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RESEARCH PAPER

The Development and Mechanism Studies of Cationic Chitosan-Modified Biodegradable PLGA Nanoparticles for Efficient siRNA Drug Delivery

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ABSTRACT

Purpose In order to improve siRNA delivery for possible clinical applications, we developed biodegradable chitosan-modified poly(D,L-lactide-co-glycolide) (CHT-PLGA) nanoparticles with positive surface charge, high siRNA loading, high transfection efficiency and low toxicity.

Methods CHT-PLGA nanoparticles were prepared, and siRNA was loaded by emulsion evaporation method with poly(vinyl alcohol) (PVA) as emulsifier. siRNA loading efficiency, particle size, and Zeta potential of nanoparticles were measured. Gel retardation and protection assays were conducted to determine the loading and binding of siRNA in the formulation. Cell transfection was performed to study *in vitro* siRNA silencing efficiency. XTT assay was used to evaluate the cytotoxicity.

Results It was found that the nanoparticle diameter and positive Zeta potential increase as the chitosan coating concentration increases. CHT-PLGA nanoparticles showed excellent siRNA binding ability and effective protection of oligos from RNase degradation. siRNA-loaded nanoparticles were successfully delivered into the HEK 293 T cell line, and the silencing of green fluorescence protein (GFP) expression was observed using fluorescent microscopy and flow cytometry. In addition, the cytotoxicity assay revealed that CHT-PLGA nanoparticles had relatively low cytotoxicity.

Conclusion This study suggests that biodegradable cationic CHT-PLGA nanoparticles possess great potential for efficient and safer siRNA delivery in future clinical applications.

KEY WORDS chitosan · nanoparticles · nanotechnology · poly (D,L-lactide-co-glycolide) (PLGA) · siRNA delivery

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INTRODUCTION

Gene therapy refers to specifically reducing or silencing the expression of deleterious genes in the targeted cells or organs (1,2); therefore, it is a powerful approach for the treatment of a wide range of diseases, including cancer, by producing bioactive agents or stopping abnormal cell functions, such as genetic disorders or uncontrollable proliferation of cells (3). Recently, short interfering RNA (siRNA) has been used to regulate gene expression in mammalian cells through RNA interference (RNAi) (4). RNAi was first demonstrated in mammalian cells by Elbashir *et al.* (5). Discovery of the RNAi mechanism by Fire, Mello and coworkers in the late 1990s provided new direction and gave renewed promise to the field of gene therapy (6).

RNAi is a simple and rapid method of silencing gene expression in a wide range of organisms by double-stranded 21– 23-nucleotides RNA duplex (siRNA) that contains 5′-phosphate and 3′- hydroxyl termini with two nucleotide overhangs (7). After cleavage of long double-stranded RNA (dsRNA) into short fragments of siRNA, these oligos are then separated into single strands and incorporated into a

RISC complex and subsequently target messenger RNA (mRNA), where they induce cleavage, preventing it from being used as a template. The RNAi gene silencing technique is widely used in biological and medical research to probe the biological role of a particular gene as a means to inhibit infection by human immunodeficiency virus, poliovirus and hepatitis C virus, and to suppress cancer cell growth by silencing virus and cancer gene expression (8–12).

Significant advancements in siRNA delivery systems will be needed to translate this nucleic acid-based therapy into reliable clinical therapies (13). Since cellular uptake of siRNA is usually very poor, and naked siRNA can be rapidly degraded by RNase enzymes and excreted by the kidney, wide distribution of siRNA in the body must be prevented in order to concentrate administered siRNA at the site of action. To overcome these challenges, there is a great need to develop efficient siRNA delivery systems. To date, both viral and non-viral vectors have been used to deliver siRNA. Even though viral vectors are frequently used due to high efficiency, their clinical applications are limited because of immunogenicity, potential infectivity, inflammation and complicated production. Therefore, non-viral vectors, such as cationic liposomes and polymers, have been used to avoid these problems and overcome intracellular and extracellular barriers (14,15). However, complexes formed with cationic liposomes are large in size, thermodynamically unstable, and can be easily cleared from bloodstream (16,17). Compared to liposomes, polymers can form more stable nano-size complexes, which can provide better protection for DNA or siRNA oligos from nuclease degradation (18). Different types of polymers have been used for this purpose, such as poly(D,L-lactide-*co*-glycolide) (PLGA), poly(lactic acid) (PLA), poly(cyanoacrylate) (PCA), chitosan, polyethylenimine (PEI), poly(L-lysine) (PLL), dendrimers, poly(vinylimidazole) and imidazole containing polymers (19–23).

PLGA is one of the most promising polymeric carriers for siRNA delivery. It has been tested for toxicity and safety in many studies, and is currently being used in humans for resorbable sutures, bone implants, artificial organs, tissue engineering, and contraceptive implants (24–27). Polyesters in nature, PLGA polymers undergo hydrolysis upon administration into the body, forming biologically compatible and metabolizable moieties (lactic acid and glycolic acid) that are eventually removed from the body by the citric acid cycle. PLGA has been successfully used to prepare siRNA-loaded nanoparticles for delivery of siRNA and silencing *in vitro* (19). However, the loading capacity of PLGA nanoparticles is not high, and the net negative charge of PLGA nanoparticles will not be favorable if higher silencing efficiency is desired. Therefore, in this project, we incorporated cationic chitosan into PLGA nanopartices in order to improve siRNA payload and its silencing efficiency.

Chitosan is a linear polysaccharide composed of β-(1–4)-linked D-Glucosamine (deacetylated unit) and N-acetylated-D-glucosamine (acetylated unit). Generally, the DDA (degree of deacetylation) of chitosan influences the characteristics such as charge density, solubility, crystallinity, and degradation of the polymer (28,29). Chitosan is biodegradable and biocompatible and has been widely investigated in the drug delivery, drug targeting, development of hemodialysis membranes and artificial skin, and in other applications. Chitosan breaks down slowly to harmless products (amino sugars), which are completely absorbed by the human body (30). Most studies of chitosan/DNA complexes have used highly deacetylated chitosan with higher oligo binding efficacy (31,32). It was reported that chitosan could be incorporated into PLGA nanoparticles to improve delivery of small molecules, such as paclitaxel (33), and to improve the delivery of DNA and antisense oligos (34–36). However, the delivery of siRNA, which is a very promising therapeutic in the future, by chitosan-modified PLGA nanoparticles to the cells has rarely been studied to date (37). Therefore, in this study, we developed cationic chitosan-modified biodegradable PLGA (CHT-PLGA) nanoparticles in order to achieve higher siRNA loading, protection of oligos from degradation, reduced cytotoxicity and enhanced transfection efficiency.

MATERIALS AND METHODS

Materials

Chitosan (deacetylation degree >85%), poly (D,L-lactide-*co*-glycolide) (L:G molar ratio = 75:25, MW 66,000–107,000), poly (vinyl alcohol) (PVA, MW 9,000–10,000, 80% hydrolyzed), Dulbecco's phosphate-buffered saline (PBS), Dulbecco's Modified Eagle's medium (DMEM) high glucose, LB medium, Penicillin-Streptomycin antibiotics and dimethylsulfoxide (DMSO, 99.5% GC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). si-GFP-RNA oligo (5′-GCAAGCUGACCCUGAAGUUCAU-3′, 3′-GCCGUUCGACUGGGACUUCAAG-5′) was purchased from Ambion (Austin, TX, USA). Lipofectamine™ 2,000, Opti-MEM-I Reduced Serum medium (1X), Fetal Bovine Serum (FBS), competent *E.coli* DH5α cells were obtained from Invitrogen (Carlsbad, CA, USA). HPLC grade dichloromethane (DCM) was purchased from EM Science (Gibbstown, NJ, USA). Plasmid extraction MIDI prep kit was purchased from Qiagen (Valencia, CA, USA). Sodium acetate buffer (pH 4.5) was prepared in accordance with USP guidelines. Human embryonic kidney cells (HEK 293 T) were obtained from Dr. Hua Gu's lab at Columbia University.

Plasmid Extraction

E.coli DH5α was incubated with plasmid DNA and allowed to grow by using LB media for transformation. A freshly streaked selective plate was taken and inoculated in a culture of LB media with antibiotics (liquid media), and then incubated for about 10 h at 37°C on a shaker incubator. The standard protocol of Qiagen's MIDI kit was followed, and the final confirmation was performed by gel electrophoresis. The concentration of extracted plasmid was determined by UV spectroscopy.

Preparation of siRNA-Loaded CHT-PLGA Nanoparticles

For the preparation of CHT-PLGA nanoparticles, the emulsion solvent evaporation method was used. Chitosan was dissolved in 20 ml of sodium acetate buffer (pH 4.5) at five different concentrations (0.00, 0.01, 0.05, 0.1, and 0.25% w/v). Then 40 mg of PLGA was dissolved in 2 ml of DCM in a flask by vigorous vortex mixing. PVA solution 1.0% (w/v) was prepared separately with sterilized water and filtered using a 0.2 μm membrane filter. PVA solution (10 ml) was added to the prepared aqueous chitosan solution of different concentrations to give five samples. The final chitosan coating concentrations in the solutions were 0.00, 0.0067, 0.033, 0.067 and 0.17% in Samples I, II, III, IV and V, respectively. The resultant polymer aqueous solution was mixed by a magnetic stirrer for 5 min at 500 rpm. The equivalent of 1,000 picomole of siRNA (50 μl) was added to the aqueous chitosan solution. PLGA solution was poured slowly into the chitosan solution, resulting in an oil-in-water (O/W) emulsion upon constant magnetic stirring. The emulsion was further processed using a Misonix probe sonicator (Farmingdale, NY, USA) in pulse mode at 1 watt for 45 s. DCM was removed by overnight stirring with a magnetic stirrer at room temperature in a sterile hood. After 24 h, nanoparticles were collected using an ultracentrifuge (Beckman LE-Ultracentrifuge, Fullerton, CA) at 10,000 rpm for 15 min, and the sediment (pellet) was resuspended in RNase-free deionized water. This process was repeated twice to remove excess amount of PVA. The control was PLGA nanoparticles not modified with chitosan, which is Sample I.

Characterization of Prepared Nanoparticles

The different samples of CHT-PLGA nanoparticles were collected and further diluted with deionized water to an appropriate concentration. The particle size and surface charge (Zeta potential) of nanoparticles were measured by a dynamic light scattering (DLS) particle sizer–Zetasizer (Malvern Instruments, Westborough, MA, USA).

Determination of siRNA Loading Efficiency

Nanoparticles were centrifuged (Beckman LE-Ultracentrifuge, Fullerton, CA, USA) at 30,000 rpm for 20 min at 22°C after solvent evaporation. The loading efficiency of siRNA in the CHT-PLGA nanoparticles was obtained from the free siRNA concentration in the supernatant recovered after ultra-centrifugation. A UV Spectrophotometer (Shimadzu UV-1,700 Pharma Spec., Columbia, MD, USA) was used to measure absorbance at 260 nm. 1.0% of PVA solution (without siRNA) was used as a blank. A standard calibration was obtained by plotting UV absorbance of a serial of siRNA solutions against nominal concentrations.

Gel Retardation Assay

The binding/condensation ability of siRNA with the CHT-PLGA nanoparticles was determined by agarose gel electrophoresis. Samples I–V of PLGA nanoparticles modified with different concentrations of chitosan were used for this study. Briefly, 10 μl of the sample were mixed with 2 μl of 6× loading dye making the final volume of 12 μl. The complexes were loaded onto 1% agarose gel and run with Tris-borate (TBE) buffer at 100 V for 45 min. Then the gel was collected and soaked with ethidium bromide (EtBr), a staining solution, for 30 min. Finally, it was removed and soaked in deionized water, a de-staining solution, for 30 min. The siRNA bands were visualized by irradiation with UV light using Kodak Gel Logic 200 imaging system (Eastman Kodak Company, Rochester, NY, USA). Naked siRNA and blank PLGA nanoparticles without chitosan were used as the controls.

Protection Assay of siRNA

Protection assays of siRNA in CHT-PLGA nanoparticles were carried out by electrophoresis according to a modified technique of Park *et al.* (38). Briefly, 0.2857 μl of RNase I (2 units) was added to 4 μl of prepared nanoparticles or 0.1 μg of naked siRNA, and then incubated at 37°C with shaking for 1 h. For inactivation of RNase I, all the samples were treated with 4 μl of EDTA (0.25 M) for 10 min and mixed with 1.0% sodium dodecyl sulfate (SDS), which was dissolved in 1 M NaOH (pH 7.2–7.5), making the final volume of 18 μl. The final samples were incubated for 1 h, and then gel electrophoresis was performed in 1% agarose gel with TBE buffer for 45 min at 100 V. Naked siRNA and PLGA nanoparticles without chitosan were used as the controls.

Cell Transfection Studies

HEK 293 T cells were cultured in DMEM supplemented with 10% of FBS and 1% of streptomycin-penicillin

antibiotics. Cells were incubated at 37°C in an atmosphere of 5% CO_2 and split using trypsin/EDTA complex solution when 80% of confluence was achieved. Then cells were seeded in 24-well plates at an initial density of 10^5 cells/well in 500 µl complete growth medium. After incubation for 24 h, the media were replaced with serum- and antibiotic-free DMEM medium. One-hundred µl of prepared CHT-PLGA nanoparticles were added to the well together with 500 µl of fresh DMEM medium. After 24 h of incubation, the medium was changed with complete DMEM medium with FBS and antibiotics, and the gene silencing effect was monitored under a fluorescence microscope (Axiovert 200 M, Carl Zeiss, Göttingen, Germany). Further fluorescence quantification for each well was performed by using fluorescence-activated cell sorting flow cytometry (FACS) (BD LSR II, BD biosciences, San Diego, CA, USA). Each transfection experiment was carried out in triplicate. Lipofectamine 2,000 mixed with Opti-MEM-I was used as the positive control. Student's *t*-test was used to compare the transfection efficiency of CHT-PLGA nanoparticles with that of the positive control Lipofectamine ($P<0.05$).

Cytotoxicity Assay

In vitro cytotoxicity tests were conducted by following the standard protocol of XTT assay kit from Sigma (XTT is a tetrazolium derivative 2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt). Cells were seeded in 24-well plates at an initial density of 10^4 cells/well in 500 µl of growth medium and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. After 24 h, growth medium was replaced by a fresh, antibiotic- and serum-free medium. Simultaneously, 100 µl of various concentrations of prepared nanoparticles were added to the assigned wells. After an additional incubation for 24 h, the medium was changed with growth medium containing reconstituted XTT in an amount equal to 20% of the culture medium volume. Finally, after further incubation for 3 h, the content of each individual well was transferred to an appropriate size cuvette, and absorbance was measured individually for each sample at wavelength of 450 nm by a UV spectrophotometer. The cell viability (%) was calculated using following equation:

$$\text{Cell viability}(\%) = \left[\text{OD}_{450(\text{sample})}/\text{OD}_{450(\text{control})}\right] \times 100$$

where $\text{OD}_{450(\text{sample})}$ is the absorbance measurement from a well treated with samples and $\text{OD}_{450(\text{control})}$ is the absorbance measurement from a well treated with XTT solution without drug.

RESULTS

Characterization of Prepared CHT-PLGA Nanoparticles

CHT-PLGA nanoparticles were successfully prepared, and the particle size distribution was determined. The schematic representation of loading siRNA into chitosan-modified PLGA nanoparticles is shown in Fig. 1. Chitosan is postulated to be inside and on the surface of PLGA nanoparticles due to the electrostatic interaction of cationic chitosan molecules with anionic PLGA polymers. Some siRNA oligos are entrapped in PLGA nanoparticles, while the rest of siRNA are coated on the surface of PLGA nanoparticles due to the electrostatic interaction of negatively charged siRNA with positively charged chitosan molecules. It was found that the mean diameter of the PLGA nanoparticle control without chitosan was 182 nm, which was smaller than chitosan-modified PLGA nanoparticles. The particle size of CHT-PLGA nanoparticles increased from 204 to 543 nm with the increase of chitosan concentration in the formulation (Fig. 2). The particle size distribution is relatively narrow, which is considered an ideal characteristic for the siRNA nanoparticle formulation, as demonstrated in a representative size distribution diagram of Sample V (Fig. 3). The surface charge or Zeta potential of the control PLGA nanoparticles was negative, −10.6 mV, whereas all CHT-PLGA nanoparticles were electropositive, and Zeta potential increased from +16.9 to +31.2 mV with the increase of initial chitosan coating concentration (Fig. 4).

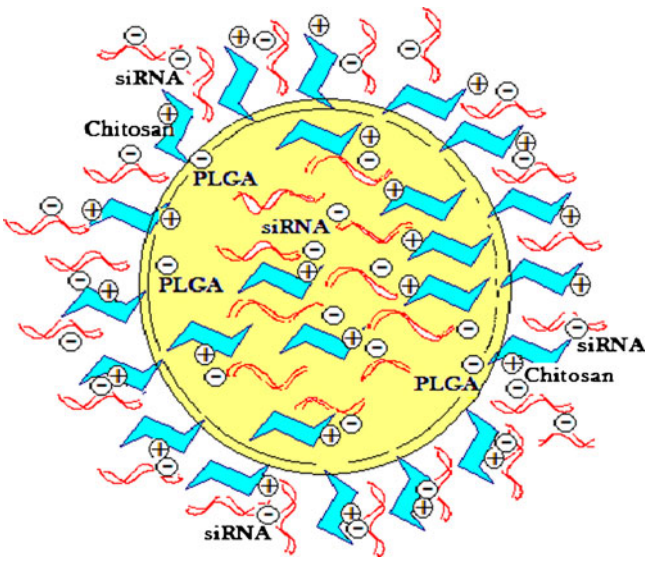

Fig. 1 Schematic representation of siRNA loaded cationic CHT-PLGA nanoparticles.

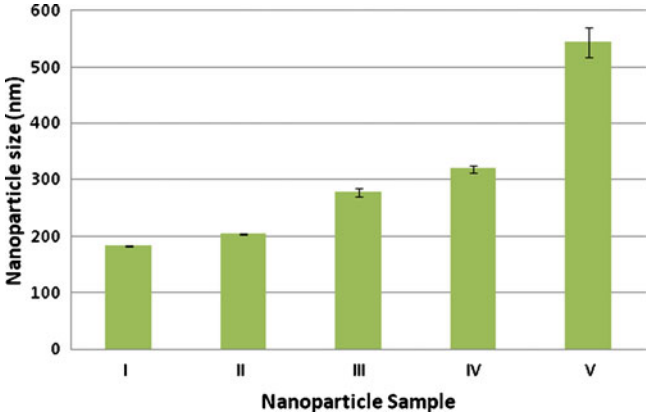

Fig. 2 Particle sizes of siRNA loaded CHT-PLGA nanoparticles ($n = 3$).

Loading Efficiency of siRNA

The loading efficiency of siRNA was the percentage of siRNA loaded in CHT-PLGA nanoparticles over total siRNA. The concentration of siRNA in supernatant was calculated from the standard calibration curve obtained ($y = 0.0003x + 0.0016$, $R^2 = 0.992$, where y is the absorbance, and x is siRNA concentration). As expected, the PLGA nanoparticle control has the lowest trapping efficiency (4.3%), due to the fact that both siRNA and PLGA are negatively charged. It was found that the loading efficiencies of CHT-PLGA nanoparticles increased accordingly with the concentration of chitosan as shown in Fig. 5. The highest siRNA loading efficiency (77.7%) was observed for Sample V nanoparticles, which were modified with the highest amount of chitosan.

Interaction of siRNA with CHT-PLGA nanoparticles

The binding efficiency of CHT-PLGA nanoparticles with siRNA was evaluated using agarose gel electrophoresis. It was found that the migration of siRNA was completely retarded in Samples III to V (Lane 4 to 6), when the chitosan concentration in the solution was above 0.033% (Fig. 6). However, modification with lower concentration of chitosan in Sample II could not prevent the migration of siRNA, due to relative weaker electrostatic interaction with siRNA. In other words, in Samples III to V modified with higher amount of chitosan, siRNA formed stable complexes with CHT-PLGA nanoparticles, which effectively prevented the migration of siRNA oligos.

Protection Assay of siRNA

The naked siRNA without incubation with RNase I showed a bright band (Lane 1), whereas siRNA incubated with RNase I showed no band (Lane 2) in Fig. 7, indicating that naked siRNA was not protected against RNase I and was degraded. Similar to previous gel retardation assays, both PLGA nanoparticles (Lane 3) and CHT-PLGA nanoparticles modified with the low concentration of chitosan (Lane 4) could not protect siRNA from RNase I degrdadation. In contrast, siRNA complexed in CHT-PLGA nanoparticles with higher chitosan concentrations in Sample III to V (Lane 5 to 7) was effectively protected from RNase I degradation. These results suggested that more integral siRNA could be delivered into cells without being degraded by enzymes, indicating better stability of siRNA in a CHT-PLGA nanoparticle delivery system.

Cell Transfection Studies

In vitro silencing was performed to investigate transfection efficiency of CHT-PLGA nanoparticles by using HEK 293 T cell line. Cells transfected with GFP plasmid but without siRNA, and cells with PLGA nanoparticles without chitosan were used as the negative controls. Cells transfected with GFP plasmid and siRNA-loaded Lipofectamine 2,000 were used as the positive control. The negative

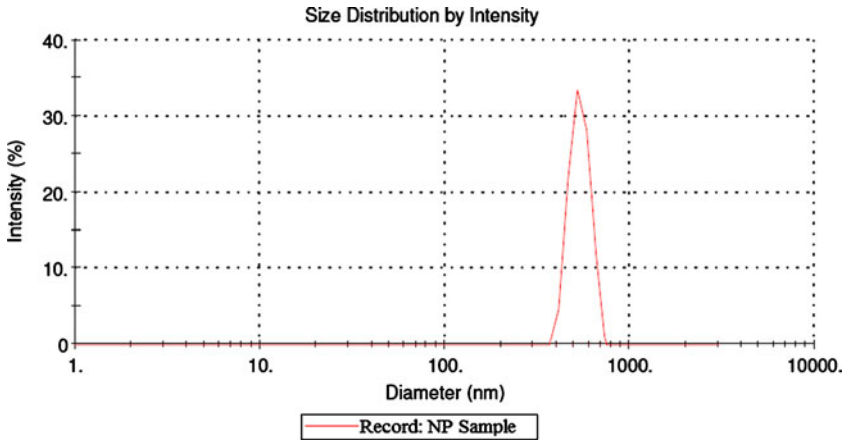

Fig. 3 A typical particle size distribution diagram of CHT-PLGA nanoparticles (Sample V).

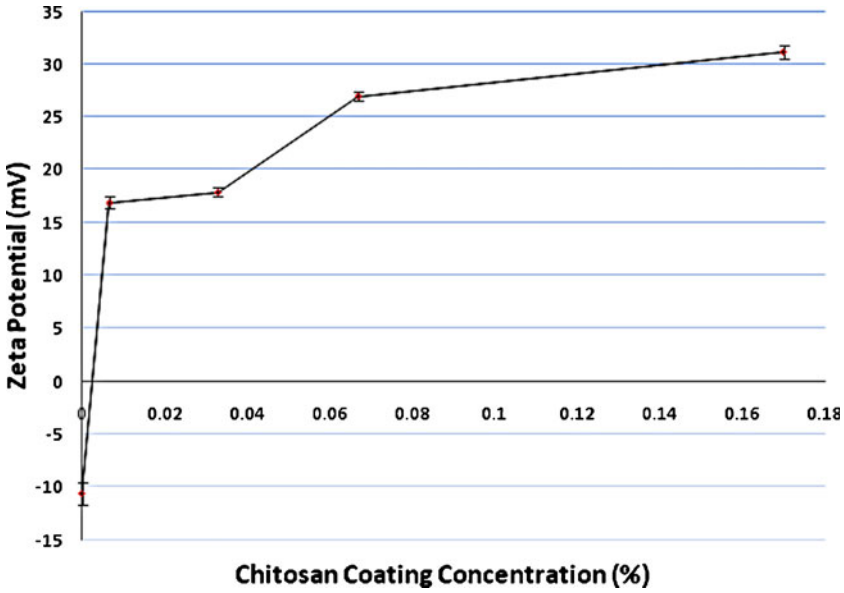

Fig. 4 Zeta potential of siRNA loaded CHT-PLGA nanoparticles ($n = 3$).

control cells showed good confluency after 24 h of incubation under bright field in Fig. 8a. Fig. 8b showed the same control cells under a fluorescence filter demonstrating extensive GFP expression in cells. For the siRNA-loaded CHT-PLGA nanoparticles, the inhibition of GFP expression and the effect of gene silencing were prominent after 24 h. In the case of Sample V, CHT-PLGA nanoparticles with 0.17% of chitosan, the GFP silencing effect was very significant as shown in Fig. 8c and d.

FACS was used to quantify the GFP expression in 293 T cells after transfection by CHT-PLGA nanoparticles. Fig. 9a shows cell viability (88.9%), and 9B indicates extensive GFP expression (98.2%) in the negative control cells transfected with GFP plasmid. Fig. 9c shows good viability (85.9%), and Fig. 9d demonstrates significantly reduced GFP expression (30.1%) after silencing by siRNA-loaded CHT-PLGA nanoparticles (Sample V). The efficiency of GFP silencing by CHT-PLGA nanoparticles with different chitosan concentration is shown in Fig. 5. The GFP gene silencing effects by CHT-PLGA nanoparticles, which are in the order of Sample V > Sample IV > Sample III > Sample II > Sample I, increased with the initial chitosan coating concentration in nanoparticle formulation. Sample V nanoparticles modified with 0.17% of chitosan showed the highest silencing effect ($63.3 \pm 5.6\%$), which was comparable to Lipofectamine positive control ($68.3 \pm 2.9\%$).

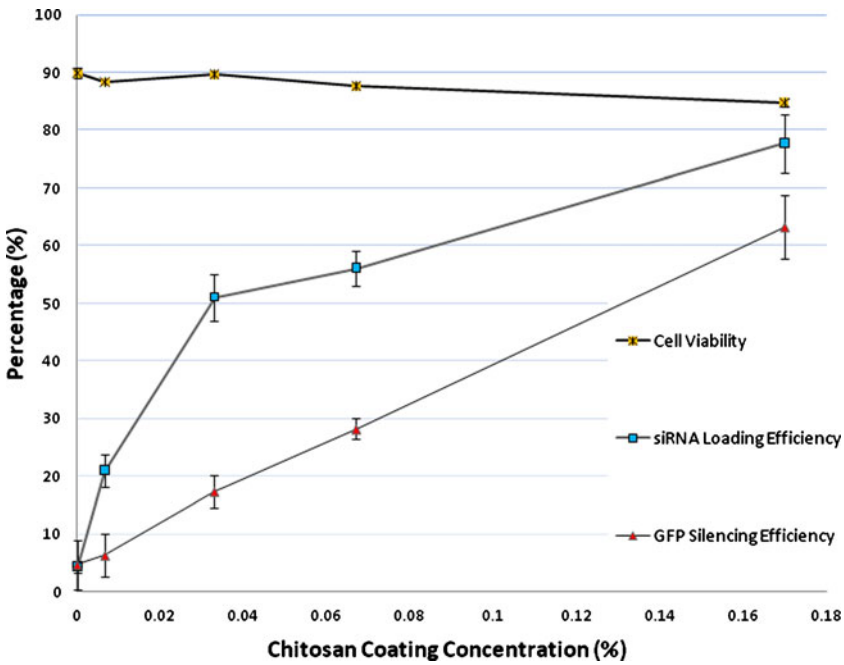

Fig. 5 GFP silencing efficiency, siRNA loading efficiency, and cell viability of CHT-PLGA nanoparticles ($n = 3$).

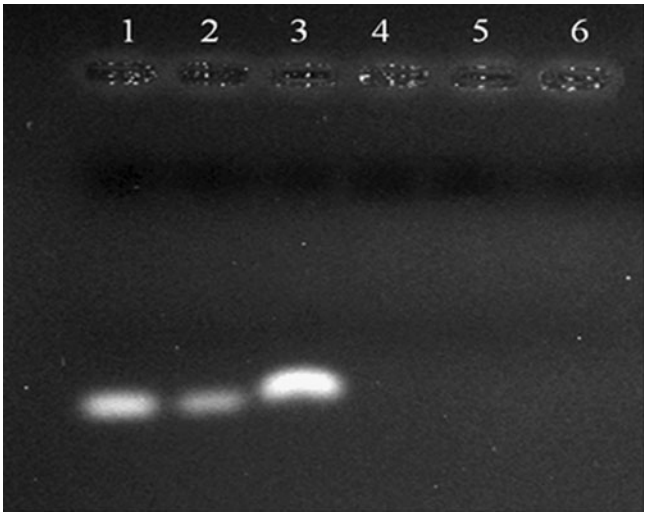

Fig. 6 Gel retardation assay of siRNA load CHT-PLGA nanoparticles to determine the degree of complexation Lane 1: Naked siRNA; Lane 2: Sample I (PLGA nanoparticle control); Lane 3: Sample II; Lane 4: Sample III; Lane 5: Sample IV; Lane 6: Sample V.

Statistical analysis by Student's *t*-test showed that they are not statistically different since the calculated $P>0.05$. The cell viability of all the nanoparticle samples was above 85% in FACS study and confirmed by the following XTT cytotoxicity study.

Cytotoxicity of CHT-PLGA Nanoparticles

Determination of live cell number is often used to assess rates of cell proliferation and to screen cytotoxic agents. XTT measures cell viability based on the activity of mitochondrial enzymes in living cells that reduce XTT to formazan, an orange-colored product. The amount of orange-colored product generated from XTT is proportional to the number of living cells in the sample and quantified by measuring absorbance at wavelength of 450 nm. An increase or decrease in cell number results in an associated change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test sample. The results showed that higher concentration of chitosan resulted in slightly lower viability. However, the difference of viability is not very significant as shown in Fig. 5. CHT-PLGA nanoparticles modified with the highest amount of chitosan in Sample V still showed an average of 84.8% viability, which is slightly lower than 89.9% viability of the PLGA nanoparticle control.

DISCUSSION

To induce RNAi, siRNA should be delivered into cells and released to the cytoplasmic compartment. However, the uptake of siRNA through fluid phase endocytosis does not deliver siRNA into the cytoplasm due to the lack of RNA-uptake machinery (39). Therefore, an appropriate carrier system is needed for successful delivery of siRNA. In this study, we use cationic CHT-PLGA nanoparticles to deliver siRNA into cells *via* endocytosis, protect siRNA from enzymatic degradation and then release it to cytoplasm after endosomal escape.

For a particulate delivery system, the size and shape play crucial roles in drug delivery to cells, and they greatly influence particle distribution inside the body. In general, the nano-sized particles have a higher intracellular uptake compared to microparticles (40,41). In the preparation of CHT-PLGA nanoparticles, 1.0% of PVA was used as the emulsifier to produce nanoparticles with relatively uniform and smaller size by preventing coalescence of the emulsion droplets. Further optimization of nanoparticles could be done by adjusting different parameters, such as stirring rate, power and time for sonication, and concentration of chitosan. The siRNA loading capability in regular PLGA nanoparticles is limited, due to negative charges on both siRNA and PLGA polymer, which may require large amount of oligos in the formulation to obtain satisfactory silencing. Therefore, cationic chitosan was employed to complex with negatively charged PLGA and siRNA *via* electrostatic interaction to improve the siRNA loading efficiency (Fig. 1). Chitosan is believed to be mainly distributed in the core or matrix of PLGA nanoparticles because of the interaction of positively charged chitosan with negatively charged PLGA polymer, which exist mainly in the matrix, and thereafter, entrapment of chitosan is inside the matrix. However, there is a layer of chitosan being coated on

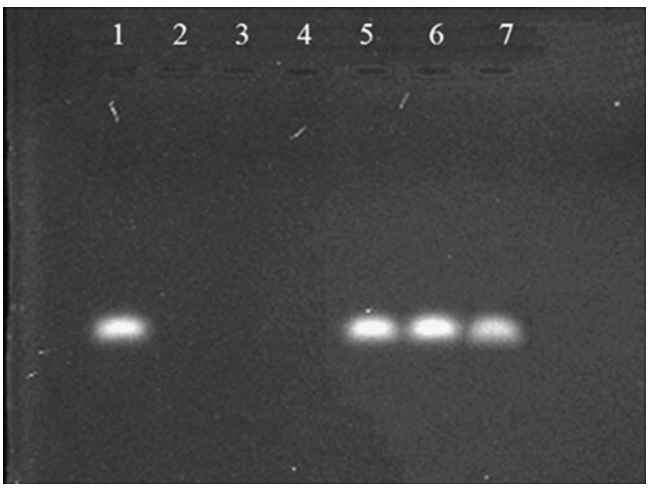

Fig. 7 Protection assay of siRNA loaded CHT-PLGA nanoparticles to determine nanoparticles protection ability Lane 1: Untreated naked siRNA; Lane 2: Naked siRNA; Lane 3: Sample I (PLGA nanoparticle control); Lane 4: Sample II; Lane 5: Sample III; Lane 6: Sample IV; Lane 7: Sample V.

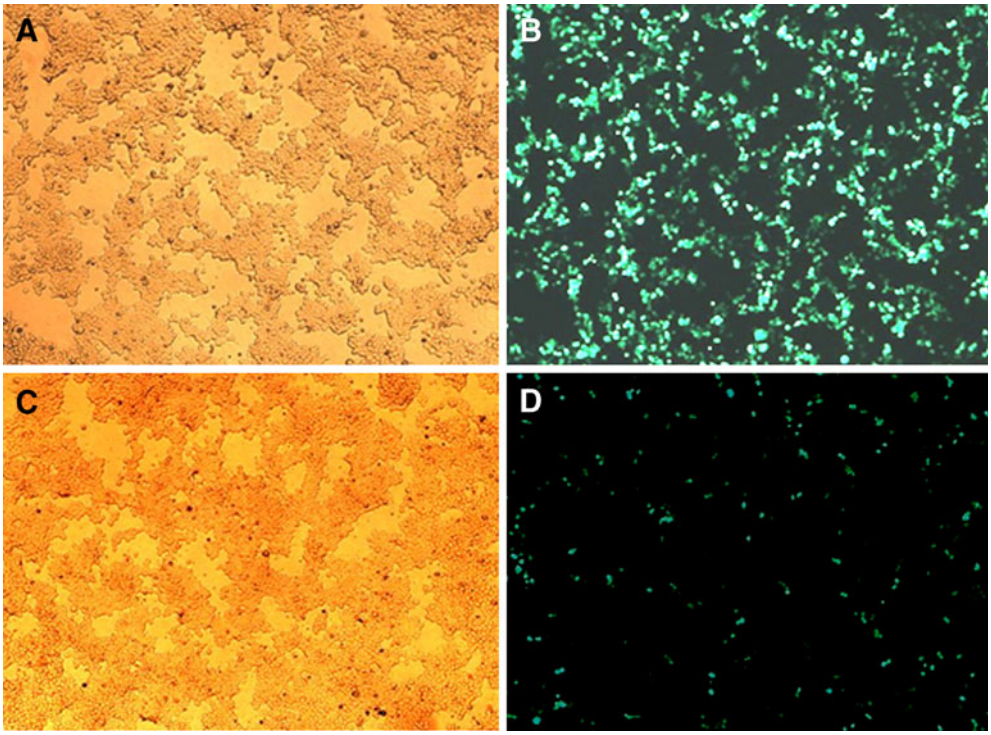

Fig. 8 The siRNA loaded CHT-PLGA nanoparticle transfection of HEK 293 T cells with GFP plasmid (**a**) The negative control 293 T cells with GFP plasmid under bright field; (**b**) The negative control 293 T cells with GFP plasmid under fluorescent filter; (**c**) The cells transfected by siRNA loaded CHT-PLGA nanoparticles (Sample V) under bright field; (**d**) The cells transfected by siRNA loaded CHT-PLGA nanoparticles (Sample V) under fluorescent filter.

the surface of PLGA nanoparticles, which changes negative Zeta potential to positive. But the increase of positive Zeta potential is not significant when chitosan concentration is above certain range as shown in Fig. 3. The positive chitosan coating on the surface of CHT-PLGA nanoparticles can also shield siRNA from degradation in the harsh environment. In addition, since the cell membrane is negatively charged, cationic CHT-PLGA nanoparticles can interact more easily with the cell membrane, therefore promoting endocytosis and increasing siRNA silencing efficiency.

In this study, the average particle size of CHT-PLGA nanoparticles increased gradually from 182 to 543 nm with the increase of initial chitosan concentration from 0.00 to 0.17% (Fig. 2). The increased particle size could be credited to the increased viscosity of chitosan, which lowered the shear stress on PLGA organic phase during sonication and produced larger emulsion droplets and subsequently larger solid nanoparticles. Another reason behind increased particle diameter was due to increasing amounts of coated chitosan on the surface of the nanoparticles. The loading of chitosan in nanoparticles is increased proportionally as evidenced by increasing nanoparticle sizes with increased chitosan concentration. However, chitosan-to-PLGA ratio in the final nanoparticles will be lower than the theoretical ratio, due to the fact that a certain amount of chitosan is not incorporated or coated on PLGA nanoparticles and remains in aqueous phase. The Zeta potential of CHT-PLGA nanoparticles increased from −10.6 to +31.2 mV with increasing amounts of chitosan in the formulation. It is also reasonable to predict that a plateau of Zeta potential can be achieved eventually, which could be indicative of saturated adsorption of chitosan on CHT-PLGA nanoparticles. In addition, the siRNA loading efficiency in CHT-PLGA nanoparticles increased with the chitosan concentration. The highest siRNA loading efficiency (77.7%) was observed in nanoparticles modified with the highest amount of chitosan in Sample V.

By using agarose gel electrophoresis, the ability of CHT-PLGA nanoparticles to interact with siRNA was investigated. siRNA migration was found for both naked siRNA and PLGA nanoparticle controls, due to the absence of complexation of siRNA with chitosan (Fig. 6). Migration was also found for CHT-PLGA nanoparticles with a lower amount of chitosan in Sample II, due to the fact that complexation of chitosan with siRNA was weak at low concentration. But beyond 0.033% of chitosan coating concentration, siRNA formed stable complexes with chitosan in nanoparticles, which effectively prevented siRNA from migrating in the gel electrophoresis. To achieve effective gene silencing *in vivo*, siRNA in delivery systems should be protected from degradation by enzymes. In the protection assay, it was also found that complexation of siRNA with chitosan also effectively protected oligos from RNase I degradation in CHT-PLGA nanoparticles with chitosan coating concentration above 0.033% (Fig. 7). For samples modified with lower chitosan, siRNA was degraded by RNase I, and the corresponding siRNA band was not observed in the gel electrophoresis. The results obtained from protection assays were in good agreement with those from the previous gel retardation assay. Since siRNA is

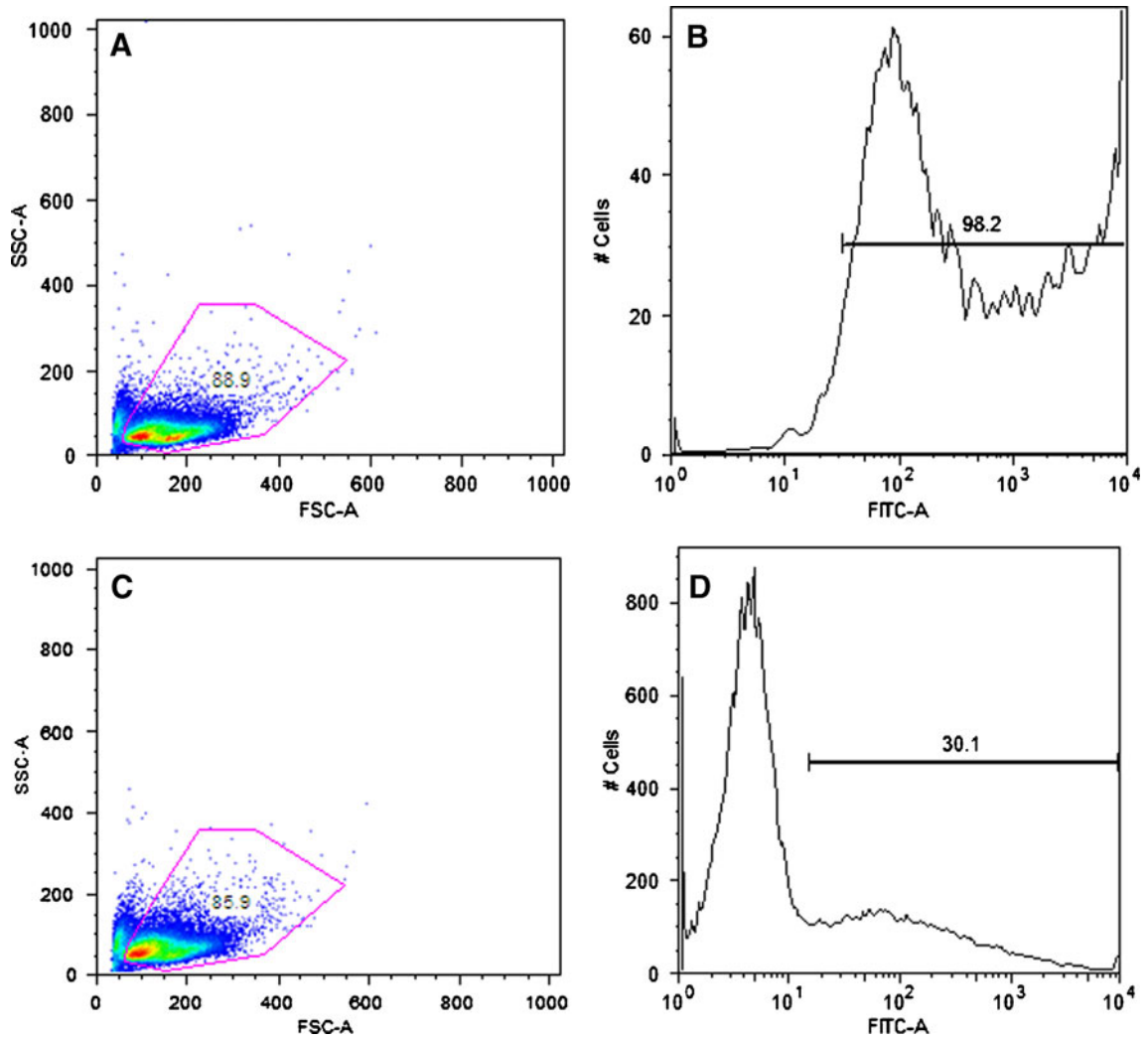

Fig. 9 FACS of the siRNA loaded CHT-PLGA nanoparticle transfection of HEK 293 T cells with GFP plasmid (**a**) Cell viability of the negative control 293 T cells with GFP plasmid; (**b**) GFP expression level of the negative control 293 T cells with GFP plasmid; (**c**) Cell viability after transfection by siRNA loaded CHT-PLGA nanoparticle (Sample V); (**d**) GFP expression level after transfection by siRNA loaded CHT-PLGA nanoparticles (Sample V).

negatively charged, it will complex with positively charged chitosan. Therefore, it exists both inside the matrix of nanoparticles and on the surface of nanoparticles. After cell uptake of nanoparticles, siRNA coated on the surface will be released at first to produce an initial burst release. Then siRNA entrapped inside the matrix will be released slowly through diffusion and polymer erosion to achieve sustained release.

Determination of gene silencing activity of prepared CHT-PLGA nanoparticles was performed in HEK 293 T cells (Fig. 8). FACS was used to quantify the percentage of GFP expression or the efficiency of gene silencing (Fig. 9). It was found that CHT-PLGA nanoparticles with highest amount of chitosan (Sample V) showed the highest GFP silencing efficiency (63.3±5.6%) (Fig. 5), which was statistically the same as that of positive control Lipofectamine 2,000 (68.3±2.9%). The GFP silencing efficiency increased significantly with the increase of siRNA loading efficiency due to the fact that more siRNA was available to be released into cytoplasm (Fig. 10). In addition, as a result of higher positive Zeta potential, CHT-PLGA nanoparticles were able to complex with more negatively charged siRNA. Therefore, the loading efficiency was increased, endocytosis of nanoparticles was enhanced, and subsequently, GFP silencing efficiency was improved (Fig. 11). The increased silencing effect by CHT-PLGA nanoparticles was a result of intertwined factors of increased siRNA loading and positive Zeta potential, instead of a single factor.

In the XTT cytotoxicity studies, it was found that all nanoparticle-treated cells showed high viability above 85%, which was in agreement with FACS results. The low cytoxicity of CHT-PLGA nanoparticles was probably due to the biocompatible and biodegradable nature of both

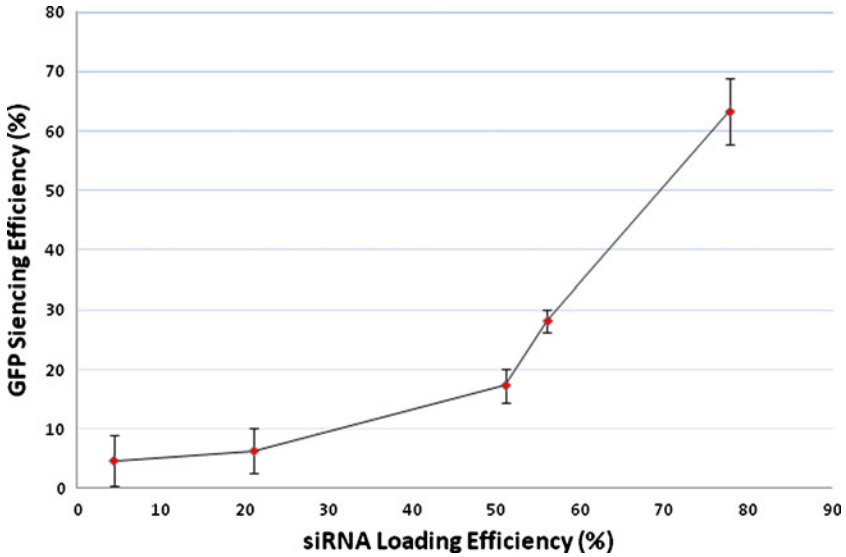

Fig. 10 The relationship between siRNA loading efficiency in CHT-PLGA nanoparticles and GFP silencing efficiency ($n = 3$).

PLGA polymer and chitosan. The attribute of low toxicity of CHT-PLGA nanoparticles is highly desirable for future *in vivo* applications.

CONCLUSION

In this study, siRNA was successfully loaded into biodegradable cationic CHT-PLGA nanoparticles, which offered enhanced cytoplasmic delivery of siRNA and effective protection for oligos from RNase I degradation. The delivered siRNA effectively silenced GFP and inhibited its expression in cultured cells. The prepared biodegradable CHT-PLGA nanoparticles may be practically useful for siRNA therapeutics in future application. However, additional optimization and *in vivo* studies should be conducted to confirm the effectiveness and safety of this delivery system before it can be possibly used in clinical settings.

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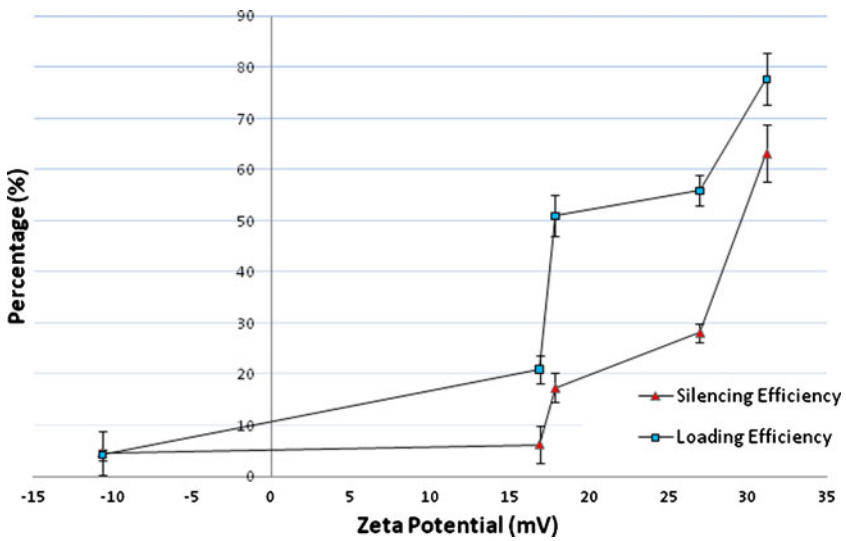

Fig. 11 The relationship between CHT-PLGA nanoparticle Zeta potential with siRNA loading efficiency and GFP silencing efficiency ($n = 3$).

REFERENCES

1. Jiang HL, Kim YK, Arote R, Nah JW, Cho MH, Choi YJ *et al.* Chitosan-graft-polyethylenimine as a gene carrier. J Control Release. 2007;117:273–80.
2. Relano-Gines A, Gabelle A, Lehmann S, Milhavet O, Crozet C. Gene and cell therapy for prion diseases. Infectious disorders drug targets. 2009;9:58–68.
3. Kima T-H, Jianga H-L, Jerea D, Parka I-K, Chob M-H, Nahc J-W *et al.* Chemical modification of chitosan as a gene carrier *in vitro* and *in vivo*. Prog Polym Sci. 2007;32:726–53.
4. Katas H, Alpar HO. Development and characterisation of chitosan nanoparticles for siRNA delivery. J Control Release. 2006;115:216–25.
5. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001;411:494–8.
6. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998;391:806–11.
7. Jones SW, Souza PM, Lindsay MA. siRNA for gene silencing: a route to drug target discovery. Curr Opin Pharmacol. 2004;4:522–7.
8. McManusand MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. Nat Rev. 2002;3:737–47.
9. Lima RT, Martins LM, Guimaraes JE, Sambade C, Vasconcelos MH. Specific downregulation of bcl-2 and xIAP by RNAi enhances the effects of chemotherapeutic agents in MCF-7 human breast cancer cells. Cancer Gene Ther. 2004;11:309–16.
10. Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature. 2000;408:325–30.
11. Gonczy P, Echeverri C, Oegema K, Coulson A, Jones SJ, Copley RR *et al.* Functional genomic analysis of cell division in C. elegans using RNAi of genes on chromosome III. Nature. 2000;408:331–6.
12. Nabatiyan A, Krude T. Silencing of chromatin assembly factor 1 in human cells leads to cell death and loss of chromatin assembly during DNA synthesis. Mol Cell Biol. 2004;24:2853–62.
13. Suri SS, Fenniri H, Singh B. Nanotechnology-based drug delivery systems. J Occup Med Toxicol. 2007;2:16.
14. Quong D, Neufeld RJ. DNA protection from extracapsular nucleases, within chitosan- or poly-L-lysine-coated alginate beads. Biotechnol Bioeng. 1998;60:124–34.
15. Smith AE. Viral vectors in gene therapy. Annu Rev Microbiol. 1995;49:807–38.
16. Pinnaduwage P, Schmitt L, Huang L. Use of a quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells. Biochim Biophys Acta. 1989;985:33–7.
17. Legendre JY, Szoka Jr FC. Cyclic amphipathic peptide-DNA complexes mediate high-efficiency transfection of adherent mammalian cells. Proc Natl Acad Sci USA. 1993;90:893–7.
18. Gao X, Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry. 1996;35:1027–36.
19. Yuan X, Li L, Rathinavelu A, Hao J, Narasimhan M, He M *et al.* SiRNA drug delivery by biodegradable polymeric nanoparticles. Journal of Nanoscience and Nanotechnology. 2006;6:2821–8.
20. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc Natl Acad Sci USA. 1995;92:7297–301.
21. Haensler J, Szoka Jr FC. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconjug Chem. 1993;4:372–9.
22. Ihm JE, Han KO, Han IK, Ahn KD, Han DK, Cho CS. High transfection efficiency of poly(4-vinylimidazole) as a new gene carrier. Bioconjug Chem. 2003;14:707–8.
23. Rolland AP. From genes to gene medicines: recent advances in nonviral gene delivery. Crit Rev Ther Drug Carrier Syst. 1998;15:143–98.
24. Montag ME, Morales Jr L, Daane S. Bioabsorbables: their use in pediatric craniofacial surgery. J Craniofac Surg. 1997;8:100–2.
25. Eppley BL, Reilly M. Degradation characteristics of PLLA-PGA bone fixation devices. J Craniofac Surg. 1997;8:116–20.
26. Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev. 2003;55:329–47.
27. Miyamoto S, Takaoka K. Bone induction and bone repair by composites of bone morphogenetic protein and biodegradable synthetic polymers. Ann Chir Gynaecol. 1993;207:69–75.
28. Aiba S. Studies on chitosan: 2. Solution stability and reactivity of partially N-acetylated chitosan derivatives in aqueous media. Int J Biol Macromol. 1989;11:249–52.
29. Tomihata K, Ikada Y. *In vitro* and *in vivo* degradation of films of chitin and its deacetylated derivatives. Biomaterials. 1997;18:567–75.
30. Chandy T, Sharma CP. Chitosan-as a biomaterial. Biomater Artif Cells Artif Organs. 1990;18:1–24.
31. Mao HQ, Roy K, Troung-Le VL, Janes KA, Lin KY, Wang Y *et al.* Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J Control Release. 2001;70:399–421.
32. Erbacher P, Zou S, Bettinger T, Steffan AM, Remy JS. Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability. Pharm Res. 1998;15:1332–9.
33. Yang R, Yang SG, Shim WS, Cui F, Cheng G, Kim IW *et al.* Lung-specific delivery of paclitaxel by chitosan-modified PLGA nanoparticles via transient formation of microaggregates. J Pharm Sci. 2009;98:970–84.
34. Nafee N, Taetz S, Schneider M, Schaefer UF, Lehr CM. Chitosan-coated PLGA nanoparticles for DNA/RNA delivery: effect of the formulation parameters on complexation and transfection of antisense oligonucleotides. Nanomedicine. 2007;3:173–83.
35. Beisner J, Dong M, Taetz S, Nafee N, Griese EU, Schaefer U, *et al.* Nanoparticle mediated delivery of 2′-O-methyl-RNA leads to efficient telomerase inhibition and telomere shortening in human lung cancer cells. Lung Cancer. 2009.
36. Taetz S, Nafee N, Beisner J, Piotrowska K, Baldes C, Murdter TE *et al.* The influence of chitosan content in cationic chitosan/PLGA nanoparticles on the delivery efficiency of antisense 2′-O-methyl-RNA directed against telomerase in lung cancer cells. Eur J Pharm Biopharm. 2009;72:358–69.
37. Kim WJ, Kim SW. Efficient siRNA delivery with non-viral polymeric vehicles. Pharm Res. 2009;26:657–66.
38. Park MR, Han KO, Han IK, Cho MH, Nah JW, Choi YJ *et al.* Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers. J Control Release. 2005;105:367–80.
39. Huang M, Fong CW, Khor E, Lim LY. Transfection efficiency of chitosan vectors: effect of polymer molecular weight and degree of deacetylation. J Control Release. 2005;106:391–406.
40. Zauner W, Farrow NA, Haines AM. *In vitro* uptake of polystyrene microspheres: effect of particle size, cell line and cell density. J Control Release. 2001;71:39–51.
41. Bivas-Benita M, Romeijn S, Junginger HE, Borchard G. PLGA-PEI nanoparticles for gene delivery to pulmonary epithelium. Eur J Pharm Biopharm. 2004;58:1–6.