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Research Paper

Thiolated Chitosan/DNA Nanocomplexes Exhibit Enhanced and Sustained Gene Delivery

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Purpose. Thiolated chitosan appears to possess enhanced mucoadhesiveness and cell penetration properties, however, its potential in gene-drug delivery remains unknown. Herein, we report on a highly effective gene delivery system utilizing a 33-kDa thiol-modified chitosan derivative.
Methods. Thiolated chitosan was prepared by the reaction with thioglycolic acid. Nanocomplexes of unmodified chitosan or thiolated chitosan with plasmid DNA encoding green fluorescenct protein (GFP) were characterized for their size, zeta potential, their ability to bind and protect plasmid DNA from degradation. The transfection efficiency of thiolated chitosan and sustained gene expression were evaluated in various cell lines *in vitro* and in Balb/c mice *in vivo*.
Results. Thiolated chitosan–DNA nanocomplexes ranged in size from 75 to 120 nm in diameter and from +2.3 to 19.7 mV in zeta potential, depending on the weight ratio of chitosan to DNA. Thiolated chitosan, CSH360, exhibited effective physical stability and protection against DNase I digestion at a weight ratio $\geq$ 2.5:1. CSH360/DNA nanocomplexes induced significantly ($P<0.01$) higher GFP expression in HEK293, MDCK and Hep-2 cell lines than unmodified chitosan. Nanocomplexes of disulphide-crosslinked CSH360/DNA showed a sustained DNA release and continuous expression in cultured cells lasting up to 60 h post transfection. Also, intranasal administration of crosslinked CSH360/DNA nanocomplexes to mice yielded gene expression that lasted for at least 14 days.
Conclusions. Thiolated chitosans condense pDNA to form nanocomplexes, which exhibit a significantly higher gene transfer potential and sustained gene expression upon crosslinking, indicating their great potential for gene therapy and tissue engineering.

KEY WORDS: chitosan; gene transfer; thiolation; crosslinking; nanocomplexes.

INTRODUCTION

Advances in genomics and proteomics have led to the discovery of numerous gene targets and the use of gene therapy for treatment of diverse human diseases (1,2). Although both viral and non-viral gene delivery systems are under investigation, virus-based gene therapy is limited by concerns about endogenous virus recombinations, oncogenic effects and immunological reactions (3–6). The non-viral, polymer- or lipid-based gene delivery agents such as polyamidoamine, polyethyleneimine, poly-L-lysine and poly (lactic-co-glycolic acid) (PLGA) copolymers, offer several advantages, including ease of production and reduced risk of immunogenicity, but their use has been limited by their relatively low transfection efficiency, non-degradability and potential toxicity (7,8).

We and others have used chitosan, a linear copolymer of *N*-acetyl-*D*-glucosamine and *D*-glucosamine linked by glucosidic linkages, as a vehicle for *in vivo* therapeutic transfer of genes and siRNAs (9–14). Chitosan has emerged as a promising candidate for gene delivery because of biocompatibility, biodegradability, favorable physicochemical properties and ease of chemical modification. The presence of positive charges from amine groups makes chitosan suitable for modification of its physicochemical and biological properties (15,16), and enables it to transport plasmid DNA (pDNA) into cells via endocytosis and membrane destability (4,11,17,18). Most studies to date have used high molecular weight chitosan (100 – 400 kDa), which exhibits aggregation, low solubility under physiological conditions, high viscosity at concentrations used for *in vivo* delivery and slow dissociation or degradation (18). However, recently a second-generation chitosan with molecular weight up to 50 kDa has been isolated and characterized (7). Chitosans less than 10 kDa, also known as oligo-chitosans have been described that form weak complexes with pDNA, resulting in physically unstable polyplexes with low transfection efficiency (7,18).

We have been investigating the potential for enhancing gene transfer by thiol modification of chitosan. Thiolated chitosan appears to possess significantly improved mucoad-

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hesiveness and to enhance the permeability properties of drugs (19,20), but the potential of thiolated chitosans for gene transfer has not been studied. Since thiolated chitosan forms inter- as well as intramolecular disulfide bonds upon oxidation (19,21), it was reasoned that thiolation may allow crosslinking of chitosan, which, in turn may allow slow sustained release of pDNA. To test this, a 33 kDa chitosan with a high degree of deacetylation (>90%) was characterized and tested for enhanced and sustained gene delivery and expression in the absence or presence of thiolation with or without crosslinking. The results indicate that thiolated chitosan forms nanocomplexes with pDNA encoding the reporter gene for the green fluorescenct protein (GFP) and allows sustained gene delivery and expression of GFP both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Preparation of Thiolated Chitosan and Plasmid DNA

Chitosan (MW 33 kDa, degree of deacetylation >90%, viscosity 2.8 cps at 0.5% solution in 0.5% acetic acid at 20°C, Taehoon Bio. Korea) of 0.5 g was dissolved in 50 ml of aqueous acetic acid solution (1.0%) to which 400 or 100 μl of thioglycolic acid (TGA) was added. In order to activate the carboxylic acid moieties of TGA, 0.5 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was added. The pH of the solution was adjusted to 5.0 using 1 mM NaOH and the chemical reaction was allowed to run at room temperature for 5 h. To eliminate unbound TGA and isolate the conjugated polymers, the reaction mixture was dialyzed (molecular weight cut-off 6 kDa). The chitosan conjugate was lyophilized at -30°C and stored at 4°C until further use. The degree of chemical modification of the chitosan–thioglycolic acid conjugate was determined spectrophotometically by measuring thiol groups at room temperature using Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid) at a wavelength of 412 nm (19,20,22). A plasmid (pEGFP-N2, 4.7 kbp, Clontech, USA) containing the human cytomegalovirus (CMV) promoter and enhanced green fluorescent protein gene was amplified in *Escherichia coli* and purified using GenElute HP Plasmid Maxprep Kits (Sigma, USA). For cellular uptake studies, the plasmid was labeled with TM-rhodamine (Mirus, WI).

Preparation and Characterization of Chitosan/pDNA Nanocomplexes

Chitosan/pDNA nanocomplexes were prepared by mixing chitosan (2 μg/μl) and pDNA (2 μg/μl) solutions in phosphate buffer at pH 6.2. The chitosan/pDNA charge ratio was determined assuming a molecular weight of pDNA of 325 g/mol and one negative charge per DNA base. Positive charge units were calculated assuming one positive charge per amine group adjusted for the degree of deacetylation of chitosan. The loss of amine groups after thiolation was not considered in the calculation of positive charges of thiolated chitosans. Nanocomplexes of thiolated chitosan with pDNA were incubated at 37°C for 12 h to oxidize thiol groups to crosslink thiolated chitosan in the nanocomplexes. Particle size and zeta potential of chitosan/pDNA nanocomplexes were measured using a Nicomp380/ZLS (Particle Sizing Systems Inc., USA) at 25°C.

MTT Cytotoxicity Assay

The evaluation of cytotoxicity of thiolated chitosan was performed by MTT assay using the HEK 293 cell line. Cells were seeded at 2.0×10^5 cells/well in a 12-well flat-bottomed tissue culture plate and incubated for 24 h. Chitosan/pDNA complexes were added and incubated for 6 h at 37°C. The transfection mixture was replaced with 500 μl of serum-free DMEM to which 150 μl MTT solution (2 mg/ml) in PBS was added. After incubation at 37°C for 4 h, the MTT-containing medium was removed and 750 μl of DMSO was added to dissolve the formazan crystals formed by cells. Cell viability was determined by measuring the absorbance at 570 nm.

Adsorption of Mucin by Chitosan/pDNA Nanoparticles

Mucoadhesiveness was calculated as the amount of mucin adsorbed by chitosan/DNA nanocomplexes (400 μg/80 μg) in a certain time period. Nanocomplex suspensions were mixed with type I-S mucin solutions (100μg/ml), vortexed, and incubated at 37°C for 2, 4, 6 h. After adsorption, the suspensions were centrifuged at $20{,}000\times g$ for 30 min and free mucin concentration was measured in the supernatant by a colorimetric method using periodic acid/Schiff (PAS) staining, as reported previously (23).

Protection Against DNase I Degradation

pDNA (1 μg) alone or chitosan/pDNA nanocomplexes with a different weight ratio were prepared in 10 μl of phosphate buffer at pH 6.2 to which 2 μl of DNase I (5U) was added and incubated at 37°C for 2 h. Then, 5 μl of 100 mM EDTA was added and the mixture was incubated at room temperature for 10 min. After incubation, 10 μl of heparin solution (5 mg/ml) was added to the mixture and incubated at room temperature for 2 h to dissociate the complexes. The integrity of DNA was examined using the agarose gel retardation assay.

In-Vitro DNA Release

Chitosan/pDNA complexes at a weight ratio of 5:1 were incubated in a transfection medium (DMEM with pH 7.0) at 37°C. After different periods of incubation, chitosan/pDNA complexes were centrifuged at $20{,}000\times g$ for 30 min and the supernatants were collected to determine the DNA content by measuring the fluorescent intensity after the addition of fluorescent nucleic acid strain (Quanti-iT™ PicoGreen®, Molecular Probes, USA). The integrity of pDNA released was also examined using the agarose gel retardation assay.

In Vitro Transfection

Transfection medium was prepared by dissolving Dulbecco's Modified Eagles' Medium (Sigma) in sterile water and adjusting pH to 7.0 by adding sodium bicarbonate. HEK 293 cells were seeded in a six-well culture plate at a density of 0.8×10^6 cells/well and incubated at 37°C in a CO_2

incubator for 24 h. The solutions of pDNA and various amount of chitosan were diluted separately in 50 μl of transfection medium. After 5 min, the two solutions were combined, mixed gently and incubated at room temperature for 5 min. Then, 900 μl of transfection medium was added to each tube containing chitosan/pDNA nanocomplexes. The formulations were mixed gently and added to cells. After 6 h incubation, the medium and chitosan/DNA nanocomplexes were replaced with fresh DMEM containing 5% FBS.

Flow Cytometry

To quantify the transfection efficiency of chitosan, transfected cells were harvested and scored for GFP-positive cells by flow cytometry (FACScan, BD Biosciences, USA) with appropriate gating and controls using the green channel FL-1H. A total of 1.5×10^4 events were counted for each sample and more than 90% of cells were gated for analysis. The percentage of positive events was calculated as the events within the gate divided by total number of events then subtracting percentage of control samples.

Immunoblotting

Proteins were extracted from transfected HEK 293 cells after various periods of incubation using lysis buffer. Electrophoresis was performed using 40 μg of cell lysate on a 12% polyacrylamide gel and proteins were transferred to PVDF membranes (Bio-Rad, USA). The blot was incubated with a rabbit anti-green fluorescent protein polyclonal antibody (Chemicon, USA) and HRP-conjugated anti-rabbit IgG (Cell Signaling, USA) which is used as a secondary antibody. Immunoblot signals were developed using Super-Signal Ultra chemiluminescent reagent (Pierce, USA).

Examination of Bronchoalveolar Lavage (BAL) Fluid

Plasmid DNA (15 μg) was combined with chitosan solution (2 mg/ml) to form nanocomplexes. These nanocomplexes were then given intranasally to 4–6 week old BALB/c mice (n=4) on day 0. Mice were sacrificed on days 3, 7, and 14 and lungs were lavaged with 500 μl of PBS introduced through the trachea. The BAL fluid was centrifuged, washed with PBS and resuspended in PBS. Aliquots of the cell suspension (150 μl) were applied to slides using cytospin apparatus. Cells were examined under a fluorescent microscope (ECLIPSE TE300 Inverted Microscope, Nikon, Japan) for GFP expression and photography. Fluorescent images were made with a fixed exposure time so that low-intensity auto-fluorescence of BAL cells was not imaged.

RESULTS

Characterization and Physicochemical Properties of Thiolated Chitosan

To synthesize and characterize the nanocomplexes of chitosan and pDNA, 33 kDa chitosan was used as a starting material. To graft thiol groups on chitosan, the primary amine groups of chitosan were utilized, as shown in Fig. 1A. Water soluble EDAC (1-ethyl-3-(3-dimethylaminopropyl)

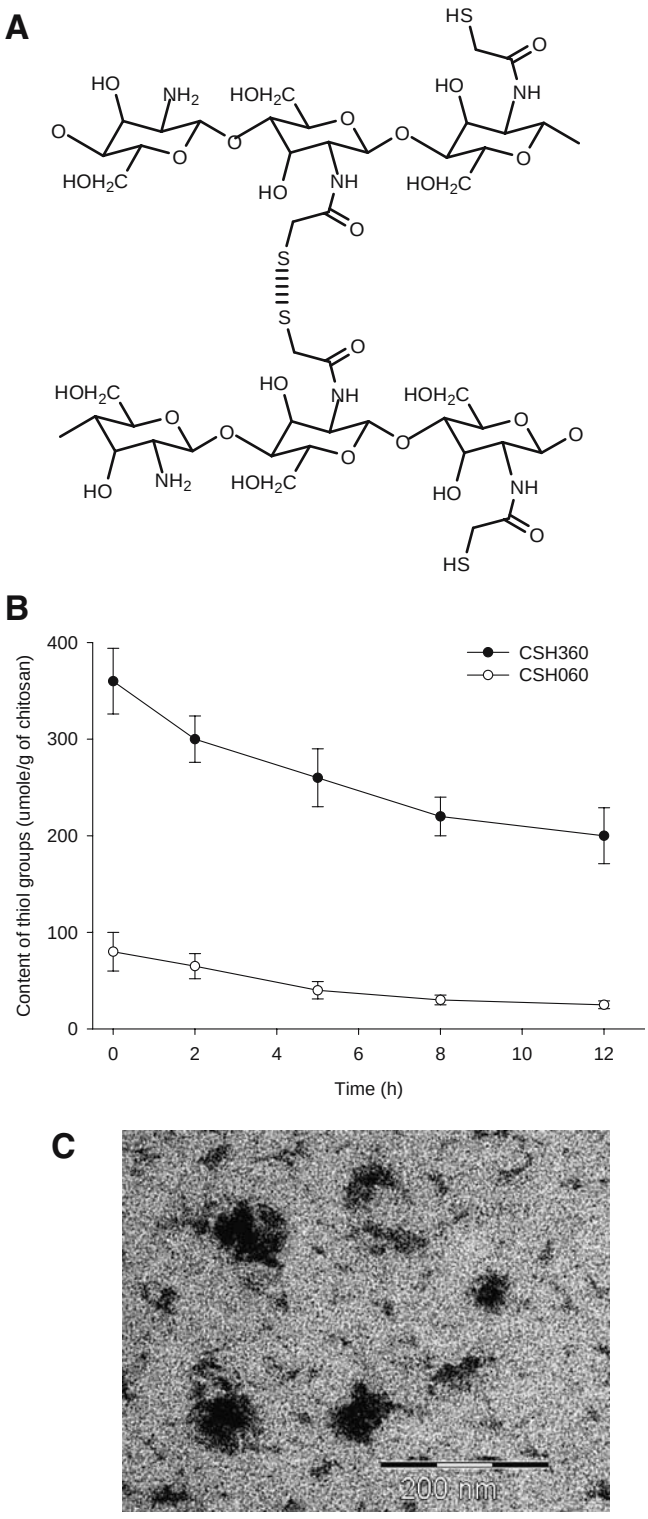

Fig. 1. Chemical structure of chitosan–thioglycolic acid conjugates and intermolecular disulfide bonding (A) and decrease of the thiol group content within CSH60 and CSH360 (B). Conjugates were dissolved in demineralized water at a final concentration of 0.2% and pH was adjusted to 6.0 with 100 mM acetate buffer. The samples were incubated at 37°C. The amount of remaining thiol groups was determined with Ellman's reagent. Indicated values are means (±S.D.) of at least three experiments. TEM micrograph of CSH360/pDNA nanocomplexes at a weight ratio 5:1 (C).

Table I. Characteristics of Nanocomplexes of Unmodified Chitosan with pDNA Expressing GFP

Weight ratio (Chitosan/pDNA)	Charge ratio (+/−)	Particle size (nm)	Zeta potential (mV)
1:1	1.8:1	75±4	2.3±0.3
2.5:1	4.5:1	92±4	7.6±1.1
5:1	9.0:1	120±7	19.7±3.9

Particle size and zeta potential of nanocomplexes were measured at pH 6.2 by dynamic light scattering and electrophoretic light scattering. Indicated values are means (±S.D.) of three experiments.

carbodiimide hydrochloride) reacted with carboxyl groups of thioglycolic acid to form active ester intermediates which reacted with free amine groups of chitosan to form amide bonds (17). Lyophilized thiolated chitosan appears as a white fibrous powder easily soluble in water. Since two equivalent carbons and two equivalent protons of thioglycolic acid appeared overlapped with those of chitosan in a NMR spectrum, UV spectrophotometer was employed to measure the thiol group content, as reported previously (19,20,22,24,25). From the spectrophotometric assay using Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid), the content of thiol groups conjugated to chitosan molecules was determined to be equivalent to 60.0 ± 10.0 or 360 ± 34 μmol per 1 g of chitosan, depending on the ratio between chitosan and thioglycolic acid. The preparations were referred to as CSH060 and CSH360, respectively. It was estimated that ~2.5 and ~12.5 thiol groups were grafted to each CSH60 and CSH360 molecule. The content of thiol groups decreased constantly during an incubation at 37°C at pH 6.0 (Fig. 1B). CSH360 and CSH060 exhibited a reduction in thiol group content by 33 and 40%, respectively, indicating the formation of inter- as well as intramolecular disulfide bonds.

Physicochemical properties of unmodified chitosan/pDNA nanocomplexes with various weight ratios were characterized in terms of size and zeta potential. The nanocomplexes ranged from 75 to 120 nm in diameter and from +2.3 to +19.7 mV in zeta potential, which was directly related to the ratio of chitosan to pDNA and hence the surface charge (Table I). The nanocomplexes of thiolated chitosans with pDNA showed a reduction in zeta potential (Table II), but the size remained similar to unmodified chitosan nanocomplexes, i.e., about 120 nm as determined by transmission electron microscopy (Fig. 1C). CSH360/pDNA nanocomplexes were incubated at 37°C for 12 h to oxidize thiol groups to crosslink thiolated chitosan through the formation of inter- as well as intramolecular disulfide bonds. Crosslinking of thiolated chitosan in the nanocomplexes increased the particle size to some extent; however, zeta potential was not altered. The biocompatibility of chitosan and its derivatives was evaluated using human embryonic kidney (HEK) 293 cells according to a standard methyl thiazole tetrazolium (MTT) cytotoxicity assay. Unmodified chitosan incubated for 6 or 12 h with HEK 293 cells exhibited no cytotoxicity at all weight ratios (data not shown). Both thiolation and crosslinking showed no influence on cell viability.

Mucin Adsorption on Chitosan/pDNA Nanocomplexes

Mucin, a glycoprotein, is the major component of the mucus that coats the cells lining the surfaces of the respiratory track and may be a barrier to transfection of epithelial cells. Since thiolation increases mucoadhesiveness, mucin adsorption by unmodified or thiolated chitosans (CSH360) was measured to examine their ability to bind the mucosal surfaces and augment gene transfer. Mucoadhesiveness was calculated as the amount of mucin adsorbed by chitosan/pDNA nanocomplexes over an 8-h time period. Unmodified chitosan/pDNA and CSH360/pDNA nanocomplexes adsorbed a similar amount of mucin after 1 h incubation (Fig. 2). Mucin adsorption on unmodified chitosan/pDNA nanocomplexes did not change during the study period. In contrast, CSH360/pDNA nanocomplexes exhibited a gradual increase in mucin adsorption and greater binding after 6 h than unmodified chitosan/pDNA nanocomplexes, indicating the enhanced mucoadhesiveness of thiolated chitosan due probably to the formation of covalent bonds between thiol groups of chitosan and cysteine-rich subdomains of mucin (26).

Thiolation Protects DNA and Allows Slow DNA Release

To examine the effect of thiolation and crosslinking of chitosan on pDNA binding capacity and protection, agarose gel electrophoresis was performed. The physical stability of the chitosan/pDNA nanocomplexes was investigated in the absence and presence of heparin (Fig. 3A). Unmodified chitosan complexed and retained pDNA completely at a weight ratio of 1:1 in the absence of anionic heparin. In the presence of heparin, a slight pDNA release was observed at a ratio of 1:1 and highly effective complexation was observed at a ratio ≥ 2.5:1. Thiolated (CSH360) chitosan released a very small fraction of pDNA at a ratio of 1:1 in the absence

Table II. Characteristics of Nanocomplexes with pDNA and Transfection Efficiency in HEK 293 Cells by Flow Cytometry at a Chitosan:pDNA Ratio of 5:1 (wt/wt)

	Content of Thiol groups (μmol/g of Chitosan)	Particle size (nm)	Zeta potential (mV)	Transfection efficiency (%) at pH 7.0
Lipofectin	N/A	N/A	N/A	16.4±2.1
Unmodified chitosan	0	120±7	19.7±3.9	5.8±1.1
Thiolated chitosan CSH360	360±34	113±7	12.5±2.1	31.2±4.3
Thiolated chitosan CSH060	60±10	103±10	15.3±3.3	12.3±2.3
Crosslinked CSH360	200±20.1	220±16	7.3±2.8	18.1±2.8

Transfection efficiency was measured at 60 h post-transfection. Indicated values are means (±S.D.) of three experiments.

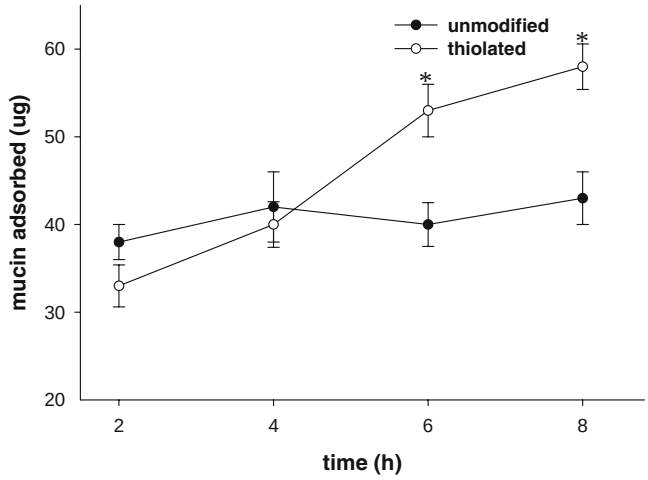

Fig. 2. Mucin adsorption on chitosan/pDNA nanocomplexes. Indicated values are means (±S.D.) of three experiments. *$P<0.01$ relative to unmodified chitosan at the same time point.

of heparin. However, upon incubation with heparin, a large fraction of pDNA was released from CSH360. As the weight ratio of chitosan to pDNA increased the fraction of pDNA released was diminished. CSH360/pDNA nanocomplexes were incubated at 37°C for 12 h to crosslink the thiolated chitosans through the formation of inter- as well as intramolecular disulfide bonds. Subsequent to crosslinking CSH360 did not release pDNA at a weight ratio of 1:1 in the absence of heparin. Upon the addition of heparin, a small fraction of pDNA was released from the crosslinked CSH360. The physical stability of the nanocomplexes was enhanced by increasing the weight ratio and/or charge ratio of the complexes. Crosslinked CSH360 released less pDNA than non-crosslinked CSH360, suggesting that the former made more stable nanocomplexes.

In an attempt to investigate the effect of thiolation and crosslinking of thiolated chitosan on pDNA protection against DNase, chitosan/pDNA nanocomplexes were treated with DNase I and then dissociated with heparin (Fig. 3B). Unmodified chitosan protected pDNA in the complexes and

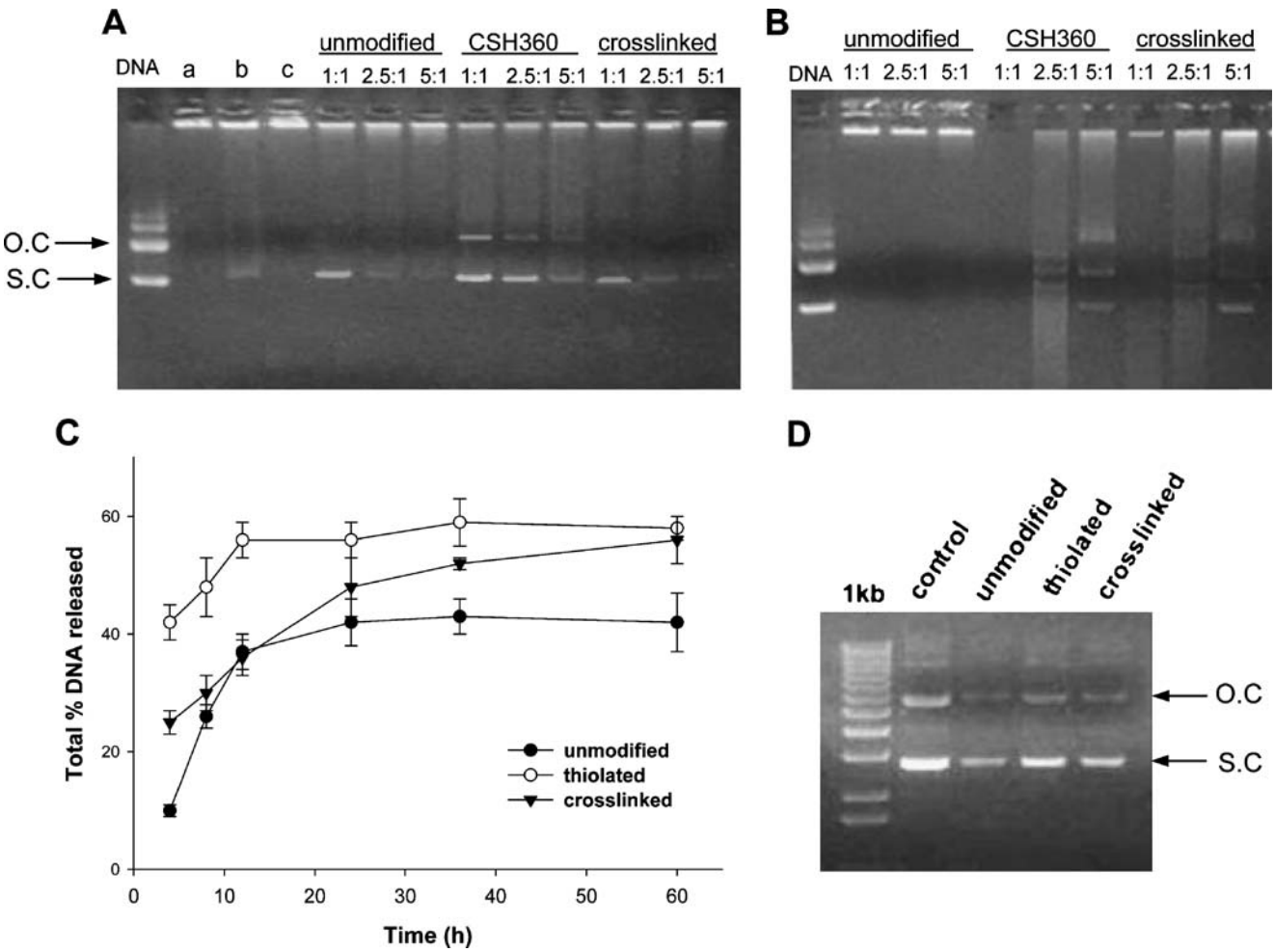

Fig. 3. Agarose gel electrophoresis and release profile of pDNA from chitosan/pDNA nanocomplexes. (A) Assay for DNA binding capacity of chitosan in the absence or presence of heparin. In the absence of heparin, pDNA nanocomplexes with unmodified chitosan (*a*), CSH360 (B) or crosslinked CSH360 at a weight ratio of 1:1 were electrophoresed. The other formulations were given heparin. (B) Assay for DNA protection against DNase I. (C) Release profiles of pDNA from chitosan/pDNA nanocomplexes at a weight ratio of 5:1. CSH360 was complexed with pDNA and then assessed before and after incubation to crosslink thiolated chitosan in the nanocomplexes with pDNA. (D) Agarose gel electrophoresis of pDNA released from chitosan/pDNA nanocomplexes. The control indicates 500 ng of pDNA. Control *S.C* and *O.C* indicate the supercoiled and open circle forms of pDNA.

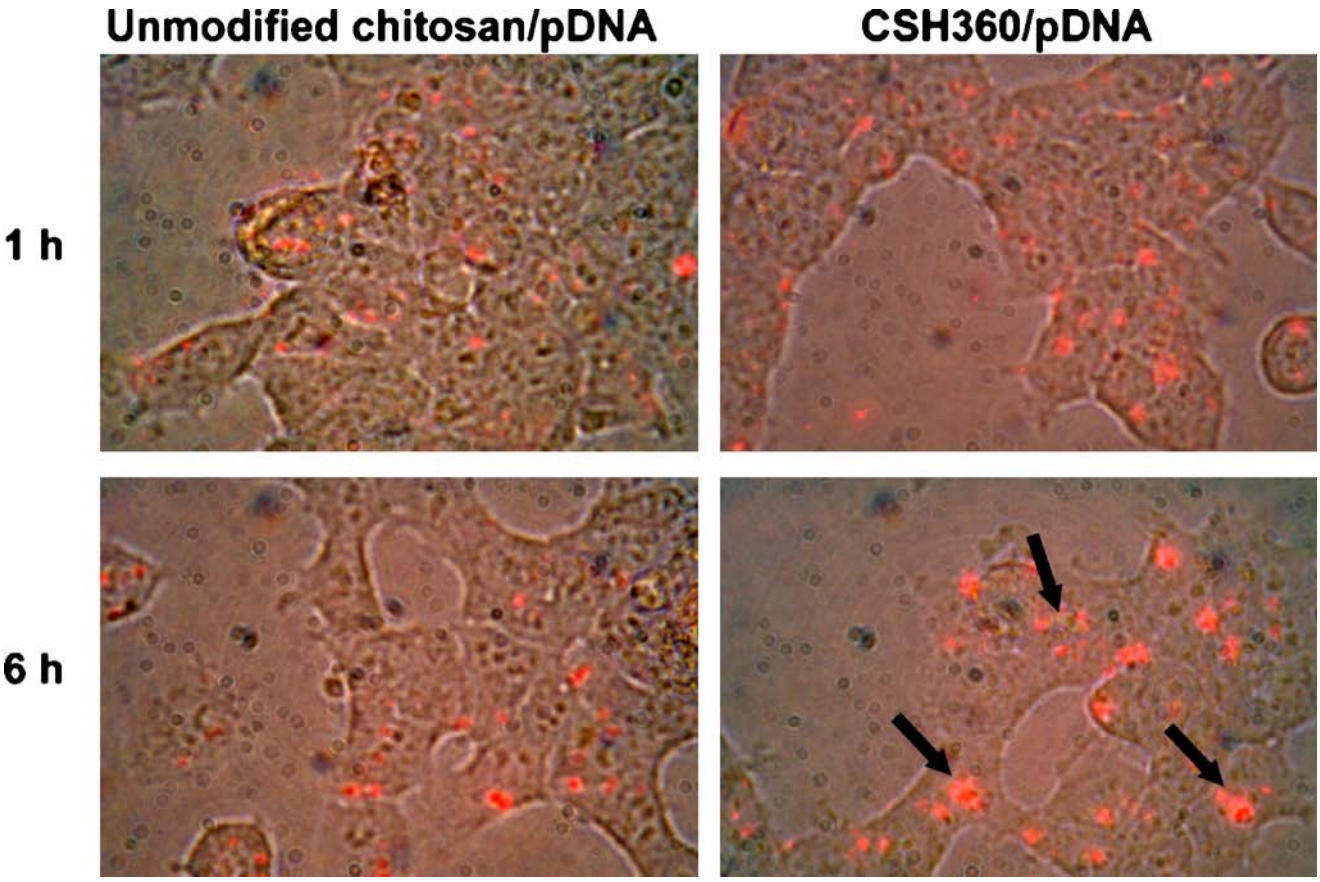

Fig. 4. Fluorescent micrographs of HEK 293 cells transfected with chitosan/rhodamine-pDNA nanocomplexes. Fluorescent images were made with the identical exposure time and merged with bright-field images using a WICF Image J program (NIH, USA). The results are expressed from the one representative experiment of two performed. *Arrows* point to rhodamine-pDNA in the nucleus.

retained pDNA completely at all weight ratios. CSH360 exhibited less protection against DNase I digestion and physical stability compared to unmodified chitosan. Effective protection against DNase I was observed at a weight ratio 5:1. In contrast, crosslinked CSH360 effectively protected and retained pDNA at a weight ratio ≥ 2.5:1, suggesting crosslinking of CSH enhanced the DNA protection ability.

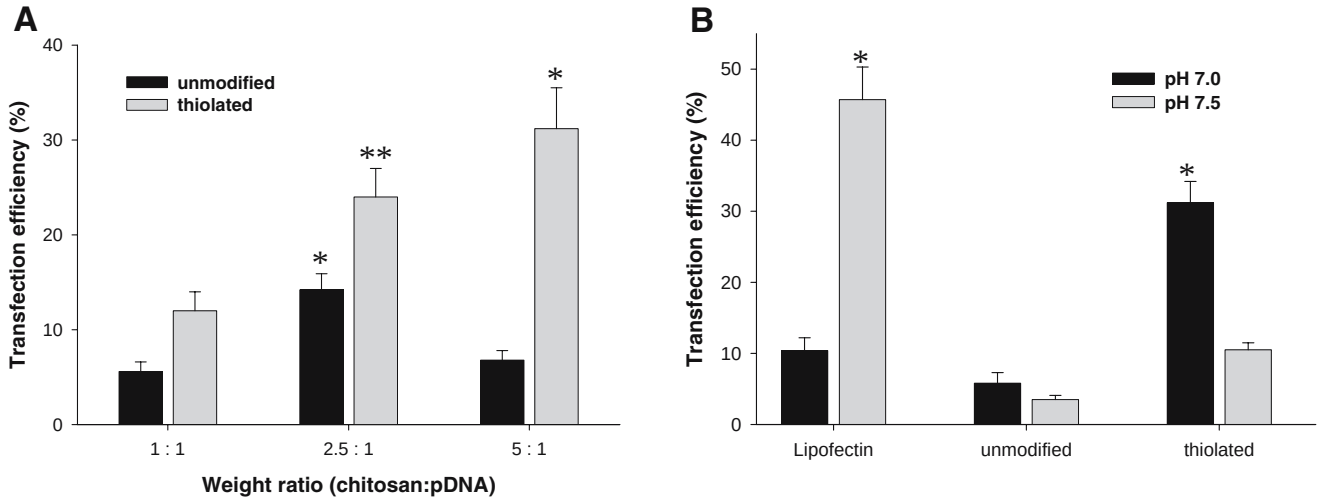

Fig. 5. The effects of weight ratio of chitosan to pDNA and pH on the transfection efficiency of chitosan. (A) The effect of weight ratio of chitosan to pDNA. Indicated values are means (±S.D.) of three experiments. **P*<0.01, **<0.05 relative to the weight ratio of 1:1. (B) The effect of pH on the transfection efficiency of unmodified and thiolated chitosan (CSH360). The weight ratio of chitosan to pDNA is 5:1. Indicated values are means (±S.D.) of three experiments. **P*<0.01.

Thiolation Enhances Transfection of Cultured Cells

To ascertain the effect of thiol groups on the cellular uptake of chitosan, HEK 293 cells were transfected with rhodamine-pDNA using unmodified chitosan and CSH360 in transfection medium (pH 7.0). After 1 h-incubation, more fluorescence was observed on the cell surface when delivered with CSH360 compared to unmodified chitosan. Also, more rhodamine-pDNA was accumulated in the cytoplasm and nucleus of cells transfected using CSH360 at 6 h post-transfection (Fig. 4). The results suggest that thiolated chitosan exhibits the enhanced cell permeation properties, leading to more cellular uptake.

To determine the *in vitro* transfection efficiency of thiolated chitosan and crosslinked thiolated chitosan, HEK 293 cells were transfected with chitosan nanocomplexes containing pDNA encoding GFP in transfection medium (pH 7.0). GFP-positive cells were scored by flow cytometry. For unmodified chitosan with 90% deacetylation, the highest transfection efficiency was obtained at a weight ratio of 2.5:1. In contrast, for thiolated chitosan (CSH360), the transfection efficiency increased with increasing weight ratio from 1:1 to 5:1 (Fig. 5A). However, a further increase in a weight ratio to 10:1 did not increase the transfection efficiency. CSH360 exhibited higher transfection efficiency than the unmodified chitosan or a liposomal transfection reagent, Lipofectin (Invitrogen, USA). It was also found that thiolated chitosan (CSH360) with a higher thiol group content exhibited a higher transfection efficiency (Table II). To further investigate the effect of thiol group content on gene transfer, thiol groups of CSH360 were oxidized to decrease the thiol group content and then mixed with pDNA to form nanocomplexes. After oxidation for 12 h, CSH360 exhibited reduced thiol group content (200±20 μmol per gram of chitosan) and a subsequent reduction in the transfection efficiency (Table II). HEK293 cells were transfected with unmodified and CSH360 nanocomplexes with GFP DNA to examine the effect of pH on the transfection efficiency of chitosan. While Lipofectin exhibited extremely high transfection efficiency at pH 7.5, unmodified and thiolated chitosan showed a significantly higher transfection efficiency at pH 7.0 than at 7.5 (Fig. 5B).

To test whether thiolation and crosslinking of thiolated chitosan/pDNA nanocomplexes affect the pDNA release profile and gene expression level, the time course of transfection was examined using HEK293 cells and the percentage of transfection was monitored for 4 days after transfection. A significant ($P<0.01$) increase in transfection efficiency was found with cells transfected with CSH360/pDNA and crosslinked CSH360/pDNA nanocomplexes compared to unmodified chitosan/pDNA nanocomplexes at 60 h post-transfection (Fig. 6). CSH360/pDNA nanocomplexes showed rapid enhanced gene expression by 60 h and reached a plateau soon after. In contrast, crosslinked CSH360/pDNA nanocomplexes exhibited a gradual increase in gene expression for 4 days. To confirm the superior transfection efficiency of CSH360, transfection was performed using two other cell lines, HEp-2 and MDCK. Transfection efficiency with each of the cell lines studied was lower than HEK293, but thiolated chitosan exhibited a higher transfection efficiency than unmodified chitosan (Fig. 7A). As a negative control, butanoyl chitosan was prepared to examine the effect of partial neutralization by the chemical reaction on the transfection efficiency of chitosan. Butanoyl chitosan showed less transfection efficiency than unmodified chitosan (Fig. 7B).

To further support sustained gene expression by thiolated chitosan/pDNA nanocomplexes after crosslinking, we extracted green fluorescent proteins synthesized in transfected cells to examine the level of gene expression using an immunoblotting assay. Thiolated chitosan (CSH360) induced more gene expression than Lipofectin and unmodified chitosan at an identical time point (Fig. 8A). While CSH360 showed rapid gene expression, crosslinked CSH360 showed a steady increase in gene expression during an observation period of 96 h (Fig. 8B, C). The results suggest that thiolation of chitosan increases the transfection efficiency and that sustained gene expression can be achieved by crosslinking the thiolated chitosan in the nanocomplexes with pDNA.

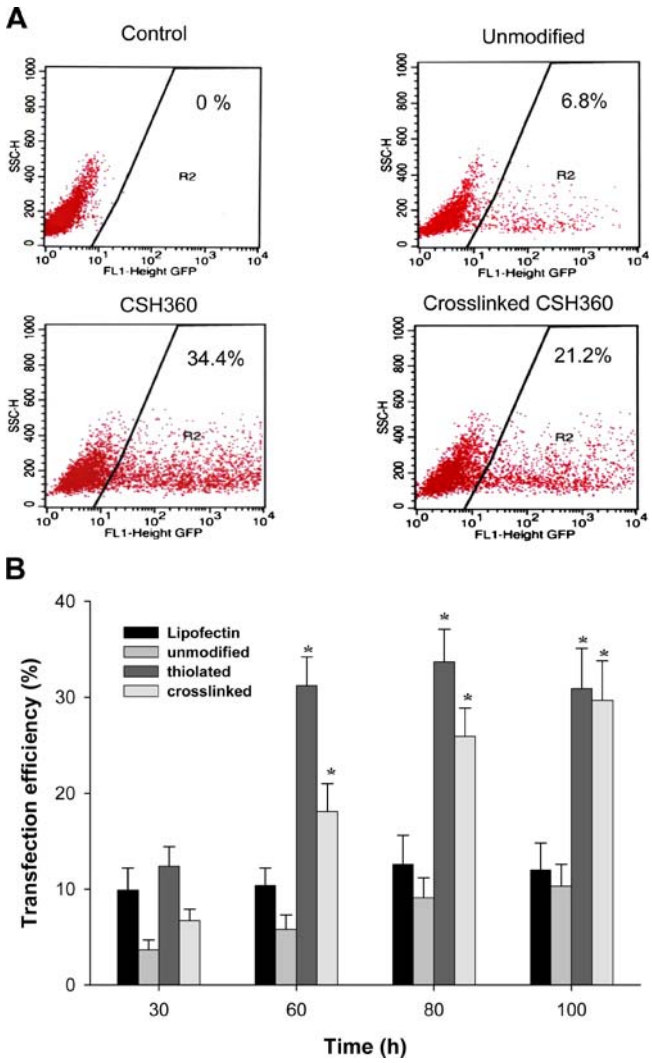

Fig. 6. Representative flow cytometric analysis of GFP-expressing cells 60 h after transfection at a weight ratio of 5:1 (A) and kinetics of gene expression (B). Transfection was performed with >50% cell confluency. Indicated values are means (±S.D.) of three experiments. *$P<0.01$ relative to unmodified chitosan at the same time point.

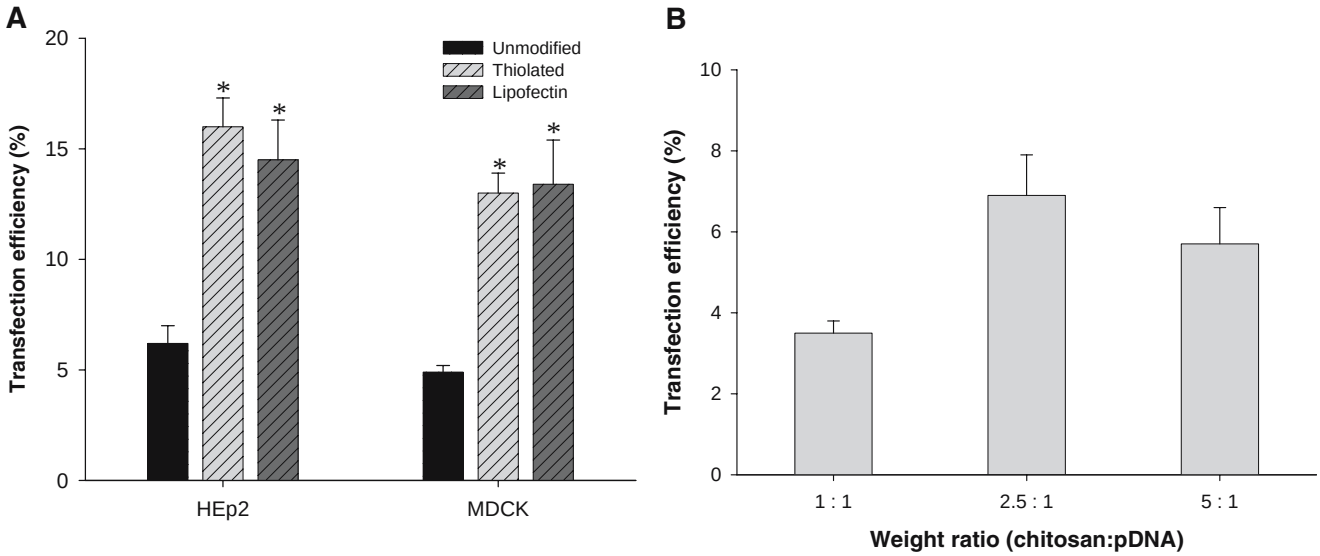

Fig. 7. The comparison of transfection efficiency in HEp2 and MDCK cells (A) and transfection efficiency of butanoyl chitosan in HEK293 cells (B). Indicated values are means (±S.D.) of three experiments. *P<0.01 relative to unmodified chitosan.

Thiolated Chitosan Enhances *In Vivo* Gene Delivery

To investigate the *in vivo* gene transfer potential of CSH360 and crosslinked CSH360, we utilized the nasal cavity for the route of nanoparticle delivery because of its greater permeability than other administration routes and avoidance of first-pass metabolism in the liver (27). The *in vivo* transfection efficiency of thiolated chitosan (CSH360) was studied after intranasal administration of pDNA to mice by observing the cells in bronchoalveolar lavage (BAL) fluid (Fig. 9). At 3 days post-intranasal administration, CSH360/pDNA nanocomplexes yielded more gene expression than that induced by unmodified or crosslinked CSH360. Crosslinked CSH360/pDNA nanocomplexes exhibited increased gene expression after 7 days in comparison to that observed after 3 days. At 14 days post-intranasal administration, crosslinked CSH360 mediated more gene expression than unmodified and CSH360. These observations suggest that thiolation of chitosan and subsequent crosslinking enhance gene delivery potential and mediate sustained gene expression.

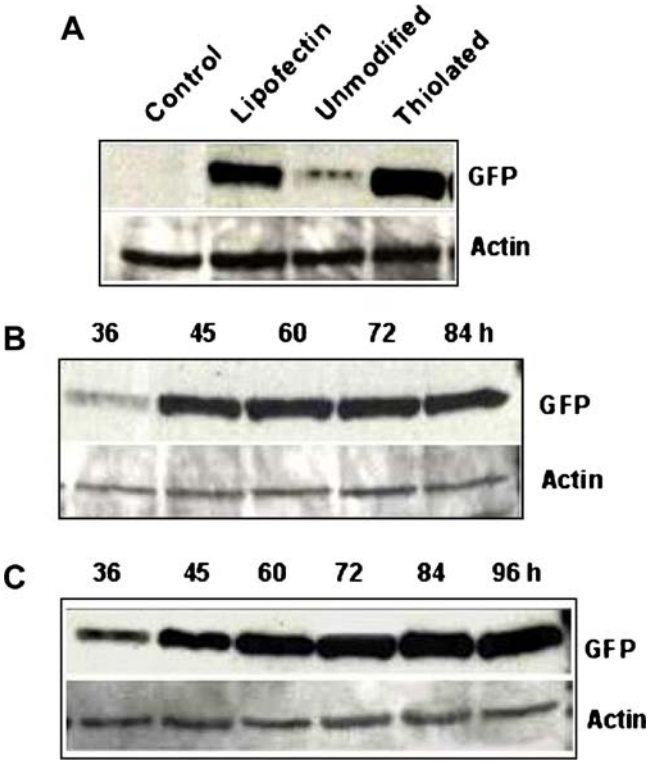

Fig. 8. Immunoblotting analysis of green fluorescent protein production due to reporter gene expression. (A) Comparison of GFP expression mediated by Lipofectin, unmodified chitosan and thiolated chitosan (CSH360) at 60 h post-transfection. Control is untransfected cells. (B) GFP expression mediated by CSH360. (C) GFP expression mediated by crosslinked CSH360.

DISCUSSION

Chitosan appears to be one of the most promising carriers of genes because of its many advantages including biodegradability, biocompatibility, non-toxicity, non-immunogenicity, and wound-healing properties. The most important limitation of chitosan as a gene carrier, which has limited its clinical potential, is its low cellular transfection efficiency. The results of our studies in this report demonstrate that thiolated chitosan represents an advanced generation of nanocomplexes that exhibit enhanced gene expression and, upon crosslinking, can generate a slow, sustained release of pDNA and gene expression both in cultured cells and in mice.

Both high and low molecular weight chitosan have their advantages and disadvantages in the application of gene transfer. It has been reported that high molecular weight chitosan forms highly stable complexes and pDNA is released from the complexes upon dissolution which is the major rate limiting step in gene expression. However, pDNA release from the complexes with low molecular weight chitosan is driven by easy dissociation (18). In this report, chitosan of moderate molecular weight (33 kDa) was chosen

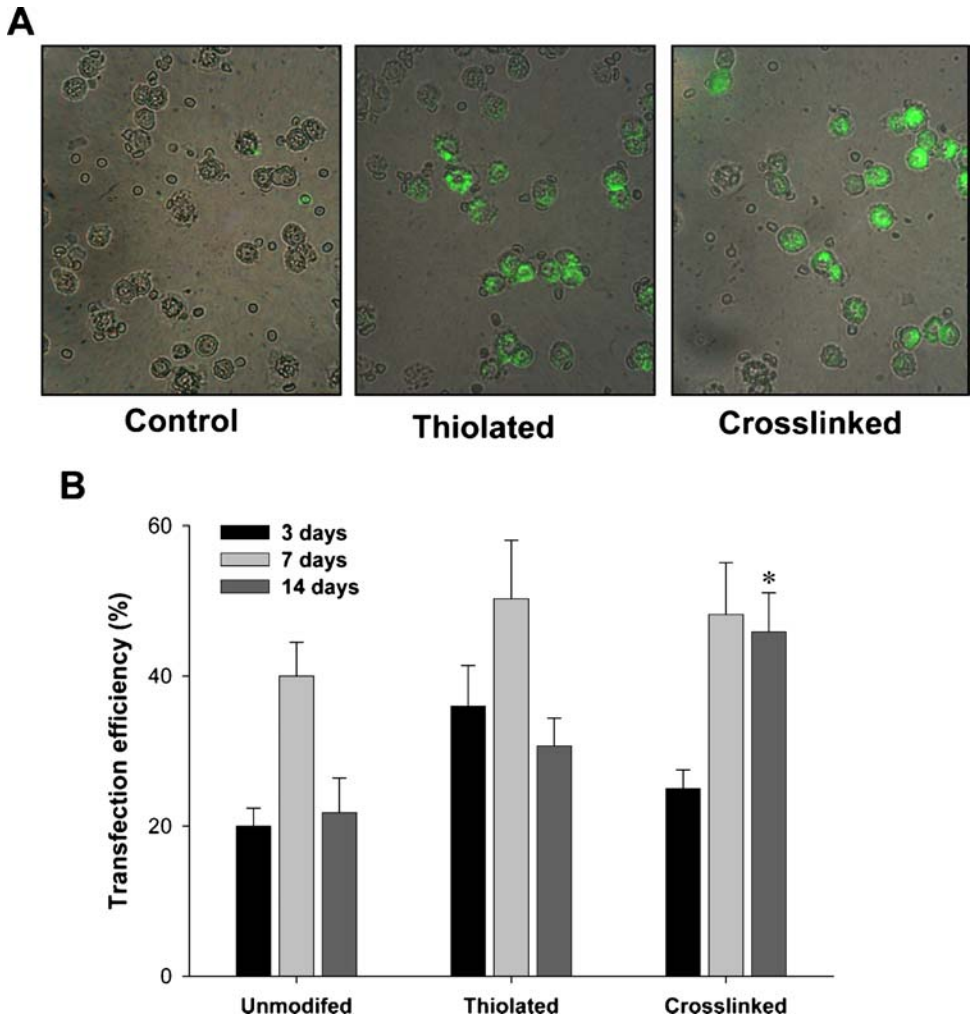

Fig. 9. Gene expression of GFP pDNA in mouse BAL cells. (A) Micrographs of BAL cells 14 days after intranasal administration. Gray bright-field images were merged with fluorescence using WICF Image J program (NIH, USA). Control is BAL cells from untreated mice. (B) The level of gene expression in BAL cells. Four different areas of each slide were examined and gene expression level was calculated by counting the number of total cells and GFP expressing cells. *$P<0.01$ relative to unmodified and thiolated chitosan at 14 days post-intranasal administration ($n=4$).

to develop chitosan/pDNA nanocomplexes and the pDNA release assay was performed in transfection medium at pH 7.0. Chitosan of 33 kDa forms nanocomplexes which are strong enough to protect pDNA, but sufficiently weak to dissociate at pH 7.0. pDNA release from the electrostatic nanocomplexes with chitosan can be explained by the dissociation of complexes at pH 7.0 at which chitosan has a slightly positive charge because of protonation of amine groups. The chemical modification of chitosan alters mainly the degree of deacetylation (28). It was also reported that at constant molecular weight, the reduction in the degree of deacetylation decreased the zeta potential and DNA binding capacity, subsequently leading to a reduction in transfection efficiency (16). Thiolation of chitosan was expected to decrease positive charge density resulting in a lower zeta potential, and hence decreased transfection efficiency. Thus, the finding that thiolated chitosan exhibits a higher *in vitro* and *in vivo* transfection efficiency is contrary to the commonly accepted notion that a higher zeta potential is required for increased transfectability. In addition, thiolated chitosan exhibited reduced transfection efficiency after the formation of intra- as well as intermolecular disulfide bonds, despite the unchanged zeta potential, which suggests that enhanced gene transfer of thiolated chitosan is mediated by the introduced thiol groups. The mechanism underlying increased transfectability of thiolated chitosan *in vitro* as well as *in vivo* is unclear. It might be due to increased mucoadhesion and cell permeation properties, as suggested previously (20,24,26). Unmodified chitosan formed very stable complexes with pDNA and delayed the pDNA release at weight ratios (>2.5:1), leading to low transfection efficiency. This is in good agreement with previous studies (18), which showed that the high physical stability of chitosan is a major rate-limiting step for the intercellular release of pDNA from complexes. One of the possible explanations for the enhanced gene delivery of thiolated chitosan is that thiolation of chitosan reduces the positive charge density and pDNA complexing capacity, resulting in more rapid pDNA

release. An additional chemical reaction of chitosan was performed with butanoic anhydride to answer the question of whether partial neutralization of positive charges increases the transfection efficiency of chitosan. Butanoyl chitosan exhibited reduced surface charges and less DNA binding capability which can result in easy and rapid pDNA release. However, butanoyl chitosan showed lower transfection efficiency than unmodified chitosan. From this observation, it can be concluded that the enhanced gene transfer capability of thiolated chitosan is not only from the reduced DNA binding capability by partial neutralization of positive charges, but also from increased mucoadhesion and cell permeation properties by introduced thiol groups. Alternatively, transfection efficiency of crosslinked thiolated chitosan might result from the thiolation-endowed physical stability of chitosan/pDNA nanocomplexes by crosslinking of thiolated chitosan through the inter- as well as intramolecular disulfide bonding, and protection of complexed pDNA from nucleases, as shown by the results of this study. The delay of pDNA release can be explained by the rationale that crosslinking of thiolated chitosan results in effective entrapping and/or immobilizing pDNA.

A major finding of these studies is that thiolated chitosan supports significantly enhanced transfection in cells, notably higher than a commercial transfection reagent, Lipofectin. It is noteworthy that transfection is highly pH-dependent, irrespective of the transfection agent. Lipofectin has extremely high transfection efficiency (>45%) at pH 7.5, but exhibits significantly diminished transfection efficiency at pH 7.0. On the other hand, both unmodified and thiolated chitosans showed significantly higher transfection efficiency at pH 7.0 than at pH 7.5. This observation is in good accordance with previous studies (29,30), in which chitosan of 40 kDa showed higher transfection efficiency at pH 7.0 than at pH 7.5. Ishii *et al*. reported that the effect of pH on the transfection capability of chitosan is presumably due to the fact that chitosan has slightly positive charges at pH 7.0. Because of protonation of amine groups, chitosan, through electrostatic interaction, forms more stable complexes with DNA and the cell membrane (31), thus perturbing the cell membrane bilayers (32) and resulting in higher transfection.

One of the most important features of thiolated chitosan is its intrinsic ability to readily oxidize its thiol groups to form inter- as well as intramolecular disulfide bonds. The results show that crosslinking of thiolated chitosan promotes extended release of pDNA both *in vitro* in cultured cells and in mice. Most likely, the thiolation feature allows sustained gene expression over several days, which is key to achieving the therapeutic efficacy of DNA delivery and expression of gene products. The mechanism underlying this is unclear. It is possible that not all thiol groups participate in inter- and intramolecular disulfide bonding. Thus, thiol groups located close to each other form disulfide bonds readily and form the networked structure through the crosslinking. The remaining thiol groups cannot oxidize without neighboring thiol groups (21) and play a role in mucoadhesion and permeability enhancement. This rationale is supported by the *in vitro* pDNA release test and the slow and continuous gene expression both *in vivo* and *in vitro*.

To our knowledge, the present work describes, for the first time, chitosan-based nanocomplexes for sustained gene delivery, adding this to a number of sustained DNA delivery systems including poly(lactide-co-glycolide) (PLG) matrices (33), collagen sponges (34), PLGA emulsion coating (35), PLGA nanoparticles (36), poly(ethylene-co-vinyl acetate) (EVAc) disks (37), gelatin nanospheres (38), and Pluronic hydrogels (39). The major limitations of these sustained delivery systems are the tedious procedures and the use of harsh chemical reagents which are toxic and difficult to remove completely (2,40). In remarked contrast to other sustained release microspheres (41) or nanoparticles (35), the crosslinking of thiolated chitosan nanocomplexes for sustained gene delivery of DNA is accomplished under very mild conditions without any chemical crosslinking agent. An ideal non-viral gene delivery system must have well-defined physicochemical characteristics and the following properties, including ease of assembly with DNA, stabilization of DNA before and after cell uptake, the capability of escaping the endocytic pathways, and adjustable expression of the therapeutic level of proteins over time (42,43). The thiolation and crosslinking of thiol groups may help chitosan fulfill many of these requirements.

In conclusion, these results demonstrate that thiolated chitosan forms nanocomplexes with pDNA and exhibits significantly improved gene delivery potential *in vitro* as well as *in vivo*. The extended pDNA release and subsequent slow gene expression were achieved by oxidation of introduced thiol groups to crosslink the thiolated chitosan, thus presenting a novel approach with great potential for enhanced and sustained gene delivery.

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