Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

European Journal of Pharmaceutics and Biopharmaceutics 74 (2010) 421–426

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Chitosan-modified poly(D,L-lactide-*co*-glycolide) nanospheres for improving siRNA delivery and gene-silencing effects

Kohei Tahara [a,b], Hiromitsu Yamamoto [a,*], Naohide Hirashima [b], Yoshiaki Kawashima [a]

[a] *Laboratory of Pharmaceutical Engineering, Aichi Gakuin University, Nagoya, Japan*
[b] *Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan*

ARTICLE INFO

Article history:
Received 27 July 2009
Accepted in revised form 16 December 2009
Available online 23 December 2009

Keywords:
siRNA
PLGA
Nanosphere
Chitosan
Emulsion solvent diffusion method

ABSTRACT

Chitosan (CS) surface-modified poly(D,L-lactide-*co*-glycolide) (PLGA) nanospheres (NS) for a siRNA delivery system were evaluated *in vitro*. siRNA-loaded PLGA NS were prepared by an emulsion solvent diffusion (ESD) method, and the physicochemical properties of NS were investigated. The level of targeted protein expression and siRNA uptake were examined in A549 cells. CS-modified PLGA NS exhibited much higher encapsulation efficiency than unmodified PLGA NS (plain-PLGA NS). CS-modified PLGA NS showed a positive zeta potential, while plain-PLGA NS were negatively charged. siRNA uptake studies by observation with confocal leaser scanning microscopy (CLSM) indicated that siRNA-loaded CS-modified PLGA NS were more effectively taken up by the cells than plain-PLGA NS. The efficiencies of different siRNA preparations were compared at the level of targeted protein expression. The gene-silencing efficiency of CS-modified PLGA NS was higher and more prolonged than those of plain-PLGA NS and naked siRNA. This result correlated with the CLSM studies, which may have been due to higher cellular uptake of CS-modified PLGA NS due to electrostatic interactions. It was concluded that CS-modified PLGA NS containing siRNA could provide an effective siRNA delivery system.

1. Introduction

Recent efforts in the design of molecular biology tools have focused on the RNA interference (RNAi) effect to knock down the expression of a target protein [1]. Small interfering RNAs (siRNA), which are small double-stranded RNA (dsRNA) oligonucleotides with or without overhangs, are substrates for the RNA-induced silencing complex [2]. When transfected into cells, synthetic siRNAs strongly inhibit the expression of a target protein in mammalian cells. The application of siRNA to human therapy has attracted much attention, although the delivery of siRNA to the appropriate cells, tissues, or organs remains a major challenge. An ideal gene delivering carrier should safely transport genetic materials without any toxic effects or inducing immune responses [3]. Most research has used viral vectors, retroviruses, and adenoviruses, or liposomes [4]. Recently, many non-viral vectors have been reported that were modified with fusogenic peptides [5], cationic lipids [6], cationic polymers [7–9], and others. However, some cationic compounds show cytotoxicity effects.

Polymeric nanospheres (NS) have been used for drug delivery due to their high stability and are easily taken up into cells by endocytosis, and they can target specific tissues or organs by adsorption or binding with ligands attached to the surface of the particles [10]. In particular, biodegradable nanospheres are available for delivering drugs and are degraded after passing a required specific site [11]. Among these, poly(lactide) (PLA) and poly(D,L-lactide-*co*-glycolide) (PLGA) have been approved by the FDA for certain human clinical uses. The degradation time of PLGA can be altered from days to years by varying the molecular weight, the lactic acid to glycolic acid ratio in the copolymer, or the nanospheres' structure. PLGA nanospheres have been suggested to be a good gene delivery carrier because of their safety and their properties of sustained release [12,13]. We have developed an emulsion solvent diffusion (ESD) method in water for preparing PLGA NS [14]. The advantages of this ESD method are that NS can be prepared by a simple process under mild conditions without sonication. A PLGA NS platform to encapsulate a wide variety of nucleic acids (e.g., plasmid DNA, antisense oligonucleotides, siRNA) for gene delivery was established using the ESD method [15].

We have also investigated the usefulness of cationically charged CS-modified PLGA NS to improve bioavailability and for a pulmonary plasmid DNA (pDNA) gene delivery system *in vitro* and *in vivo* [manuscript submitted]. A number of pulmonary diseases are candidates for pulmonary gene delivery. Lung cancer, influenza virus infection, respiratory syncytial virus infection (RSV), and severe acute respiratory syndrome (SARS) have attracted particular attention as targets of siRNA therapy [16,17].

* Corresponding author. Laboratory of Pharmaceutical Engineering, School of Pharmacy, Aichi Gakuin University, 1-100, Kusumoto, Chikusa, Nagoya, Aichi 464-8650, Japan. Tel.: +81 52 757 6771; fax: +81 52 757 6799.
E-mail address: hiromitu@dpc.agu.ac.jp (H. Yamamoto).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.12.007

CS-modified PLGA NS that was previously tested for pDNA delivery for pulmonary gene therapy has been adapted for siRNA because the barriers to delivery are similar. The aims of this study are to evaluate siRNA-loaded PLGA NS for cellular uptake and RNAi effects using human lung adenocarcinoma cells *in vitro*.

2. Materials and methods

2.1. Materials

PLGA (lactide:glycolide = 75:25, MW = 5000) was purchased from Wako (Osaka, Japan). Polyvinylalcohol (PVA) was purchased from Kuraray (Osaka, Japan). Chitosan (MW 20,000; deacetylation degree 84.2%) was obtained from Katakurachikkarin (Tokyo, Japan). The fluorescent dye coumarin 6, laser grade, [3-(2-benzothiazolyl)-7-(diethylamine) coumarin] (6-coumarin), was purchased from MP Biomedicals (Solon, OH). A549 human lung adenocarcinoma cells (A549) were purchased from RIKEN Gene Bank (Ibaraki, Japan). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Basel, Switzerland). D(−)-Mannitol (Kishida Chemical Co., Ltd., Osaka, Japan) was used as a cryoprotectant for the reconstitution of siRNA-loaded PLGA NS when redispersed in aqueous medium. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP, Sigma, St. Louis, MO) was used as a cationic complexing agent for the preparation of siRNA-loaded PLGA NS. As a model siRNA, we used annealed siRNA targeting pGL3 firefly luciferase (Luc-siRNA, sense: 5′-CUUACGCUGAGUACUUCGAdTdT-3′, antisense: 3′-dTdTGAAUGCGACUCAUGAAGCU-5′, Dharmacon Inc., Chicago, IL) and Cy3-labeled siRNA targeting the same protein (sense: 5′-CUUACGCUGAGUACUUCGAdTdT-3′, antisense: 3′-dTdTGAAUGCGACUCAUGAAGCU-5′-Cy3, Dharmacon). The annealed siRNA targeting protein kinase c alpha (PKC-siRNA, sense: 5′-GGACAUAUCAAAAUUGCUGdTdT-3′, antisense: 3′-dTdTCCUGUAUAGUUUUAACGAC-5′, Ambion, Austin, TX) was used as a control siRNA. All other chemicals were obtained commercially at the highest available analytical grade.

2.2. Cell lines and cell culture

A549 cells were grown in DMEM supplemented with 10% FBS and 50 μg/ml penicillin and streptomycin at 37 °C in a humidified incubator with 5% CO_2. After confluent growth, A549 cells were trypsinized and seeded in plates for each experiment. To establish A549 cells that stably expressed firefly luciferase (A549-Luc), A549 cells were transfected with plasmid DNA encoding firefly luciferase under the control of cytomegalovirus immediate early promoter complexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) [18]. The cells were then treated with the medium containing 1 mg/mL G418 (Geneticin, Sigma), and G418-resistant cells were picked and examined for their luciferase activity as described below. There was no significant difference in the growth rates of A549 and A549-Luc cells *in vitro*. In addition, they were microscopically identical.

2.3. Preparation of siRNA-loaded PLGA NS by the emulsion solvent diffusion method

siRNA-loaded PLGA NS were prepared by the emulsion solvent diffusion method in water [15]. First, to prepare siRNA/DOTAP complexes, 50 μl of Milli-Q water containing 300 μg of siRNA was added to the same volume of Milli-Q water containing 300 μg of DOTAP solution with stirring. The PLGA (50 mg) and siRNA/DOTAP complex solution was dissolved in acetone (2 ml). For the preparation of fluorescence-labeled PLGA NS, 100 μg of 6-coumarin was added to this organic solution, which dissolved both PLGA and siRNA. The resultant organic solution was poured into 25 ml of an aqueous PVA solution (2%, w/v) and stirred at 400 rpm using a propeller-type agitator with three blades. The entire dispersed system was then centrifuged (43,400*g* for 10 min) and resuspended in Milli-Q water (10 ml). The same volume of mannitol solution (5%, w/v) as a cryoprotectant was added to the NS suspension. The resultant dispersion was dried by freeze drying. For the preparation of CS-modified PLGA NS, chitosan (0.05%, w/v, in 0.5 M acetate buffer, pH 4.4)-PVA (1%, w/v, in distilled water) mixed solution was used as the dispersing phase for the emulsion solvent diffusion process.

2.4. Preparation of siRNA complexes

In this study, 2 siRNA complexes (siRNA/DOTAP and siRNA/CS-modified PLGA NS) were evaluated *in vitro*. To prepare a siRNA/DOTAP complex, 50 μl of Milli-Q water containing 50 μg of siRNA was added to the same volume of Milli-Q water containing 300 μg of DOTAP solution with stirring. For the siRNA/NS complex, 100 μl of Milli-Q water was used to suspend 1 mg of blank (empty) CS-modified PLGA NS (10 mg/ml) that was added rapidly to an equivalent volume of Milli-Q water containing siRNA (1 mg/ml) with stirring.

2.5. Analysis of NS physicochemical properties

Particle size and zeta potential measurements were performed using Zetasizer® 3000 HSA (Malvern Instruments, UK). Particle size was measured by photon correlation spectroscopy (PCS). Zeta potential determinations were based on electrophoretic mobility of the NS in an aqueous medium. The surface topology and shape of a nanosphere were observed by scanning electron microscope (SEM, JSM-T330A, Nihon Denshi, Japan). The amount of Cy3-labeled siRNA entrapped in the NS was analyzed by dissolving the NS (2 mg) in acetonitrile (1 ml), to which acetate buffer (pH 4.4, 0.5 ml) containing 0.1% (w/v) SDS was added to precipitate the polymer and dissolve the siRNA in the resultant aqueous mixture. This solution was centrifuged (43,400*g* for 10 min), and the siRNA contents in the supernatant were determined by Cy3 fluorescence measurement with a fluorescence spectrophotometer (F-3010, excitation wavelength 550 nm, emission wavelength 565 nm; Hitachi, Tokyo, Japan). The loading efficiency of siRNA was calculated from the following equation:

$$\text{Loading efficiency } (\%) = \frac{\text{weight of nucleic acids in NSs}}{\text{weight of nucleic acids formulated in the system}} \times 100$$

2.6. siRNA release studies

The release properties of Cy3-labeled siRNA from NS were investigated *in vitro*. A 10 mg sample of NS was dispersed in 5 ml PBS (pH 7.4) in a test tube shaken horizontally at 37 °C. At different residence times, the buffer was separated from the NS by centrifugation (43,400 *g* for 10 min) and analyzed for the amount of released Cy3-labeled siRNA. After each determination, the NS were resuspended in fresh medium.

2.7. Microscopic studies

A549 cells were grown on Lab-Tek® II Chamber Slides (Nalge Nunc International, Rochester, NY) at a density of 2.0×10^4 cells/well. Experiments were conducted once the cells had formed confluent monolayers as determined by light microscopy. The growth medium was replaced with a suspension of different Cy3-

siRNA preparations in serum-free DMEM, and then the system was incubated for 4 h at 37 °C. After incubating cell monolayers with different siRNA preparations, monolayers were fixed with 4% paraformaldehyde. After washing with PBS, cover slips were mounted on slides using the SlowFade anti-fade kit (Molecular Probes, Eugene, OR). The fixed cells were observed with a confocal laser scanning microscope (Carl Zeiss LSM 510, Goettingen, Germany) equipped with a Zeiss Plan-Neofluar×100/1.3 oil immersion objective lens, using an argon-ion laser (458–514 nm) and a helium/neon laser (543 nm). For F-actin staining, fixed cells were permeabilized in 0.1% Triton X-100 in PBS for 5 min and incubated with Alexa Fluor® 488-conjugated phalloidin (Molecular Probes) in PBS for 60 min at room temperature. After washing with PBS, the cover slips were mounted on slides as above.

2.8. Measurement of luciferase activity

A549-Luc cells were seeded 24 h before transfection at a density of 2.0×10^5 cells per well in 12-well plates. The experimental schedule is shown in Fig. 1. The growth medium was replaced with a suspension of different siRNA preparations in serum-free DMEM (50 nM siRNA), and the system was incubated for 48 h at 37 °C. The NS suspension was replaced with FBS containing DMEM, and the system was incubated for 0–3 days. Culture medium was renewed once two days. At various times after transfection, cells were rinsed three times with ice-cold PBS, solubilized with 0.2 ml of reporter cell lysis reagent (Promega, Madison, WI), and centrifuged at 17,970*g* for 2 min. Supernatants were collected and analyzed for luciferase activity. In a typical experiment, 100 μl of luciferase assay buffer (Promega) was added to 20 μl of supernatant in the tubes. The luciferase activity of these samples was measured with a Glomax 20/20 luminometer (Promega). Cellular protein contents were determined with a BCA Protein Assay (Pierce, Rockford, IL) to convert the data into luciferase activity (RLU) per milligram of protein. Results were expressed as a percentage of control.

3. Results and discussion

3.1. siRNA-loaded PLGA NS characterization

Five siRNA preparations were evaluated *in vitro*: naked siRNA, siRNA/DOTAP complex, siRNA/DOTAP-loaded unmodified PLGA NS (plain-PLGA NS), siRNA/DOTAP-loaded PLGA NS modified with chitosan (CS-modified PLGA NS), and siRNA/CS modifying *blank*-PLGA NS complex (siRNA/NS complex). Various formulation factors and physicochemical properties of the NS play key roles in biological applications. The most important factors that can influence the transfection and cellular uptake are the particle size and the surface properties of the NS [19]. Physicochemical properties of the siRNA preparations are shown in Table 1. DOTAP, a commercial cationic lipid used as a transfecting reagent, could easily form a submicron-sized complex with siRNA, and this complex had a high positive zeta potential (44.7 mV).

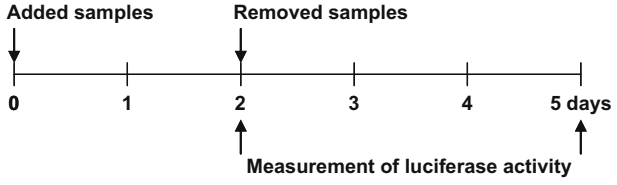

Fig. 1. Experimental schedule for the evaluation of luciferase activity in A549-Luc cells.

siRNA-loaded PLGA NS were prepared using the ESD method. This technique is a simple process using mild conditions without sonication, which may degrade nucleic acids. We prepared siRNA-loaded PLGA NS by complexation with a cationic compound using the ESD method [15]. The PLGA NS prepared by the emulsion solvent diffusion method gave a yield of NS in excess of 90%. The particle size of PLGA NS prepared by the ESD method with siRNA-loaded PLGA NS was about 300 nm. As shown in Fig. 2, the topology of siRNA-loaded PLGA NS observed by SEM was spherical, smooth, and the surface was homogenous on the submicron-size range. The siRNA-loaded PLGA NS, without adding mannitol as a cryoprotectant, aggregated after lyophilization (data not shown). There was little disparity in the sizes and size distributions of nanoparticles containing cryoprotectant before and after lyophilization. To provide for the dispersibility of lyophilized NS into aqueous solution, mannitol is required as a cryoprotectant.

The zeta potential of plain-PLGA NS was negative due to dissociation of the carboxyl group of PLGA. The negative zeta potential of siRNA-loaded PLGA NS proved that the siRNA/DOTAP complexes, which have a positive zeta potential, were encapsulated in the PLGA NS, but were not adsorbed on the surface of PLGA NS. CS-modified PLGA NS could be prepared by adding a CS solution into the outer phase with a PVA solution. The CS-modified PLGA NS had a positive charge due to protonation of the amino group. The zeta potential shifted to positive values due to the effect of CS adsorbed on the surface of PLGA NS. The particle size of CS-modified PLGA NS increased because the molecular layers of CS modified the surface of PLGA NS. We have also checked the composition of CS-modified PLGA NS using FITC-conjugated CS. CS adsorbed on NS surface was 8% (w/w) in the NS (data not shown). After modifying with CS, the encapsulation efficiency was increased. The effect of modifying with CS on the loading efficiency might be caused by an ionic interaction between siRNA and CS and prevented leakage of siRNA from emulsion droplets during the diffusion process. The zeta potential of the siRNA/NS complex decreased compared with CS-modified PLGA NS because the siRNA had a negative charge derived from a dissociated phosphate group.

3.2. In vitro siRNA release profiles from PLGA NS

The drug release from a nanoparticulate matrix system, in which the drug is uniformly enclosed, generally occurs by diffusion or erosion of the matrix. Several parameters also affect the drug release rate from matrix systems. For example, molecular weight and lactide to glycolide ratio of the polymer used as the matrix-forming system have an important impact on the drug release. As shown in Fig. 3, siRNA release exhibited a biphasic release pattern, which was characterized by an initial release followed by a slower release phase. The initial burst releasing 60% of the siRNA from plain-PLGA NS was shown. After the initial burst, drug release was significantly prolonged for more than five days, where 20% of the siRNA still remained in the plain-PLGA NS. The sustained release at the later stage was due to the diffusion of siRNA through the PLGA matrix, as well as the erosion of NS.

Modifying the NS with chitosan did not alter the drug release pattern. However, the PLGA NS modified with chitosan reduced the initial burst of siRNA release and prolonged the release at the later stage. The interaction of siRNA with chitosan might have caused some of the siRNA to remain at the surface of the PLGA NS. Therefore, different nanostructure designs of siRNA-loaded PLGA NS might be required to control for a more desirable release pattern by changing the surface properties. However, adsorption of siRNA on the surface of CS-modified PLGA NS is unlikely, as it was found that no siRNA was adsorbed on blank NS after 24 h.

There were some reports about siRNA-controlled release carriers using PLGA. Especially, many experts published the researches

Table 1
Physicochemical properties of the different siRNA preparations. Results are means ± SD of three samples.

	Particle size (nm)	Polydispersity	Zeta potential (mV)	siRNA loading efficiency (%)
Plain-PLGA NS	317.6 ± 5.8	0.102 ± 0.01	−31.1 ± 2.3	28.3 ± 4.5
CS-modified PLGA NS	352.8 ± 10.3	0.229 ± 0.03	11.2 ± 1.9	44.5 ± 6.6
siRNA/NS complex	325.3 ± 14.7	0.216 ± 0.02	6.6 ± 3.1	–
siRNA/DOTAP complex	225.4 ± 3.9	0.097 ± 0.02	44.7 ± 3.0	–

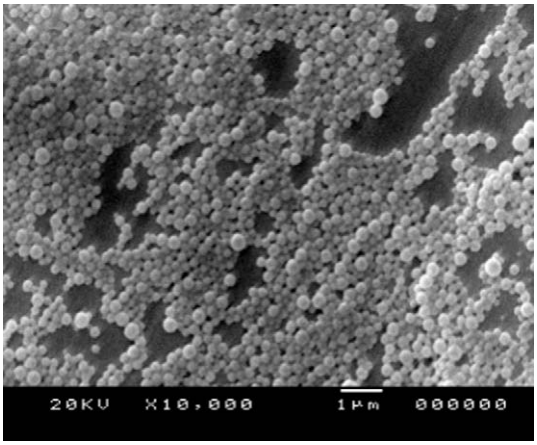

Fig. 2. Scanning electron micrograph of siRNA-loaded plain-PLGA NS (magnification × 10,000, scale bar = 1 μm).

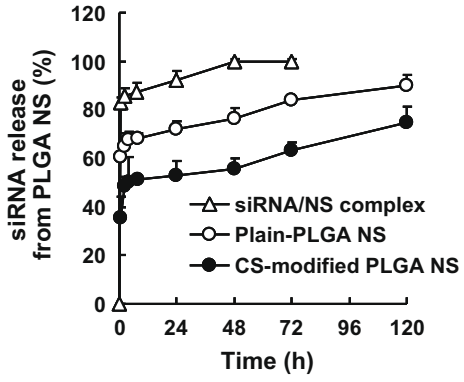

Fig. 3. Cumulative release (% of amount loaded) of siRNA from PLGA NS. Release was studied in PBS (pH 7.4) at 37 °C. Results are the means ± SD of three samples.

of PLGA microsphere which is capable of long-term drug release. siRNA release of PLGA microsphere can be prolonged for a few months in comparison with submicron-sized NS [20]. However, over micro-sized particle was difficult to penetrate deep into tissue and uptake into the cells. These microspheres may have limited applications due to the large size.

3.3. Effects of chitosan modification on cellular uptake of PLGA NS

The cellular uptake of the different Cy3-labeled siRNA (red fluorescence) preparations were evaluated visually using confocal laser scanning microscopy (CLSM), as shown in Figs. 4 and 5. After naked siRNA was added to the A549 cells, Cy3-siRNA fluorescence was not observed in the cells, as shown in Fig. 4A. This was because siRNA is a hydrophilic polymer, and the strong negative charge derived from phosphate group makes it very difficult for penetration of naked siRNA into cell without a transfecting reagent. Cy3-siRNA could be observed in the cells that were stained with Alexa Fluor® 488-conjugated phalloidin to clarify the location of cells using DOTAP as a transfecting reagent (Fig. 4B). DOTAP is a cationic lipid that can easily form electrostatic complexes with siRNA.

CLSM of A549 cells exposed to fluorescence (6-coumarin)-labeled PLGA NS loaded with Cy3-labeled siRNA demonstrated fluorescence activity in the cells during the incubation with the PLGA NS suspension (Fig. 5). The green fluorescence of the 6-coumarin-labeled PLGA NS in the images was changed to a yellow color to provide a better illustration of the co-localization of PLGA NS and siRNA. By *in vitro* cytotoxicity tests, PLGA NS did not negatively affect the viability of A549 cells during the uptake experiments [19]. The images shown are *z*-sections through the center of the cells, which indicated that the fluorescence observed was the result of PLGA NS localization inside the cells.

We found after 4-h incubation that the cellular uptake of siRNA using different siRNA formulations was increased with all the delivery systems compared to naked siRNA, which showed only negligible cellular uptake. This suggested the hypothesis that cellular uptake is the most restraining factor for siRNA therapeutics and, in general, colloidal drug delivery systems appear to be potential candidates for improving this situation. The uptake fluorescence for plain-PLGA NS and CS-modified PLGA NS could only be observed in the cytosol after analyzing the CLSM images of the A549 cells, suggesting that PLGA NS were internalized by the A549 cells. Cy3-labeled siRNA could also be observed in the cytosol, the same as PLGA NS. The co-localization of PLGA NS and Cy3-siRNA fluorescence, appearing as yellow fluorescence, was as a consequence of PLGA NS internalization. Therefore, siRNA-loaded PLGA NS were taken up into the A549 cells with the siRNA internally encapsulated so as not to release siRNA from NS before uptake.

CS-modified PLGA NS showed a higher uptake into A549 cells than plain-PLGA NS. The zeta potential of CS-modified PLGA NS proved to be positively charged. These results suggested that cationic CS on the surface of NS enhanced the association between CS-modified PLGA NS and negatively charged cell membranes by electrostatic interactions and that NS cellular uptake might be increased. In the case of the siRNA/NS complex, cellular uptake of Cy3-siRNA was decreased compared to CS-modified PLGA NS, because siRNA release from the siRNA/NS complex was rapid, as shown in Fig. 3. Therefore, Cy3-siRNA could not be observed in the cytosol as with PLGA NS. However, further investigations with regard to the mechanism of nanoparticle uptake and the kinetics of drug uptake and retention in the A549 cells will be helpful in order to establish the efficiency of PLGA NS for siRNA delivery.

3.4. Effects of chitosan modification on the gene-silencing effect

Normal A549 cells showed no significant luciferase activity (<150 RLU/10 μL of sample). Luciferase activity of A549-Luc cells proportionally increased according to the number of the cells, indicating that the luciferase activity could be used as an indicator of the number of cells. As shown in Fig. 6A, the luciferase gene-silencing efficiency of CS-modified PLGA NS was higher and more prolonged than that of plain-PLGA NS. This result that correlated with the CLSM studies might have been caused by the higher cellular uptake ability of CS-modified PLGA NS due to electrostatic interactions. In contrast, siRNA effect of plain-PLGA NS was not observed. Because

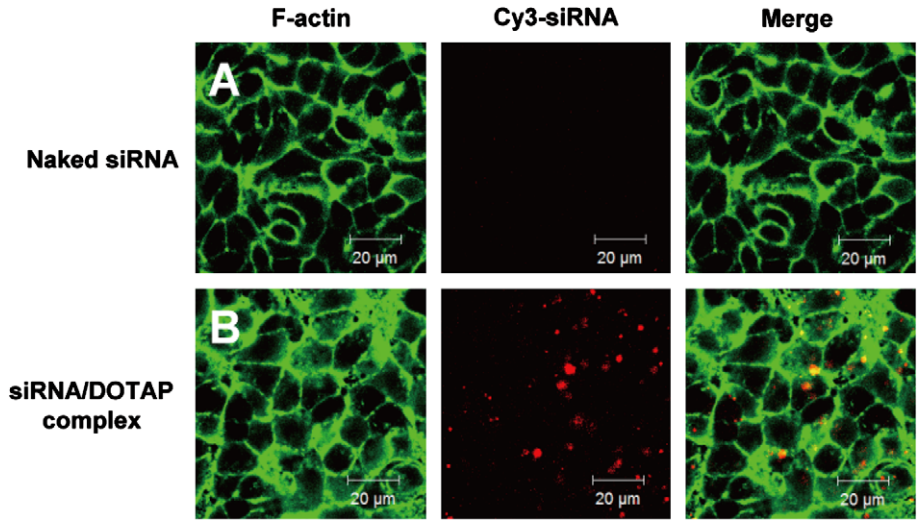

Fig. 4. Confocal laser microscopic images of A549 cells following 4-h uptake of Cy3-labeled siRNA (50 nM) preparations. (A) Naked siRNA and (B) siRNA/DOTAP complexes were used to treat A549 cells. F-actin in A549 cells was stained with Alexa Fluor® 488-conjugated phalloidin; green fluorescence. Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

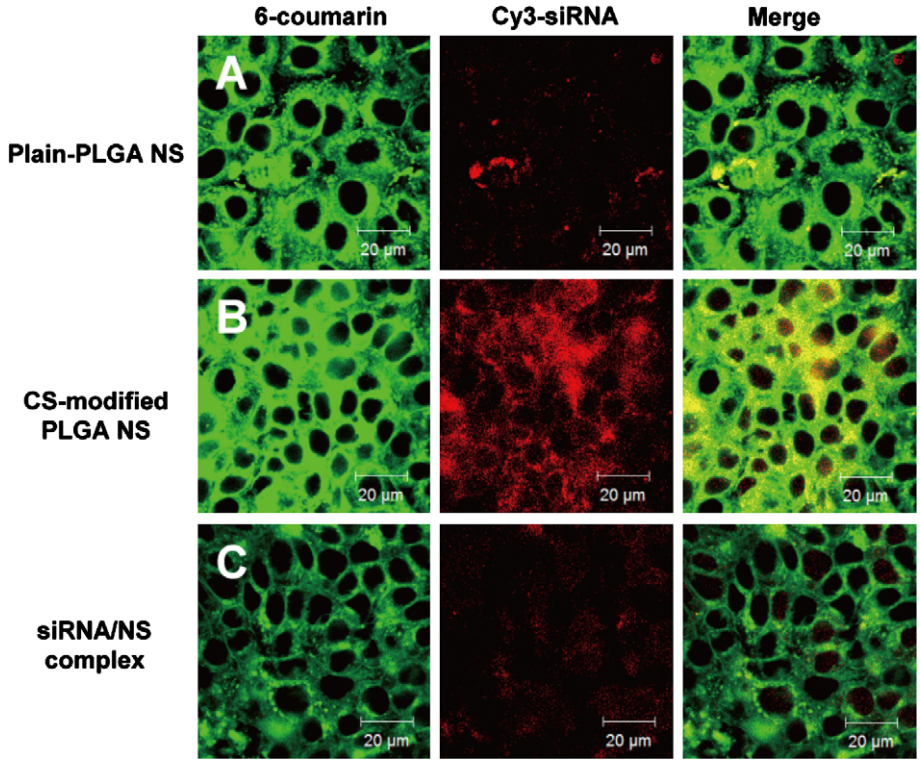

Fig. 5. Confocal laser microscopic images of A549 cells following 4-h uptake of the different PLGA NS preparations containing Cy3-labeled siRNA (50 nM): (A) Plain-PLGA NS, (B) CS-modified PLGA NS, (C) siRNA/NS complexes. PLGA NS were stained with 6-coumarin as a fluorescence marker (green fluorescence). Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

siRNA encapsulated in plain-PLGA NS taken up by the cells was little because almost siRNA (60%) encapsulated in plain-PLGA NS was released as an initial burst. Furthermore, after initial burst, siRNA did not easily released form plain-PLGA NS. Naked siRNA did not down-regulate the production of the luciferase gene.

The siRNA/DOTAP complex, used as a positive control, showed strong initial suppression, after which the down regulation effect became gradually weaker. This suppression pattern was quite different with CS-modified PLGA NS. The suppression pattern by siRNA-loaded CS-modified PLGA NS was initially weak and then gradually became stronger. These phenomena could be accounted for by the sustained siRNA release from PLGA NS in the cytosol. A sustained release profile of siRNA from CS-modified PLGA NS was confirmed in Fig. 3. Adsorption of siRNA on the PLGA NS's surface (siRNA/NS complex) did not maintain the gene-silencing effect. siRNA release from PLGA NS's surface was very rapid (Fig. 3), resulting in a low, non-sustained gene-silencing effect. In contrast, control siRNA-loaded PLGA NS and empty CS-modified PLGA NS did not show any significant difference for luciferase activity compared to no treatment A549-Luc cells, as shown in Fig. 6B.

Though siRNA/cationic lipid complex (lipoplex) is easily transfected, some of the transfection reagents available commercially

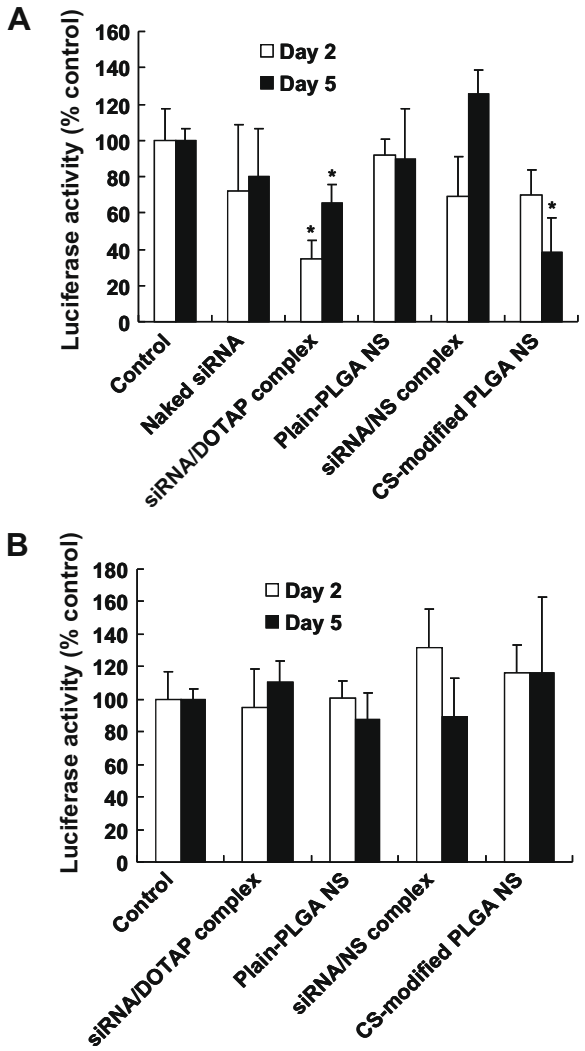

Fig. 6. Inhibition of luciferase expression in A549-Luc cells by different siRNA preparations. Luciferase activity was measured at 48 and 120 h after siRNA addition. (A) A549-Luc cells were treated with different Luc-siRNA preparations. (B) A549-Luc cells were treated with different control-siRNA preparations and blank (empty) CS-modified PLGA NS. Results are the means ± SD (n = 3–6), $^*p < 0.01$, significantly different compared with control.

may have cytotoxicity and cannot provide controlled or sustained release [21]. For example, some cationic lipids are known to be toxic effects or inducing immune responses and unstable at high ionic conditions. PLGA have been approved by the US Food and Drug Administration (FDA) for limited clinical use. Chitosan as a surface modifier enhances the transport of NS into the cells and is biocompatible and biodegradable [19]. We previously found that CS-modified PLGA NS did not show any cytotoxic effects for A549 cells, in contrast to pDNA/DOTAP complexes [manuscript submitted]. It is possible that PLGA NS can be used in high doses for siRNA therapy due to a lack of side effects.

This system has also the advantage of siRNA protection against the RNase during drug release. siRNA release pattern can be controlled by loading in PLGA NS [15]. siRNA effects with varying patterns can be created by combining those carriers that show the effect during the first stage with those carriers showing the effect at later stages. Furthermore, NS storage for a long time is possible because the powder of PLGA NS after freeze drying have high stability [22]. This suggests the possibility that CS-modified PLGA NS can be applied to the therapeutics for various diseases using siRNA. CS-modified PLGA NS might be developed as a non-viral vector for siRNA delivery. Further investigation is required to optimize the siRNA effect before applying the present system to gene therapy.

4. Conclusions

siRNA-loaded PLGA NS for a pulmonary gene delivery system were prepared, and the *in vitro* activity was evaluated. CS-modified PLGA NS can be highly recommended as carrier for siRNA delivery due to their high interactions with cells and safety in terms of cytotoxicity. These non-toxic formulations will be further evaluated for their *in vitro* and *in vivo* abilities to provide siRNA carriers for the purposes of gene therapy.

References

[1] J. Yano, K. Hirabayashi, S. Nakagawa, T. Yamaguchi, M. Nogawa, I. Kashimori, H. Naito, H. Kitagawa, K. Ishiyama, T. Ohgi, T. Irimura, Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer, Clin. Cancer Res. 10 (2004) 7721–7726.
[2] 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.
[3] J. Smith, Y. Zhang, R. Niven, Toward development of a nonviral gene therapeutic, Adv. Drug. Deliv. Rev. 26 (1997) 135–150.
[4] H. Büning, S.A. Nicklin, L. Perabo, M. Hallek, A.H. Baker, AAV-based gene transfer, Curr. Opin. Mol. Ther. 5 (2003) 367–375.
[5] C. Rudolph, C. Plank, J. Lausier, U. Schillinger, R.H. Müller, J. Rosenecker, Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells, J. Biol. Chem. 278 (2003) 11411–11418.
[6] C.M. Wiethoff, J.G. Koe, G.S. Koe, C.R. Middaugh, Compositional effects of cationic lipid/DNA delivery systems on transgene expression in cell culture, J. Pharm. Sci. 93 (2004) 108–123.
[7] W.T. Godbey, K.K. Wu, A.G. Mikos, Poly(ethylenimine) and its role in gene delivery, J. Control. Release 60 (1999) 149–160.
[8] W.J. Kim, S.W. Kim, Efficient siRNA delivery with non-viral polymeric vehicles, Pharm. Res. 26 (2009) 657–666.
[9] A. Maheshwari, R.I. Mahato, J. McGregor, S. Han, W.E. Samlowski, J.S. Park, S.W. Kim, Soluble biodegradable polymer-based cytokine gene delivery for cancer treatment, Mol. Ther. 2 (2000) 121–130.
[10] R. Lobenberg, L. Araujo, J. Kreuter, Body distribution of azidothymidine bound to nanoparticles after oral administration, Eur. J. Pharm. Biopharm. 44 (1997) 127–132.
[11] A. Belbella, C. Vauthier, H. Fessi, J. Devissaguet, F. Puisieux, In vitro degradation of nanospheres from poly(D,L-lactides) of different molecular weights and polydispersities, Int. J. Pharm. 129 (1996) 95–102.
[12] S.K. Sahoo, J. Panyam, S. Prabha, V. Labhasetwar, Residual polyvinyl alcohol associated with poly(D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake, J. Control. Release 82 (2002) 105–114.
[13] S. Prabha, W.Z. Zhou, J. Panyam, V. Labhasetwar, Size dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles, Int. J. Pharm. 244 (2002) 105–115.
[14] Y. Kawashima, H. Yamamoto, H. Takeuchi, T. Hino, T. Niwa, Properties of a peptide containing D,L-lactide/glycolide copolymer nanospheres prepared by novel emulsion solvent diffusion methods, Eur. J. Pharm. Biopharm. 45 (1998) 41–48.
[15] K. Tahara, H. Yamamoto, H. Takeuchi, Y. Kawashima, Establishing chitosan coated PLGA nanosphere platform loaded with wide variety of nucleic acid by complexation with cationic compound for gene deliver, Int. J. Pharm. 354 (2008) 210–216.
[16] M. Thomas, JJ. Lu, J. Chen, AM. Klibanov, Non-viral siRNA delivery to the lung, Adv. Drug Deliv. Rev. 59 (2007) 124–133.
[17] N. Durcan, C. Murphy, S.A. Cryan, Inhaleable siRNA: potential as a therapeutic agent in the lungs, Mol. Pharm. 5 (2008) 559–566.
[18] K. Hyoudou, M. Nishikawa, Y. Umeyama, Y. Kobayashi, F. Yamashita, M. Hashida, Inhibition of metastatic tumor growth in mouse lung by repeated administration of polyethylene glycol-conjugated catalase: quantitative analysis with firefly luciferase-expressing melanoma cells, Clin. Cancer Res. 10 (2004) 7685–7691.
[19] K. Tahara, T. Sakai, H. Yamamoto, H. Takeuchi, N. Hirashima, Y. Kawashima, Improved cellular uptake of chitosan-modified PLGA nanospheres by A549 cells, Int. J. Pharm. 382 (2009) 198–204.
[20] A. Khan, M. Benboubetra, P.Z. Sayyed, K.W. Ng, S. Fox, G. Beck, I.F. Benter, S. Akhtar, Sustained polymeric delivery of gene silencing antisense ODNs, siRNA, DNAzymes and ribozymes: in vitro and in vivo studies, J. Drug Target. 12 (2004) 393–404.
[21] H. Lv, S. Zhang, B. Wang, S. Cui, J. Yan, Toxicity of cationic lipids and cationic polymers in gene delivery, J. Control. Release 114 (2006) 100–109.
[22] H. Yamamoto, W. Hoshina, H. Kurashima, H. Takeuchi, Y. Kawashima, T. Yokoyama, H. Tsujimoto, Engineering of poly(D,L-lactic-co-glycolic acid) nanocomposite particles for dry powder inhalation dosage forms of insulin with the spray-fluidized bed granulating system, Adv. Powder Technol. 18 (2007) 215–228.