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.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Chitosan-modified poly(3-lactide-co-glycolide) nanospheres for improving siRNA delivery and gene-silencing effects

Kohei Tahara, Hiromitsu Yamamoto, Naohide Hirashima, Yoshiaki Kawashima

ABSTRACT

Chitosan (CS) surface-modified poly(3-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 laser 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 delivery 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(3-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].
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). (−)-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'–CUUACCCUG AGUACUUGAdTdT-3', antisense: 3'-dTdTGAAUGCCGACUAGAA GCU-5', DHarmaco Inc., Chicago, IL) and Cy3-labeled siRNA targeting the same protein (sense: 5’–CUUACCCUGAGUACUUGAdTdTT-3', antisense: 3’–dTdTGAUUAGCCGACUAAGGGCU-5’-Cy3, DHarmaco). The annealed siRNA targeting protein kinase c alpha (PKC-siRNA, sense: 5'–GGACAUUAACAAUUUGCAdTdT-3', antisense: 3’–dTdTCCUGUAAUUGUUUAACGAC-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% CO2. 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 (43,400g 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 acetic 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 (43,400g for 10 min), and the siRNA contents in the supernatant were determined by Cy3 fluorescence measurement with a fluorescence spectrophotometer (F-3010, excitation wavelength 530 nm, emission wavelength 565 nm; Hitachi, Tokyo, Japan). The loading efficiency of siRNA was calculated from the following equation:

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

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 × 10^3 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 every 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 17,970g 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.
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 phallolidin 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 constraining 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.

### 3.3. 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 phallolidin 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 constraining 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
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 release from 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
may have cytotoxicity and cannot provide controlled or sustained release [21]. For example, some cationic lipids are known to be toxic effects or induce 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 biocompatible and biodegradable [19]. We previously found that toxicity 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 biocompatible and biodegradable [19]. We previously found that 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. Roseneker, 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. Wiertz, B. van Nostrum, P.C. Taminiau, Composition of cationic lipids/DNA delivery systems on transgene expression in cells, J. Pharm. Sci. 93 (2004) 108–123.
[7] W.T. Godbey, K.K. Wu, A.G. Mikos, Poly(ethyleneimine) 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 vectors, 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 nanoparticles from poly(ω-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 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.

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.