modification) were applied to NCI-H322 cells. Complexes of siTMPR4/25 K polyethyleneimine and siTMPR4/lipofectamine 2000 (LF2K) were used as controls. After 48 h of post-transfection, we harvested the transfected cells and used an RT-PCR to monitor the TMPRSS4 gene knockdown. The total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized with reverse transcriptase. A PCR amplification was performed with TMPRSS4-specific primers (5′-CCGATGTGTTCAACTGGAAG-3′ and 5′-CCCATCCAATGATCCAGAGT-3′ for a 237 bp product; 5′-GATCCACACTGCAGGTGCTG-3′ and 5′-CCACAGACGTGCTGTTTGTC-3′ for a 364 bp product) (Bioneer, Daejeon, Korea). A fragment of β-actin (5′-GCTCGTCGTCGACAACGGCTC-3′ and 5′-CAAACATGATCTGGGTCATCTTCTC-3′) was also amplified as an internal control. The PCR was performed at annealing temperature of 58 °C for TMPRSS4 and 55 °C for β-actin.

2.12. Cell invasion and cell migration assays

The siTMPR4-transfected NCI-H322 cells (4×10^4) were plated in a serum-free medium on 24-well Transwell inserts (Costar, Corning, NY, USA) coated with 25 μg of Matrigel (BD Biosciences, USA). The underside of the insert was pre-coated with 2 μg of Collagen I (Sigma, USA). After 48 h of incubation at 37 °C and 5% CO_2, the inserts were fixed with 3.7% paraformaldehyde/phosphate-buffered saline and stained with 2% crystal violet. Representative high-power (×200) fields were photographed, and the number of cells that had invaded or migrated was counted in 6 to 8 representative fields per Transwell insert. Cell migration assays were performed in a similar manner but without the Matrigel coating.

3. Results

3.1. Liposome preparation, size and siRNA encapsulation

The reverse-phase evaporation method is suitable for high encapsulation of a target molecule [28], but the uniform lipid system make difficulty achieving an encapsulation level of more than 70%. Although the positive charge of cationic liposomes promotes nucleic acid encapsulation, *in vivo* target gene delivery to specific cells is difficult due to the nonspecific interaction of positive lipids with the negatively charged cell surface. To overcome these problems, we developed ALPs with different components in their outer and inner lipid layers. Fig. 1 shows the overall scheme for the preparation of ALPs with highly efficient siRNA encapsulation and a negatively charged surface. After the evaporation of the organic solvents, the liposome solution was dialyzed with a 10 K dialysis membrane in HBS. The nonencapsulated (free) siRNA cannot penetrate the 10 K dialysis membrane; thus, the nonencapsulated siRNA and the total siRNA in the ALPs can be estimated by means of agarose gel-electrophoresis in the absence or presence of 1% NP40, respectively.

To produce ALPs, we initially prepared two different inverted micelles. We used DODAP/DOPE (total: 1.5 μmol; molar ratio: 9:1) with small head groups for the inner inverted micelle. The inner lipid film was hydrated with a 150 mM citrate buffer (pH 4.0) containing siRNA to induce the strongly positive charge of DODAP [23]. Under these conditions, we achieved an encapsulation level of more than 90% (Fig. 2A). However, changing the inner pH to 5.0 and using DOTAP instead of DODAP prevent almost siRNA encapsulation (Fig. 2A). The exclusive use of DODAP (100%) prevented the inverted micelle formation and reduced the siRNA encapsulation, whereas a DODAP/DOPE mixture with a ratio of 1:1 achieved an siRNA encapsulation level of approximately 90% (data not shown). For the outer inverted micelle, we used DSPC, DOPE, PEG-PE, and Chol. As shown in Fig. 2B, we tested various derivatives of phosphatidylcholine and phosphatidylethanolamine (PE) and found that the DSPC/DOPE combination produces the best results (that is, a higher level of siRNA encapsulation and smaller particles). Hydration of the outer lipids in 200 μl HBS (pH 7.5) forms aggregates, but the aggregates can be prevented by adding 120 μl of ethanol. The molar lipid ratio of the outer versus inner layer was set at approximately 1.06 to 1.07 to provide a larger surface area for the outer lipid layer. Finally, we fixed the outer lipid composition as DSPC/DOPE/PEG-PE/Chol (total: 1.6 μmol; molar ratio: 3:3:2:4 or 4:3:1:4) and the inner lipid composition as DODAP/DOPE (total: 1.5 μmol; molar ratio: 9:1) with an inner pH level of 4.0. The siRNA encapsulation efficiency under optimized conditions was almost 95%; and the average particle size was about 200 nm (Fig. 2B and Supplementary Table 1). A TEM image of the ALPs shows that the particles have a similar size (Fig. 2C). The unencapsulated siRNA could be removed by dialysis using a 300 K dialysis membrane (Fig. 2D). The ALPs have highly negative zeta potentials (Fig. 2B) and the inside acidic pH (the pH of the liposome solution

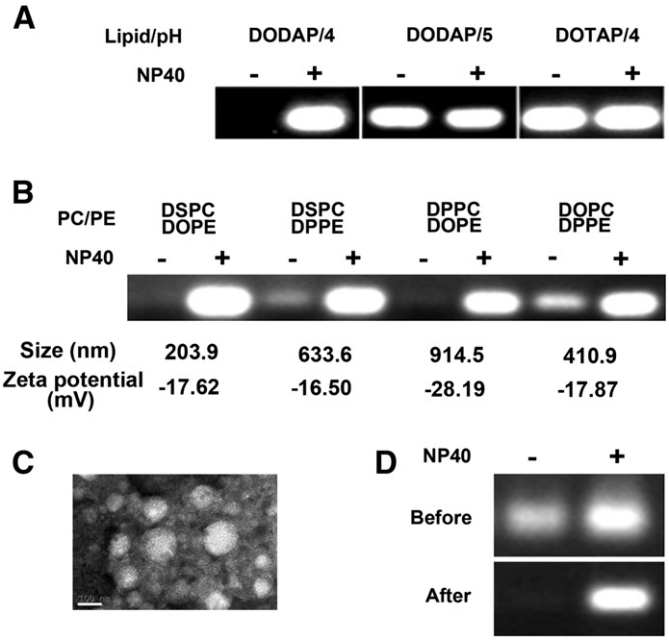

Fig. 2. The siRNA encapsulation in ALPs in relation to the inner and outer lipid compositions and inner pH levels. The encapsulation efficiency levels of the ALPs were monitored by using 4% agarose gel electrophoresis with a TBE buffer. The encapsulated siRNA and the total amount of siRNA were monitored in the absence and presence of 1% NP40, respectively. (A) The inner lipid compositions and pH levels dramatically affect the encapsulation efficiency of siRNA. (B) The outer lipid compositions slightly affect the siRNA encapsulation and particle sizes. (C) The negatively stained ALPs with 2% sodium tungstate (pH 7.0). The outer lipid compositions of (A) were used for DSPC/DOPE/mPEG-PE/Chol (molar ratio 3:3:2:4), and the inner lipid compositions of (B) were used for DODAP/DOPE (9:1).

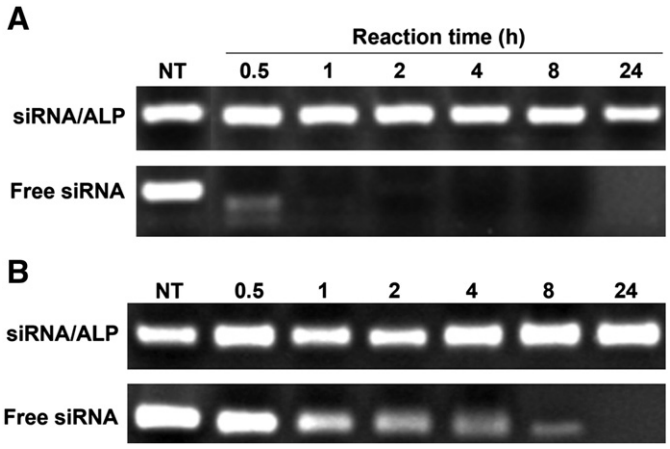

Fig. 3. Protection of the siRNA encapsulated in the ALPs from RNase A and mouse serum. The RNase A (A) and 50% serum (B) were applied to the ALPs that encapsulated the siRNA and the free siRNA at the indicated time points. The remaining siRNA was monitored by means of agarose gel electrophoresis in the presence of 1% NP40. NT refers to the non-treated RNase A.

changed from 7.5 to a range of 5.0 to 5.5 after the ALPs were disrupted with 1% NP40). In addition, the ALPs encapsulated the siRNA to an extremely high level, these results indicate that ALPs may have inside and outside asymmetric distributions of lipids, siRNA, and buffer solutions.

3.2. Stability and toxicity of siRNA/ALPs

Free siRNA degrades rapidly on account of nucleases in the bloodstream and body fluids. It is therefore important to protect siRNA from nucleases. Potent RNase A was used to investigate the stability of the siRNA encapsulated in ALPs. Although RNase A completely degrades the free siRNA within 1 h, the siRNA encapsulated in ALPs was protected from enzyme degradation for up to 24 h (Fig. 3A). ALPs also protect siRNA from 50% mouse sera (Fig. 3B), which means the lipid bilayers of ALPs protect the nuclease access to the encapsulated siRNA.

The cationic lipids in liposomes induce aggregation through electrostatic interaction between liposomes and the negatively charged species in serum, and the aggregation prevents liposome circulation in blood [31]. No aggregation was found in the mixture of siRNA/ALPs and serum (Supplementary Fig. 2), which means the negatively charged and PEGylated surface of ALPs prevents the aggregation.

In general the cationic liposomes display the toxicity at the high lipid concentration. However, the siRNA/ALPs showed almost no toxicity even at the 6.4 μmol/ml lipid concentration prepared by mixing 80 μl cell culture medium and 20 μl ALP solution in HBS. The viability for NSCLC cells and NIH-3T3 cells was 97–100% and 90% after 24 h incubation, respectively (data not shown).

3.3. Polyarginine-mediated uptake of siRNA/ALPs

FITC-siRNA was used to monitor the cellular uptake of the siRNA encapsulated in ALPs. More than 90% of the FITC-siRNA was also encapsulated in ALPs (data not shown). The liposomes with a positively charged surface cannot totally prevent the liposome uptake, even with PEGylated lipids [32]. The miPEG-PE on the surface of the ALPs was introduced to conjugate ligands with the sulfhydryl groups. The siRNA encapsulation, particle size, and zeta potential of the FITC-siRNA/ALPs with miPEG-PE are compatible to those of the ALPs shown in Fig. 2. The ALPs with negatively charged surface showed no uptake into cells without surface modification (Fig. 4). It has been reported that polyarginine effectively delivers liposome into cells and induces siRNA-mediated gene-silencing [33]. In this study, the R12-modified FITC-siRNA/ALPs were prepared by conjugation between the sulfhydryl group of R12 and the maleimide group of miPEG-PE on the ALPs. The R12-modified ALPs are effectively internalized into all the cells in the presence of 10% FBS (Fig. 4). The internalized FITC-siRNA is predominantly located at the endosome (Fig. 4), which means the R12-modified FITC-siRNA may be internalized by the endocytotic process as reported previously [34,35]. This pattern is similar to the pattern of LF2K-mediated FITC-siRNA transfection (data not shown).

3.4. Antibody-mediated cell-specific uptake of siRNA/ALPs

The NSCLC cell lines have been reported to overexpress EGFR [36]. The anti-EGFR antibody Erbitux induces internalization of the antibody in cervical cancer cells [37]. A recent report indicates that the anti-EGFR antibody can be used to deliver siRNA/liposomes to breast cancer cells that overexpress EGFR [32]. In this study, we initially tested the EGFR expression on the surface of the NSCLC cells (A549, NCI-H322, and NCI-H322) and the NIH-3T3 cells. As expected, the antibody effectively interacts with all the NSCLC cell lines but not with the NIH-3T3 cells (data not shown). The FITC-modified antibody is internalized into the NSCLC cells by mean of the EGFR-mediated endocytosis but not into the NIH-3T3 cells, and predominantly localize at the cytoplasm after overnight incubation (Supplementary Fig. 3).

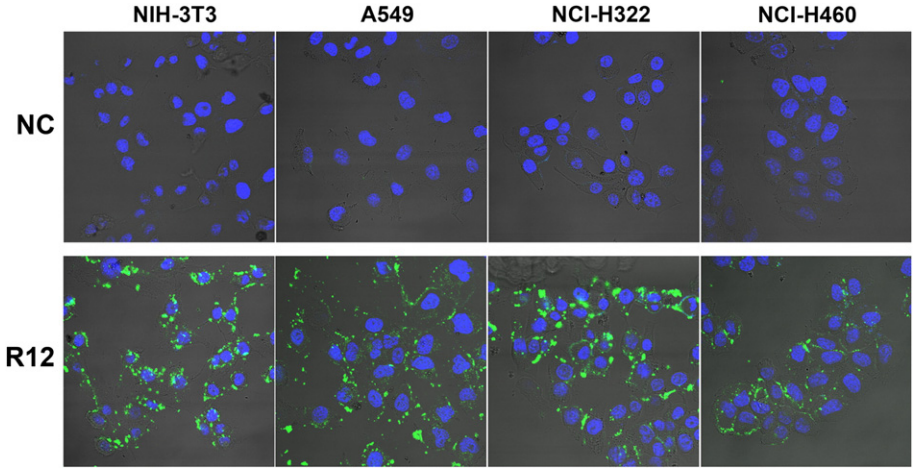

Fig. 4. Cellular uptake of ALPs with and without R12 modification. The four cell lines NIH-3T3, A 549, NCI-H322, and NCI-H 460 were transfected with FITC-siRNA/APLs with and without R12 modification on their outer layers. The non-conjugated ALPs (NC) show no penetration into any of the cell lines, whereas the R12-modified ALPs display a high uptake efficiency in all the cell lines because R12 induces a nonspecific cellular uptake.

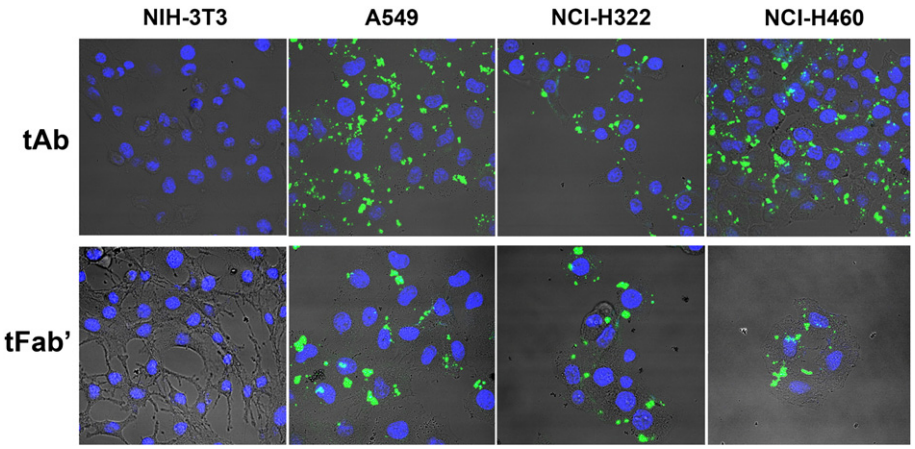

Fig. 5. Cellular uptake of ALPs modified with a thiolated antibody and Fab′. The four cell lines NIH-3T3, A 549, NCI-H322, and NCI-H 460 were transfected with FITC-siRNA/APLs modified with a thiolated anti-EGFR (tAb) or Fab′ (tFab′) on their outer layers. All the modified ALPs display a high uptake efficiency in the NSCLC cell lines due to the human EGFR expression on their surface but not in the NIH-3T3 cells in which there is no human EGFR expression.

We successfully purified the Fab′ (Supplementary Fig. 1B) and tried to conjugate the Fab′ with the free thiol group to the maleimide groups on the ALPs. Unfortunately, the conjugation of the Fab′ to the ALPs failed (data not shown), even though we incubated the mixture of Fab′ and ALPs overnight at room temperature. We consequently used 2-imminothiolane to thiolate the anti-EGFR antibody and Fab′ [32], and named tAb and tFab′, respectively. Four to five free thiol groups per a molecule of antibody or Fab′ were formed after the 2-imminothiolane treatment. Then tAb was incubated with biotin maleimide (Sigma, USA) to test whether the thiolation changes the antibody property. The results show that the biotinylated tAb was effectively internalized into the NSCLC cells but not into the NIH-3T3 cells (Supplementary Fig. 3), which suggests that the thiolation does not change the antibody-mediated endocytosis. Finally tAb or tFab′ was conjugated to the FITC-siRNA/ALPs. Because the antibody is hard to remove by dialysis, we used buoyant density gradient centrifugation with sucrose gradients to remove any unattached antibody or Fab′. Most of the unattached antibody was found at the bottom fraction (37% sucrose), but no siRNA appeared in the bottom fraction (data not shown). The ALPs were localized in the 9% sucrose regardless of the conjugating molecules. The purified ALPs possess the encapsulated siRNA as well as the antibody that is attached to the miPEG-PE (Supplementary Fig. 4A and 4B, respectively). In addition, the density gradient method was found to be useful for eliminating the non-encapsulated siRNA. The tAb-modified and tFab′-modified FITC-siRNA/ALPs were internalized into all three NSCLC cells but not into the NIH-3T3 cells (Fig. 5).

3.5. Gene-silencing of siRNA/ALPs

Because siRNA induces gene-silencing in cytoplasm, the intracellular siRNA delivery does not reflect its function in cells. In this study, we used siGL3 and siTMPR4 as model systems to investigate the gene-silencing function of siRNA/ALPs in cells. Unmodified siGL3/ALPs show almost no ability to prevent luciferase activity, whereas tAb-modified ALPs elicit a similar level of inhibition with LF2K (Fig. 6). However, particles with scrambled siRNA fail to prevent luciferase activity.

TMPRSS4 is upregulated in many cancer cells and induces many oncogenic functions, including tumor cell adhesion, migration, invasion and *in vitro* growth. These functions can be reversed with a TMPRSS4 knockdown induced by siTMPR4 [30]. The results of our analysis, which involved in TMPRSS4 mRNA depletion with both commercially available transfecting reagents LF2K and siTMPR4/25 K polyethyleneimine, confirm the gene-silencing effect of siTMPR4 (Fig. 7A and B, respectively). In addition, compared with a previous report on scrambled siRNA [30], the TMPRSS4 knockdown significantly suppresses NCI-H322 cell invasion and NCI-H322 cell migration (Fig. 7A and B, respectively).

As shown in Fig. 7C and D, the unmodified siTMPR4/ALPs (NC), which cannot penetrate the cells, show no ability to prevent TMPRSS4 transcription and any cell migration and invasion. However, the R12-modified siTMPR4/ALPs significantly inhibit the TMPRSS4 transcription as well as the cell migration and invasion (Fig. 7C). The results indicate that the internalized siTMPR4/ALPs effectively induce siRNA-dependent TMPRSS4 gene-silencing.

The tAb-modified and tFab′-modified siTMPR4/ALPs significantly inhibit the TMPRSS4 transcription as compared with the unmodified siTMPR4/ALPs (Fig. 7D). Interestingly, the TMPRSS4 knockdown was also induced by treatment with tAb-modified and tFab′-modified scrambled siRNA/ALPs. The TMPRSS4 depletion was synergistically enhanced by the siTMPR4. The synergistic effects of siTMPR4 and the anti-hEGFR antibody (tAb) or Fab′ (tFab) also observed in the study of the cell migration and invasion in correlation with the TMPRSS4 knockdown (Fig. 7D). The anti-EGFR antibody (Erbitux) evokes tumoricidal activity in cancer cells expressing EGFR. We therefore assume that the inhibition of the EGFR function by the antibody may also inhibit the TMPRSS4 expression, though further study of this assumption is needed.

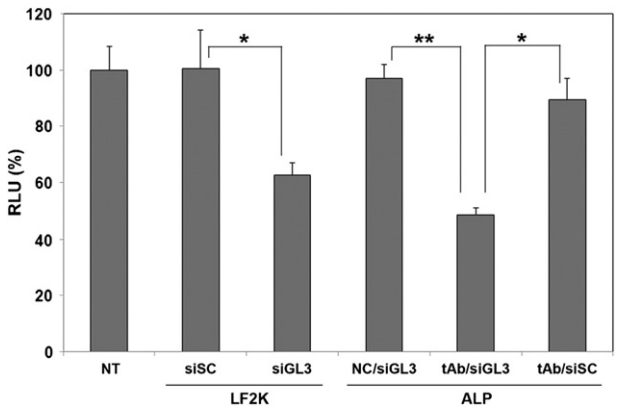

Fig. 6. *In vitro* luciferase gene-silencing by siGL3. NCI-H322 cells first were transfected by pGL3 — control vector were treated with either siGL3 or scrambled siRNA (siSC) in a complex with lipofectamine 2000 (LF2K) or encapsulated in ALPs with or without tAb conjugation (NC or tAb, respectively). The luciferase activities were measured 48 h after the transfection of siRNA. NT refers to no siRNA treatement.

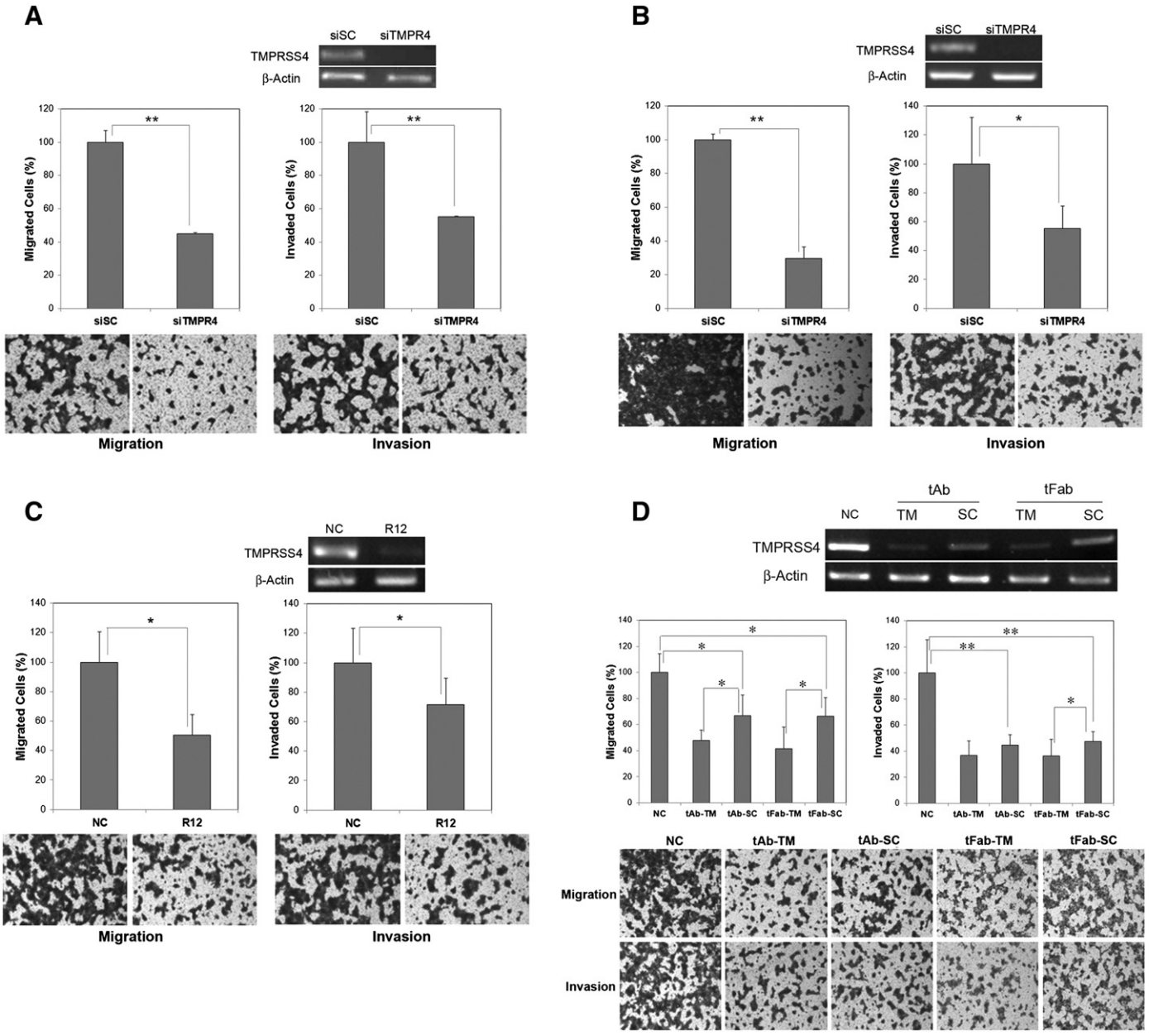

Fig. 7. TMPRSS4 knockdown and inhibitions of cell migration and invasion by siTMPR4. NCI-H322 cells were treated with siTMPR4 and siSC under various conditions. After 48 h of incubation, the cells were harvested and subjected to total RNA extraction and invasion as well as cell migration assays. The TMPRSS4 mRNA levels were determined by RT-PCR, and β-Actin was used as an internal control. For invasion and migration assays, 4×10^4 cells were allowed to migrate or invade Matrigel for 48 h. Representative high-power (×200) fields (HPFs) were photographed, and the number of cells that had migrated or invaded was counted in six to eight representative HPFs per Transwell insert. The siSC complex with LF2K or PEI25K and the siTMPR4/ALPs without conjugation (NC) were used as controls. The values are the mean plus or minus the standard deviation (*$P<0.05$ and **$P<0.01$). The samples of siSC and siTMPR4 were transfected by using (A) LF2K, (B) PEI25K, (C) ALPs conjugated with R12, and (D) ALPs conjugated with tAb or tFab′.

4. Discussion

Our aim in this study is to develop a novel method of producing ALPs that satisfy two goals: a higher siRNA encapsulation efficiency and a negatively charged surface that provides less nonspecific absorption [38,39]. Lipids with small head groups can stabilize an inverted micelle structure, whereas lipids with large head groups destabilize an inverted micelle structure. We assume that an unstable outer inverted micelle has the potential to quickly disrupt and surround the outside of the inner inverted micelle during organic solvent evaporation as shown in Fig. 1.

We initially focused on DODAP. Depending on the pH level, DODAP can participate in a strong interaction with siRNA. The siRNA encapsulation was successful when we used a combination of DODAP and DOPE (9:1) at pH 4.0 to make the inner inverted micelle. Unexpectedly, the increase of pH to 5.0 induced almost no siRNA encapsulation (Fig. 2A), and the complete removal of the inner DOPE prevents the formation of the inner inverted micelle structure (data not shown). A possible explanation for this behavior is that the dimethylammonium group of DODAP can be more strongly hydrogenated at pH 4.0 than pH 5.0 and the interaction between the hydrogen atom of DODAP and the oxygen atom(s) of the phosphate group of DOPE (Supplementary Fig. 5) may have stabilized the inner compartment of ALPs. In this aspect, DOTAP without ionizable hydrogen cannot form the hydrogen interaction with DOPE, and this and its large head group may make the unstable inner compartment. We assume that the property of DOTAP may induce almost no encapsulation of siRNA (Fig. 2A). The outer lipids are less critical than the

inner lipids but the chain length and fatty acyl tail saturation affect the particle size and encapsulation (Fig. 2B).

We subsequently studied the ALP delivery in cells. The fact that there is no cellular delivery of the ALPs without any surface modification, as shown in Fig. 4, is not surprising because the particles have a negatively charged and PEGylated surface. In other words, the ALPs without any nonspecific delivery have great potential for being developed as cargo for the target-specific delivery of siRNA after being conjugated with a target-specific ligand. Indeed, the conjugation of the ligands (R12 and anti-EGFR antibody) to ALPs remarkably enhances the cellular uptake of ALPs (Figs. 4 and 5). Moreover, the anti-EGFR conjugation induces the target cell-specific delivery (Fig. 5).

The direct conjugation of the Fab′ sulfhydryl group to the maleimide groups on ALPs was not successful (data not shown). One possible explanation is that the cysteine sulfhydryl group in Fab′ may be insufficient to access the maleimide group on huge ALPs. In contrast, as previously reported [32], when 2-imminothiolane is used for the thiolation at the lysine residues of the antibody, there is enough space for the ALPs. SDS-PAGE was used to demonstrate the multiple conjugations of miPEG-PE to tAb and tFab′ (Supplementary Fig. 4), and the multiple conjugations indicate the need to control the attachment of the thiolated antibody. Although nonspecific thiolation hinders the uniform conjugation of the antibody or Fab′ to the ALPs, the conjugation can induce an antibody-specific ALP uptake (Fig. 5).

An endosomal escape of siRNA is required for the siRNA action because the target gene-silencing takes place in a cytoplasm. Although we primarily observed the siRNA in the endosome compartment (Figs. 4 and 5), the ALP uptake capability was clearly depending on the gene-silencing efficiency (Figs. 6 and 7). One possible explanation is the time and siRNA differences in the experiments. For a delivery assay, the cells were monitored 24 h after being treated with FITC-siRNA. And for a gene-silencing assay, the cells were collected 48 h after being treated with siRNA in the absence of any fluorescence dye. In addition, we found similar distribution in the FITC-siRNA transfected by LF2K which is generally used for siRNA-mediated gene-silencing (data not shown), supporting our explanation. We assume that the endosomal escape of FITC-siRNA is slower than that of non-labeled siRNA.

The anti-EGFR antibody Erbitux evokes anticancer activity for EGFR-bearing cells by inhibiting EGFR autophosphorylation [40]. It considered as an effective treatment for NSCLC as a supplement to platinum-based chemotherapy [41]. According to this view, the antibody and Fab′-mediated anticancer activity may be related to significant reduction of the TMPRSS4 transcription as well as the inhibition of the cell migration and invasion regardless of the siRNA content. Because the antibody did not inhibit the β-actin transcription (Fig. 7D), the TMPRSS4 knockdown can possibly be explained in terms of the association with its anti-EGFR function, such as the inhibition of EGFR autophosphorylation. The fact that the antibody does not inhibit the luciferase expression supports this assumption (Fig. 6). The TMPRSS4 knockdown is synergistically enhanced by the siTMPR4 (Fig. 7D). The co-treatment of the anti-EGFR antibody and siTMPR4 may be useful for cancer therapy of those cells that express both proteins, and the ALPs can be used as cargo for the antibody and siRNA.

In conclusion, we developed a method of preparing ALPs with a high siRNA encapsulation efficiency as well as a negatively charged surface that prevents nonspecific uptake of the ALPs. The ALP internalization can be induced by conjugation of R12 or the EGFR antibody, and the siRNA in the internalized ALPs can successfully induce siRNA-dependent gene-silencing activity. Taken together, these results demonstrate that ALPs may be useful cargo for the delivery of target-specific siRNA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2012.03.016.

References

[1] D.M. Dykxhoorn, J. Lieberman, Running interference: prospects and obstacles to using small interfering RNAs as small molecule drugs, Annu. Rev. Biomed. Eng. 8 (2006) 377–402.
[2] D.M. Dykxhoorn, D. Palliser, J. Lieberman, The silent treatment: siRNAs as small molecule drugs, Gene Ther. 13 (2006) 541–552.
[3] M.T. McManus, P.A. Sharp, Gene silencing in mammals by small interfering RNAs, Nat. Rev. Genet. 3 (2002) 737–747.
[4] J.W. Shiver, T.M. Fu, L. Chen, D.R. Casimiro, M.E. Davies, R.K. Evans, Z.Q. Zhang, A.J. Simon, W.L. Trigona, S.A. Dubey, L. Huang, V.A. Harris, R.S. Long, X. Liang, L. Handt, W.A. Schleif, L. Zhu, D.C. Freed, N.V. Persaud, L. Guan, K.S. Punt, A. Tang, M. Chen, K.A. Wilson, K.B. Collins, G.J. Heidecker, V.R. Fernandez, H.C. Perry, J.G. Joyce, K.M. Grimm, J.C. Cook, P.M. Keller, D.S. Kresock, H. Mach, R.D. Troutman, L.A. Isopi, D.M. Williams, Z. Xu, K.E. Bohannon, D.B. Volkin, D.C. Montefiori, A. Miura, G.R. Krivulka, M.A. Lifton, M.J. Kuroda, J.E. Schmitz, N.L. Letvin, M.J. Caulfield, A.J. Bett, R. Youil, D.C. Kaslow, E.A. Emini, Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity, Nature 415 (2002) 331–335.
[5] E. Song, S.K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, J. Lieberman, RNA interference targeting Fas protects mice from fulminant hepatitis, Nat. Med. 9 (2003) 347–351.
[6] P.D. Zamore, T. Tuschl, P.A. Sharp, D.P. Bartel, RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals, Cell 101 (2000) 25–33.
[7] T.P. Chendrimada, R.I. Gregory, E. Kumaraswamy, J. Norman, N. Cooch, K. Nishikura, R. Shiekhattar, TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing, Nature 436 (2005) 740–744.
[8] M.A. Behlke, Progress towards *in vivo* use of siRNAs, Mol. Ther. 13 (2006) 644–670.
[9] S. Kawakami, M. Hashida, Targeted delivery systems of small interfering RNA by systemic administration, Drug Metab. Pharmacokinet. 22 (2007) 142–151.
[10] D.V. Morrissey, J.A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C.S. Shaffer, L.B. Jeffs, A. Judge, I. MacLachlan, B. Polisky, Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs, Nat. Biotechnol. 23 (2005) 1002–1007.
[11] R.E. Donahue, S.W. Kessler, D. Bodine, K. McDonagh, C. Dunbar, S. Goodman, B. Agricola, E. Byrne, M. Raffeld, R. Moen, et al., Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer, J. Exp. Med. 176 (1992) 1125–1135.
[12] K.A. Howard, Delivery of RNA interference therapeutics using polycation-based nanoparticles, Adv. Drug Deliv. Rev. 61 (2009) 710–720.
[13] D. Schaffert, E. Wagner, Gene therapy progress and prospects: synthetic polymer-based systems, Gene Ther. 15 (2008) 1131–1138.
[14] P.R. Dash, M.L. Read, K.D. Fisher, K.A. Howard, M. Wolfert, D. Oupicky, V. Subr, J. Strohalm, K. Ulbrich, L.W. Seymour, Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin, J. Biol. Chem. 275 (2000) 3793–3802.
[15] D.E. Owens III, N.A. Peppas, Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles, Int. J. Pharm. 307 (2006) 93–102.
[16] M. Ogris, S. Brunner, S. Schuller, R. Kircheis, E. Wagner, PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery, Gene Ther. 6 (1999) 595–605.
[17] P. Opanasopit, M. Nishikawa, M. Hashida, Factors affecting drug and gene delivery: effects of interaction with blood components, Crit. Rev. Ther. Drug Carrier Syst. 19 (2002) 191–233.
[18] T. Ren, Y.K. Song, G. Zhang, D. Liu, Structural basis of DOTMA for its high intravenous transfection activity in mouse, Gene Ther. 7 (2000) 764–768.
[19] Y. Zhang, P. Cristofaro, R. Silbermann, O. Pusch, D. Boden, T. Konkin, V. Hovanesian, P.R. Monfils, M. Resnick, S.F. Moss, B. Ramratnam, Engineering mucosal RNA interference *in vivo*, Mol. Ther. 14 (2006) 336–342.
[20] W.C. Zamboni, Liposomal, nanoparticle, and conjugated formulations of anticancer agents, Clin. Cancer Res. 11 (2005) 8230–8234.
[21] W. Li, F.C. Szoka Jr., Lipid-based nanoparticles for nucleic acid delivery, Pharm. Res. 24 (2007) 438–449.
[22] S.D. Li, Y.C. Chen, M.J. Hackett, L. Huang, Tumor-targeted delivery of siRNA by self-assembled nanoparticles, Mol. Ther. 16 (2008) 163–169.
[23] S.C. Semple, S.K. Klimuk, T.O. Harasym, N. Dos Santos, S.M. Ansell, K.F. Wong, N. Maurer, H. Stark, P.R. Cullis, M.J. Hope, P. Scherrer, Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures, Biochim. Biophys. Acta 1510 (2001) 152–166.

[24] S.Y. Wu, L.N. Putral, M. Liang, H.I. Chang, N.M. Davies, N.A. McMillan, Development of a novel method for formulating stable siRNA-loaded lipid particles for *in vivo* use, Pharm. Res. 26 (2009) 512–522.
[25] S. Chono, S.D. Li, C.C. Conwell, L. Huang, An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor, J. Control. Release 131 (2008) 64–69.
[26] S.C. Semple, A. Akinc, J. Chen, A.P. Sandhu, B.L. Mui, C.K. Cho, D.W. Sah, D. Stebbing, E.J. Crosley, E. Yaworski, I.M. Hafez, J.R. Dorkin, J. Qin, K. Lam, K.G. Rajeev, K.F. Wong, L.B. Jeffs, L. Nechev, M.L. Eisenhardt, M. Jayaraman, M. Kazem, M.A. Maier, M. Srinivasulu, M.J. Weinstein, Q. Chen, R. Alvarez, S.A. Barros, S. De, S.K. Klimuk, T. Borland, V. Kosovrasti, W.L. Cantley, Y.K. Tam, M. Manoharan, M.A. Ciufolini, M.A. Tracy, A. de Fougerolles, I. MacLachlan, P.R. Cullis, T.D. Madden, M.J. Hope, Rational design of cationic lipids for siRNA delivery, Nat. Biotechnol. 28 (2010) 172–176.
[27] S.C. Semple, S.K. Klimuk, T.O. Harasym, M.J. Hope, Lipid-based formulations of antisense oligonucleotides for systemic delivery applications, Methods Enzymol. 313 (2000) 322–341.
[28] F. Szoka Jr., D. Papahadjopoulos, Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 4194–4198.
[29] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[30] H. Jung, K.P. Lee, S.J. Park, J.H. Park, Y.S. Jang, S.Y. Choi, J.G. Jung, K. Jo, D.Y. Park, J.H. Yoon, D.S. Lim, G.R. Hong, C. Choi, Y.K. Park, J.W. Lee, H.J. Hong, S. Kim, Y.W. Park, TMPRSS4 promotes invasion, migration and metastasis of human tumor cells by facilitating an epithelial–mesenchymal transition, Oncogene 27 (2008) 2635–2647.
[31] W. Zhao, S. Zhuang, X.R. Qi, Comparative study of the *in vitro* and *in vivo* characteristics of cationic and neutral liposomes, Int. J. Nanomedicine 6 (2011) 3087–3098.
[32] J. Gao, W. Liu, Y. Xia, W. Li, J. Sun, H. Chen, B. Li, D. Zhang, W. Qian, Y. Meng, L. Deng, H. Wang, J. Chen, Y. Guo, The promotion of siRNA delivery to breast cancer overexpressing epidermal growth factor receptor through anti-EGFR antibody conjugation by immunoliposomes, Biomaterials 32 (2011) 3459–3470.
[33] C. Zhang, N. Tang, X. Liu, W. Liang, W. Xu, V.P. Torchilin, siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene, J. Control. Release 112 (2006) 229–239.
[34] A. El-Sayed, I.A. Khalil, K. Kogure, S. Futaki, H. Harashima, Octaarginine- and octalysine-modified nanoparticles have different modes of endosomal escape, J. Biol. Chem. 283 (2008) 23450–23461.
[35] S.M. Fuchs, R.T. Raines, Pathway for polyarginine entry into mammalian cells, Biochemistry 43 (2004) 2438–2444.
[36] M. Haeder, M. Rotsch, G. Bepler, C. Hennig, K. Havemann, B. Heimann, K. Moelling, Epidermal growth factor receptor expression in human lung cancer cell lines, Cancer Res. 48 (1988) 1132–1136.
[37] M. Eiblmaier, L.A. Meyer, M.A. Watson, P.M. Fracasso, L.J. Pike, C.J. Anderson, Correlating EGFR expression with receptor-binding properties and internalization of 64Cu-DOTA-cetuximab in 5 cervical cancer cell lines, J. Nucl. Med. 49 (2008) 1472–1479.
[38] J. Heyes, L. Palmer, K. Chan, C. Giesbrecht, L. Jeffs, I. MacLachlan, Lipid encapsulation enables the effective systemic delivery of polyplex plasmid DNA, Mol. Ther. 15 (2007) 713–720.
[39] D. Peer, E.J. Park, Y. Morishita, C.V. Carman, M. Shimaoka, Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target, Science 319 (2008) 627–630.
[40] M.S. Kies, P.M. Harari, Cetuximab (Imclone/Merck/Bristol-Myers Squibb), Curr. Opin. Investig. Drugs 3 (2002) 1092–1100.
[41] R. Pirker, J.R. Pereira, A. Szczesna, J. von Pawel, M. Krzakowski, R. Ramlau, I. Vynnychenko, K. Park, C.T. Yu, V. Ganul, J.K. Roh, E. Bajetta, K. O'Byrne, F. de Marinis, W. Eberhardt, T. Goddemeier, M. Emig, U. Gatzemeier, Cetuximab plus chemotherapy in patients with advanced non-small-cell lung cancer (FLEX): an open-label randomised phase III trial, Lancet 373 (2009) 1525–1531.