

# Degradable copolymer based on amphiphilic N-octyl-N-quatenary chitosan and low-molecular weight polyethylenimine for gene delivery

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**Background:** Chitosan shows particularly high biocompatibility and fairly low cytotoxicity. However, chitosan is insoluble at physiological pH. Moreover, it lacks charge, so shows poor transfection. In order to develop a new type of gene vector with high transfection efficiency and low cytotoxicity, amphiphilic chitosan was synthesized and linked with low-molecular weight polyethylenimine (PEI).

**Methods:** We first synthesized amphiphilic chitosan–N-octyl-N-quatenary chitosan (OTMCS), then prepared degradable PEI derivatives by cross-linking low-molecular weight PEI with amphiphilic chitosan to produce a new polymeric gene vector (OTMCS–PEI). The new gene vector was characterized by various physicochemical methods. We also determined its cytotoxicity and gene transfection efficiency in vitro and in vivo.

**Results:** The vector showed controlled degradation. It was very stable and showed excellent buffering capacity. The particle sizes of the OTMCS–PEI/DNA complexes were around 150–200 nm with proper zeta potentials from 10 mV to 30 mV. The polymer could protect plasmid DNA from being digested by DNase I at a concentration of 2.25 U DNase I/μg DNA. Furthermore, they were resistant to dissociation induced by 50% fetal bovine serum and 1100 μg/mL sodium heparin. OTMCS–PEI revealed lower cytotoxicity, even at higher doses. Compared with PEI 25 kDa, the OTMCS–PEI/DNA complexes also showed higher transfection efficiency in vitro and in vivo.

**Conclusion:** OTMCS–PEI was a potential candidate as a safe and efficient gene vector for gene therapy.

**Keywords:** nonviral gene vector, polyethylenimine, transfection efficiency, cytotoxicity

## Introduction

Based on genetic engineering and molecular genetics, gene therapy is a new method for the cure of cancer.<sup>1</sup> It has become the most promising field because it directly aims at the biological foundation of the occurrence and development of cancers. The nature of gene therapy is to feed therapeutic genes into target cells with gene vectors, and the key is to find a reliable and effective gene delivery system.<sup>2</sup> A perfect gene vector has to meet the following conditions: (1) be able to condense DNA effectively; (2) be stable in body fluid; (3) be able to target the specific cells; and (4) be able to cross membranes and release efficiently.<sup>3</sup>

Presently, viral vectors and nonviral vectors are two kinds of common gene vectors. Viral vectors are frequently used in the field of medical research because of their relatively high transfection efficiency, but their clinical utility has been seriously limited by their fatal disadvantages, such as immunogenicity, oncogenic effects,

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and poor loading capacity.<sup>4</sup> By contrast, nonviral gene vectors provide a significant supplement to viral vectors. Nonviral gene vectors are nonimmunogenic, noninfectious, easier to prepare, and capable of carrying large amounts of genetic materials.<sup>5-7</sup> Nonviral gene vectors have attracted more and more attention.<sup>8</sup>

Among nonviral vectors, the cationic polymers have been widely explored in gene delivery research. Polyethylenimine (PEI) and its derivatives are the most extensively investigated because of their “proton sponge” effect.<sup>9,10</sup> PEI, with abundant positive charges, condenses DNA by electrostatic interaction to compact complexes, which enables breaking through the various barriers to the nucleus of target cells.<sup>11</sup> DNA is condensed as nanoparticles so that it is not easily degraded by nuclease, or gathered as precipitate, which results in high transfection efficiency.<sup>12,13</sup> However, it has been reported that the molecular weight of PEI has a strong influence on its transfection efficiency and cytotoxicity. PEI with high molecular weight performs not only with high transfection efficiency, but with high cytotoxicity. To the contrary, PEI with low-molecular weight performs with low cytotoxicity but also with poor transfection efficiency. In addition, there is an inconsistency between its stability in body fluid and its cellular uptake.<sup>14</sup> It has been proved that PEI derivatives obtained by cross-linking low-molecular weight PEI with degradable materials display higher transfection efficiency and lower cytotoxicity.<sup>15</sup>

Chitosan, obtained by alkaline N-deacetylation of chitin, is one kind of the most widely used natural cationic polysaccharides.<sup>16</sup> Chitosan shows particularly high biocompatibility and fairly low cytotoxicity,<sup>17</sup> therefore in fact, can be used as a gene vector.<sup>18,19</sup> Nevertheless, chitosan is insoluble at physiological pH, and lacks charge. These major drawbacks limit its use for gene delivery.<sup>20</sup>

Based on the above analysis, we first synthesized amphiphilic chitosan, N-octyl-N-quaternary chitosan (OTMCS), then cross-linked the amphiphilic chitosan with low-molecular weight PEI. In this way we acquired a new type of degradable amphiphilic chitosan-PEI derivative with high transfection efficiency and low cytotoxicity.

The new gene vector was characterized in terms of its chemical structure and biophysical parameters. We also investigated the cytotoxicity and gene transfection efficiency of this vector in vitro and in vivo. The objective of this work was to reduce the cytotoxicity of PEI and improve its transfection efficiency, thereby enhancing the therapeutic effect of gene therapy on cancers.

## Materials and methods

### Materials

Branched PEI (MW 2000), heparin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, MO). DNase I was purchased from the Worthington Company (Worthington Company, Lakewood, NJ). RPMI 1640 culture medium and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad CA). Luciferase assay system for in vitro transfection assay and pGL3-Control vector with SC-40 promoter and enhancer encoding firefly (*Photinus pyralis*) luciferase were obtained from Promega Corp (Madison, WI). The plasmid encoding the enhanced green fluorescent protein (*pEGFP-N2*) was kindly provided by the Institute of Life Science and Technology of Tongji University in China. The plasmids were amplified using *Escherichia coli* DH5 $\alpha$  and prepared using the Tiangen End-free Plasmid Mega Kit (DP117; Qiagen GmbH, Hilden, Germany). The purity of the purified and concentrated DNA was determined by measuring its UV absorbance value at 260 nm and 280 nm respectively.

Chitosan (MW 2000, degree of deacetylation > 90%), benzene, dichloromethane, triphosgene, N-hydroxysuccinimide, triethylamine, ethyl acetate, and absolute ethyl alcohol were purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). Dialysis bag (MWCO 7000) was purchased from Spectrum Chemical & Instruments (Plainfield, IL).

### Preparation of amphiphilic chitosan (OTMCS)<sup>21</sup>

Chitosan (12 g) and octaldehyde were added into methanol with stirring at 30°C for 12 hours. Then  $\text{KBH}_4$  (6 g) dissolved in 60 mL of water was slowly added to the solution. After a further 24 hours of continuous stirring, the reaction solution was filtered, and the product was precipitated with methanol. The precipitate was washed repeatedly with water and methanol. The product, N-octyl chitosan, was dried under a vacuum at 50°C overnight.

N-octyl chitosan (0.96 g), N-methylpyrrolidone (15 mL), KI (2.4 g), NaOH (15%, 5 mL), and  $\text{CH}_3\text{I}$  (5.2 mL) were added, in turn, into a three-necked bottle. This mixture was reacted at 60°C for 1 hour. Then the reaction solution was cooled and centrifuged for 30 minutes, and the precipitate gathered and dissolved in water. At last the solution was dialyzed (MWCO 7000) against distilled water and lyophilized. The product OTMCS was obtained.

## Synthesis of OTMCS–PEI

### Activation of OTMCS

Before the experiments, all the reagents were dehydrated by coevaporation. First, 3.86 g OTMCS and the same mole of triphosgene were dissolved in benzene/dichloromethane (3:1, 40 mL) with magnetic stirring for one night. Next, the same mole of N-hydroxysuccinimide as chitosan and plenty of triethylamine was added dropwise into benzene/dichloromethane (2:1, 30 mL) with stirring for 4 hours after evaporating the primary solvent. After rotary evaporating the solvent again, 50 mL of ethyl acetate was added and centrifuged (8000 rpm, 15 minutes). At last, the supernatant was evaporated and the residue was collected.

### Linking OTMCS with PEI 2 KDa

Activated OTMCS (0.386 g, 2.14 mmol) was dissolved into 50 mL of dichloromethane. Dehydrated PEI 2 KDa (42.8 g, 21.4 mmol) was dissolved into 40 mL of alcohol. These two solutions were contemporarily added into 20 mL of dichloromethane, and stirred for one night. After evaporating solvent, the residue was dissolved in water, dialyzed, and then lyophilized. The polymer OTMCS–PEI was obtained.

## The buffering capacity of the OTMCS–PEI polymer<sup>22</sup>

The OTMCS–PEI polymer solution was prepared in a 50 mL flask (0.2 mg/mL, 30 mL) and pure water was used as a control. After adjusting the initial pH to 10.0 with 0.1 M NaOH, 25  $\mu$ L increments of 0.1 M HCl were titrated into the solution while measuring the pH response with a pH electrode. The recorded pH varied from 10.0 to 3.0.

## Characterizations of OTMCS–PEI (NMR, GPC)

First, 10 mg of OTMCS–PEI was dissolved in 0.6 mL of deuterium oxide ( $D_2O$ ) in an NMR tube, and the  $^1H$  NMR spectrum was recorded using a Varian 300 MHz spectrometer (Varian Medical Systems Inc, Palo Alto, CA) at room temperature.

The molecular weight and its distribution of the polymer was measured by gel permeation chromatography with multiangle laser light scattering (GPC-MALLS) (LC-20AD; Shimadzu Corp, Kyoto, Japan) (690 nm laser wavelength) using a TSK-GEL G5000PW<sub>XL</sub> column from TOSO (Tokyo, Japan) (temperature 40°C) operated at a flow rate of 0.4 mL/min. Ammonium acetate (0.2 M) was used as a mobile phase.

## Degradation of OTMCS–PEI

Degradation of OTMCS–PEI was estimated by the measurement of molecular weight. First, 0.5 g of the polymer was dissolved in 10 mL of phosphate-buffered solution (PBS) (0.1M, pH = 7.4), and then incubated at 37°C, with shaking at 100 rpm. After incubation for 0, 10, 20, 30, 40, 50, and 60 hours, respectively, solutions of the polymers were lyophilized, and the molecular weights of the lyophilized samples were measured by GPC-MALLS with a 690 nm laser wavelength.

## Preparation of OTMCS–PEI/DNA complexes

The ratio of an amino group to a phosphate group is hereafter defined as the charge ratio. We controlled charge ratios of OTMCS–PEI/DNA by regulating the weight ratio of OTMCS–PEI and DNA. DNA solution and polymer solution were mixed to form self-assembly complexes with desired weight/weight (w/w) ratios. The complexes were allowed to stand at 4°C for 30 minutes before they were used in the next experiments.

## Measurement of particle size, zeta potential and morphologic observation

The complexes were prepared at designed weight ratios and incubated at 4°C for 30 minutes before their particle sizes and zeta potentials were measured by an electrophoretic light-scattering spectrophotometer (Zetasizer Nano ZS90, MAN0317 Issue 5.0; Malvern Instruments Ltd, Malvern, UK). All the experiments were conducted in triplicate.

OTMCS–PEI/DNA complexes were prepared according to 2.7. The concentration of DNA was 50  $\mu$ g/mL and the w/w ratio of the polymer to DNA was 10. A drop of the complex solution was placed on a copper grid. Transmission electron microscope (JEM 2100F; JEOL Ltd, Tokyo, Japan) was used to observe the morphological characteristics of the micelle after the sample was dried.

## Agarose gel retardation assay

Before electrophoresis, 2  $\mu$ L of 6  $\times$  loading buffer was added into 10  $\mu$ L of prepared complex solution (250 ng of plasmid DNA). Electrophoresis was carried out on 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer, at 120 V for about 40 minutes. Then the gel was dyed with ethidium bromide for 15 minutes, and illuminated on an ultraviolet illuminator to show the DNA migration patterns.

## Resistance to DNase I digestion

In 0.5 mL Eppendorf tubes, the designed amount of DNase I solutions were added to 10  $\mu$ L of prepared complex solutions (250 ng of plasmid DNA), and incubated at 37°C for 15 minutes. Then, 2  $\mu$ L of 250 mM EDTA was added to each tube, and incubated at room temperature for 10 minutes to inactivate DNase I. At last, 10  $\mu$ L of 10 mg/mL sodium heparin solution was added, and incubated at room temperature for 2 hours to dissociate the complex. After that, the resistance capacity of OTMCS–PEI/DNA to DNase I was evaluated by electrophoresis.

## Resistance to serum and sodium heparin

In one tube, 10  $\mu$ L of the prepared complex solution (250 ng of plasmid DNA) was added. Different volumes of FBS solution or 2  $\mu$ L of different concentrations of sodium heparin solution were then added, and the mixed solution incubated at 37°C for 30 minutes. After that, the resistance capacities of OTMCS–PEI/DNA to serum and sodium heparin were evaluated by electrophoresis.

## Cytotoxicity assay

The cytotoxicity of the polymer OTMCS–PEI was measured using the MTT assay. First of all, a 0.22  $\mu$ m filtration membrane was used to sterilize the OTMCS–PEI polymer. HeLa cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA). To begin,  $5 \times 10^3$  cells were seeded in 200  $\mu$ L of growth medium per well in 96-well plates, and incubated to 80% confluence. Then the culture medium was replaced with 200  $\mu$ L of serum-free medium containing various concentrations of OTMCS–PEI, PEI 25 KDa, and PEI 2 KDa, and incubated for 72 hours. Next, the medium was replaced with 20  $\mu$ L of MTT (5 mg/mL) and 180  $\mu$ L of growth medium, and left to incubate for another 4 hours. After, the MTT solutions were replaced, 150  $\mu$ L of dimethyl sulfoxide was added and kept in agitation for 10 minutes. The absorbance value at 570 nm was determined by an ELISA plate reader (Model 680; Bio-Rad Laboratories, Hercules, CA). Cell viability (%) was calculated according to these data and the following equation:<sup>23</sup>

$$\text{Cell Viability (\%)} = (A_{\text{test}}/A_{\text{control}}) \times 100$$

$$(\text{Mean} \pm \text{Standard Deviation, } n = 6), \quad (1)$$

where  $A_{\text{test}}$  is the absorbance value of OTMCS–PEI or PEI treated cells and  $A_{\text{control}}$  is the absorbance value of the untreated cells.

## Gene transfection efficiency

### In vitro gene transfection

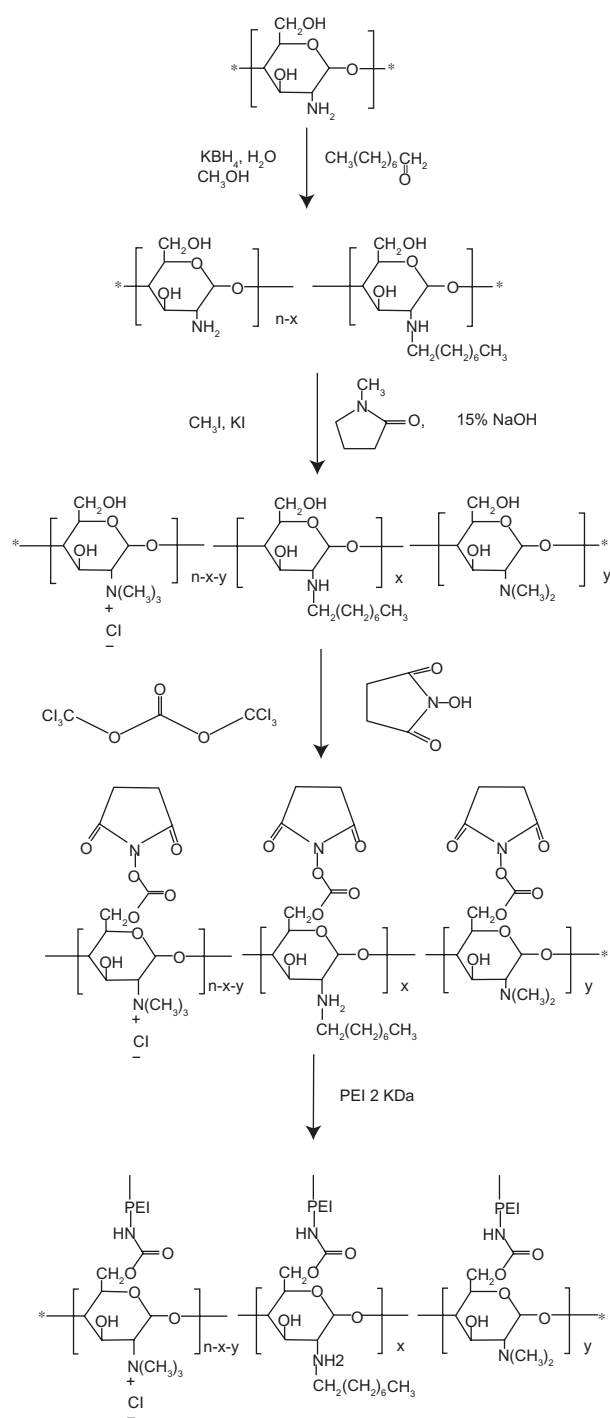
The transfection efficiency of OTMCS–PEI/DNA complexes in HeLa cells was examined using the plasmid pEGFP-N2 and pGL3–Control. Complexes were sterilized via a 0.22  $\mu$ m filtration membrane. Cells were seeded in 500  $\mu$ L of growth medium per well in 24-well plates and incubated to 80% confluence. Before transfection, the medium was replaced with 100  $\mu$ L of complex solution at various weight ratios and 400  $\mu$ L of serum-free RPMI 1640 medium. Cells were incubated for 4 hours at 37°C in a 5% CO<sub>2</sub> atmosphere before the medium was replaced with 500  $\mu$ L of medium containing 10% FBS and incubated for another 48 hours. After that, the pEGFP-N2 expression was observed with an inverted fluorescent microscope (AE-31; Motic Corporation, Wetzlar, Germany). Then the cells were trypsinized, centrifuged, and resuspended in PBS to determine the transfection efficiency by flow cytometry (BD FaCS Aria; Becton Dickinson, Franklin Lakes, NJ). The data analysis was conducted using the CellQuest software (Becton Dickinson).

The luciferase assay was conducted according to the manufacturer's specifications. The medium was replaced with 100  $\mu$ L of cell culture lysis reagent (CCLR) and shaken for 30 minutes. After mixing with substrate, luciferase activity was examined by a luminometer (Turner Designs Luminometer Model TD-20/20; Promega Corp) as soon as possible. A bicinchoninic acid (BCA) protein assay kit (PP1001; Biotek Corporation, Beijing, China) was used to measure protein contents. Transfection efficiency for the pGL3–Control was calculated by the relative light units (RLUs) against the corresponding protein contents.

### In vivo gene transfection<sup>24,25</sup>

Male BALB/C mice, 4–5 weeks and weighing 18–22 g, were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch (Shanghai, China), and maintained under specific pathogen-free condition for in vivo transfection study. All animal procedures were approved by the Committee for Animal Research of Shanghai Ocean University, China (SCXK (HU) 2007-0003) and carried out according to the Guide for the Care and Use of Laboratory Animals.

The animals were divided into three groups (five rats per group). B16 cells were injected subcutaneously into the mice to establish the tumor models. When the subcutaneous transplanted tumors increased to 10 mm in diameter, the mice were injected with 250  $\mu$ L of the sterilized complex solution



**Figure 1** Synthetic scheme of OTMCS-PEI. First the N-octyl-N-quaternary chitosan (OTMCS) was synthesized. Then the OTMCS was crosslinked with PEI by N-hydroxysuccinimide.

**Abbreviation:** OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine.

containing 30  $\mu\text{g}$  of the pGL3-Control reporter gene through the tail veins. Details are listed as follows: Group 1, OTMCS-PEI/DNA complexes, w/w 10; Group 2, OTMCS-PEI/DNA complexes, w/w 30; Group 3, PEI 25 KDa/DNA complexes, N/P 5. At 24 hours postinjection, mice were sacrificed by cervical dislocation, and the major tissues and the subcutaneous

transplanted tumors were removed. The tissues and tumors were collected and homogenized in a cell lysis buffer. The cell lysate was centrifuged for 10 minutes at 10,000  $\times\text{g}$  (9800 rpm). Luciferase activity in the extracts was examined with a luciferase assay kit in a signal-well luminometer. The relative light units were detected against protein concentration in the cell tissue extracts, which was measured with the Biotek PP1001 protein assay kit.

## Results and discussion

### Synthesis and characterization of OTMCS-PEI

Figure 1 shows the synthetic scheme of OTMCS-PEI. Figure 2A shows the structure of chitosan, and Figure 2B shows the  $^1\text{H}$  NMR spectra of OTMCS in  $\text{D}_2\text{O}$ . Compared with the structure of chitosan,  $-\text{CH}_2-$  proton peaks appear at 1.213 ppm and  $-\text{CH}_3$  proton peaks appear at 0.802 ppm, which could demonstrate that OTMCS was synthesized successfully. Figure 2C shows the  $^1\text{H}$  NMR spectra of PEI in  $\text{D}_2\text{O}$ . Figure 2D shows the  $^1\text{H}$  NMR spectra of OTMCS-PEI in  $\text{D}_2\text{O}$ . Compared with the spectra of PEI and OTMCS, the proton peaks of OTMCS-PEI moved to the lower magnet field due to the production of the groups with electronic screening effect. The performances of characteristic peaks have changed.  $-\text{CH}_2-$  and  $-\text{CH}_3$  in OTMCS were merged and moved to higher magnet field.  $-\text{H}_2\text{CNH}_2-$  and  $-\text{NCH}_2\text{CH}_2\text{NH}-$  in PEI were also changed. These results indicate that OTMCS-PEI was successfully prepared.

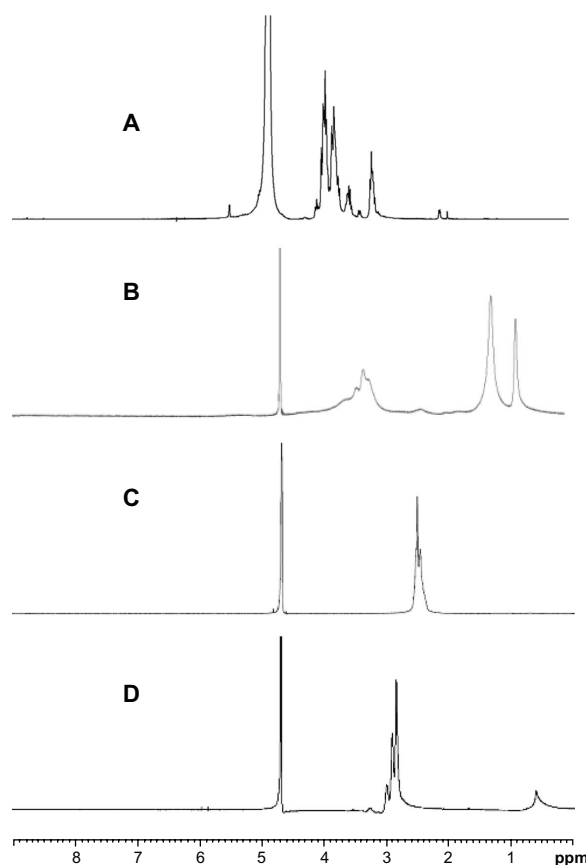
From the integral ratio of the signal at 3.136 ppm, which corresponds to the proton of glucosamine from the OTMCS to the signal at 2.719–2.824 ppm of the  $-\text{CH}_2\text{CH}_2\text{NH}-$  from the PEI, the level of OTMCS substitution was calculated to be 81% to 90% (Figure 2D).

### The buffering capacity of the OTMCS-PEI polymer

The buffering capacity of the gene vector is vital to the molecules escaping the endosomes of cells, because the molecules entering cells will experience a drop of pH from neutral to about 5. The proton sponge effect of the polymer ensures buffering in the endosomes, resulting in the degradation of the lysosomes so that genes can be protected. Figure 3 shows that the OTMCS-PEI polymer had relatively high buffering capacity in pH ranging from 4 to 7, compared with pure water. This result demonstrated that the polymer was sufficiently suitable for gene transfection.

### Degradation studies

Degradation of the gene vector is important for gene transfection. Degradable polymers can be degraded and



**Figure 2** Representative  $^1\text{H}$  NMR spectra of (A) chitosan, (B) OTMCS, (C) PEI, and (D) OTMCS-PEI, in  $\text{D}_2\text{O}$  at room temperature.

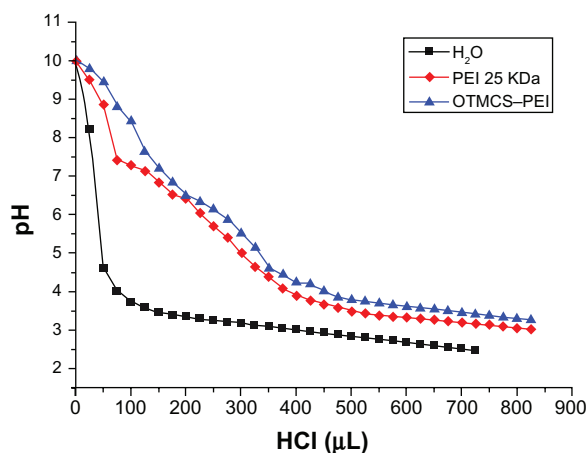
**Abbreviations:** OTMCS, amphiphilic chitosan; PEI, low-molecular weight polyethylenimine; OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine;  $\text{D}_2\text{O}$ , deuterium oxide.

secreted to the extracellular environment to show lower cytotoxicity on cells. Owing to its ester bond hydrolysis reaction at physiological conditions, OTMCS-PEI is a degradable vector (as compared with the nondegradable PEI 25 KDa) and was seen to degrade into low-molecular weight PEI and OTMCS, both of which were eliminated from cells easily, thereby reducing cytotoxicity. As is shown in Figure 4, OTMCS-PEI degraded slowly and the degradation was completed after 60 hours.

In order to obtain meaningful information from this degradation figure, the degradation figure was fitted to three kinds of kinetic models. The degradation figure of the OTMCS-PEI polymer was well described by a zero-order model. According to the model, the half-life of OTMCS-PEI was found to be 30 hours.

### Particle size, zeta potential measurements and morphological characteristics

The particle sizes and zeta potentials of the OTMCS-PEI/DNA complexes were examined at different w/w ratios.



**Figure 3** Determination of the buffering capacity of PEI 25 KDa and the OTMCS-PEI polymer by acid-base titration.

**Note:** Solution containing the polymer (0.2 mg/mL) was adjusted to pH 10.0, and then titrated with HCl from 10.0 to 3.0.

**Abbreviation:** OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine.

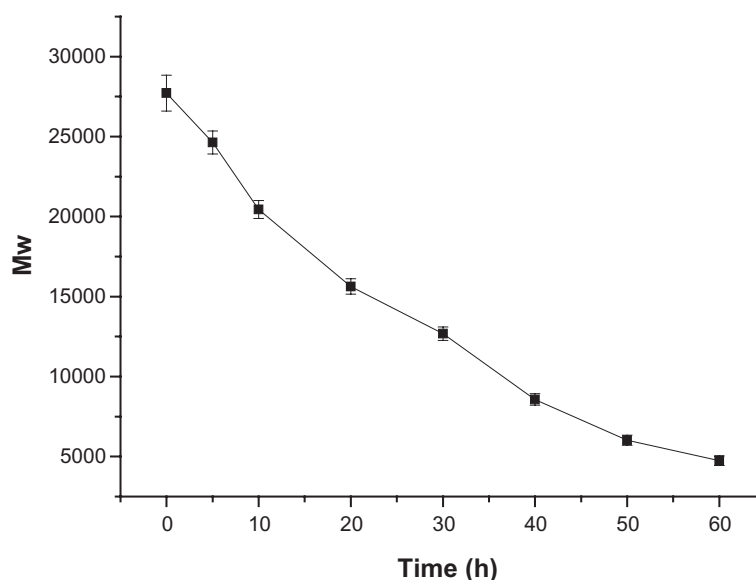
From in the range of w/w ratios studied, no precipitation was observed (Figure 5A). It was seen that the OTMCS-PEI polymer could concentrate pDNA into nanoparticles. Depending on the composition of the complexes, their average particle sizes ranged from 150 nm to 200 nm, which were suitable for an efficient gene delivery in vivo. With the increase of OTMCS-PEI/DNA weight ratios, the particle size decreased. This result showed that a polymer with more charges could condense DNA more effectively.

The OTMCS-PEI/DNA complexes are able to bind successfully to cell membranes with negative charges because the complexes contain positive charges. It is important for a gene vector to rapidly enter into cells via endocytosis, however too strong cationic charges will lead to high cytotoxicity. Zeta potential allows the measurement of overall surface charge of the nanoparticles, and Figure 5B shows that the zeta potentials of all the complexes increased with the increase of their weight ratios. At low w/w ratios, zeta potential increased rapidly, to about 27 mV from a slight positive value. After w/w 20, the increase became slow. The increase of zeta potentials resulted from the increase of OTMCS-PEI with positive charges.

In Figure 5C, the OTMCS-PEI/DNA complexes are shown as spherical and the core/shell structure is obvious. The particle size was about 100 nm. This result demonstrated the micelle was formed successfully.

### Condensation status of plasmid DNA by OTMCS-PEI polymer

Agarose gel retardation was applied to determine the DNA condensation capacity of OTMCS-PEI at various ratios. As



**Figure 4** Degradation of OTMCS-PEI.

**Notes:** The polymer was dissolved in 0.1 M PBS (pH = 7.4) and incubated at 37°C and 100 rpm. Determination of molecular weight (MW) was measured by gel permeation chromatography with multiangle laser light scattering (GPC-MALLS) (n = 3).

**Abbreviations:** OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine; PBS, phosphate-buffered solution.

the proportion of OTMCS-PEI increased, the movement of the plasmid DNA was retarded. When the DNA was completely condensed by OTMCS-PEI, it stopped migrating to the anode. Figure 6A shows that OTMCS-PEI was able to condense DNA effectively and retard it completely at w/w ratio of 0.6. When the w/w ratios of the polymer and DNA exceeded 0.6, the complexes contained positive charges and stopped migrating to the anode.

### Protection of OTMCS-PEI on plasmid DNA

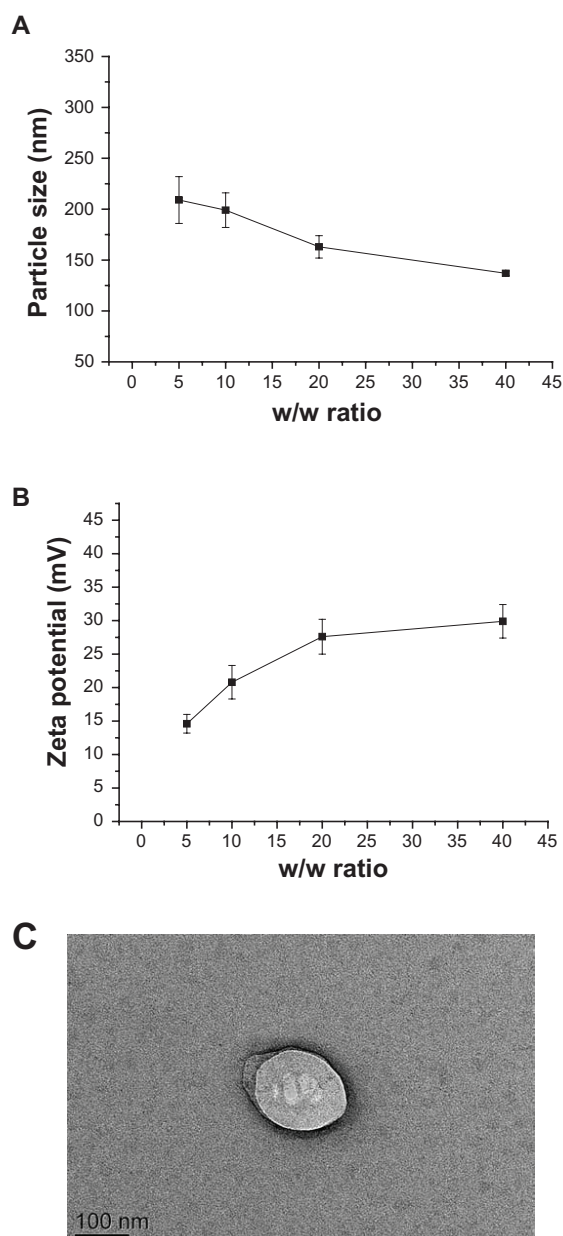
When the complex solution was incubated with DNase I, the polymer protected DNA from digestion by DNase I. At last, intact DNA was released completely by the action of sodium heparin. Figure 6B shows that with the amount of DNase I increasing, most DNA was still protected from digestion until the concentration of DNase I reached 3 U DNase I/ $\mu$ g DNA, which was higher than with other gene vectors. Under the same experimental conditions, DNA was digested by DNase I at the concentration of 0.08 U DNase I/ $\mu$ g DNA.<sup>26</sup>

Naked DNA is also easily digested by blood components. Serum and sodium heparin were used to evaluate the stability of OTMCS-PEI/DNA complexes in vivo. As shown in Figure 6C, except for the DNA in serum itself, OTMCS-PEI/DNA complexes were not digested until the concentration of serum reached 50%, from which we inferred that OTMCS-PEI might protect DNA from dissociation by blood serum.

Sodium heparin was used to simulate molecules with negative charges in the blood, and dissociated the gene vector with its negative charges. At a w/w of 20, the OTMCS-PEI/DNA complexes were partially dissociated at a concentration of 1200  $\mu$ g/mL (Figure 6D). They were completely dissociated once the concentration exceeded 1300  $\mu$ g/mL. The capacity of OTMCS-PEI/DNA complexes to resist sodium heparin was very strong.

### Cytotoxicity assay

As high-molecular weight PEI showed relatively high cytotoxicity, we attempted to introduce a natural compound to form degradable PEI derivatives. In this paper, the cytotoxicity of OTMCS-PEI was evaluated in HeLa cells using the MTT assay and compared with PEI 25 KDa. Figure 7 shows that PEI 25 KDa caused great cytotoxicity on cells at the concentration of 8  $\mu$ g/mL, where more than 50% of the cells died. Cell viability fell to lower than 20% at the concentration of 48  $\mu$ g/mL. By contrast, OTMCS-PEI showed much higher cell viability than PEI 25 KDa at any concentration ( $P < 0.01$ ). At the highest concentration of 48  $\mu$ g/mL, more than 60% of the cells survived under the influence of the OTMCS-PEI polymer. This indicated that OTMCS-PEI was quite suitable as a gene vector. Amino group density is lower in OTMCS-PEI than in PEI 25 KDa, which results in lower cytotoxicity to the cells. Also the polymer showed favorable degradability. OTMCS is a natural compound. These factors contribute to the lower cytotoxicity.



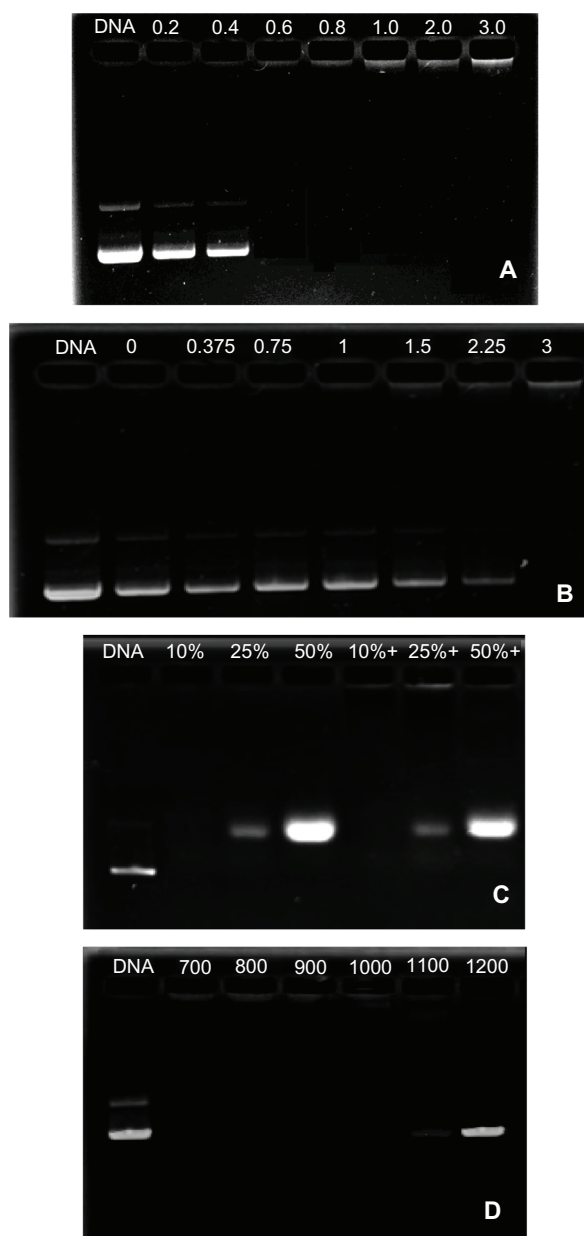
**Figure 5** (A) Particle sizes (nm) of the OTMCS-PEI/DNA complex at w/w ratios of 5, 10, 20, and 40. (B) Zeta potentials (mV) of the OTMCS-PEI/DNA complex at w/w ratios of 5, 10, 20, and 40. The data were expressed as mean values ( $\pm$ standard deviations,  $n = 3$ ). (C) Transmission electron micrograph of OTMCS-PEI/DNA nanoparticles.

**Abbreviation:** OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine.

## Transfection efficiency

### In vitro transfection efficiency

The transfection activity of the newly synthesized OTMCS-PEI polymer was investigated in HeLa cells using pEGFP-N2 and pGL3-Control reporter genes. The OTMCS-PEI/DNA complexes were prepared at various weight ratios ranging from 5 to 30. PEI 25 KDa and PEI 2 KDa polyplexes were prepared at the same weight ratios as controls.

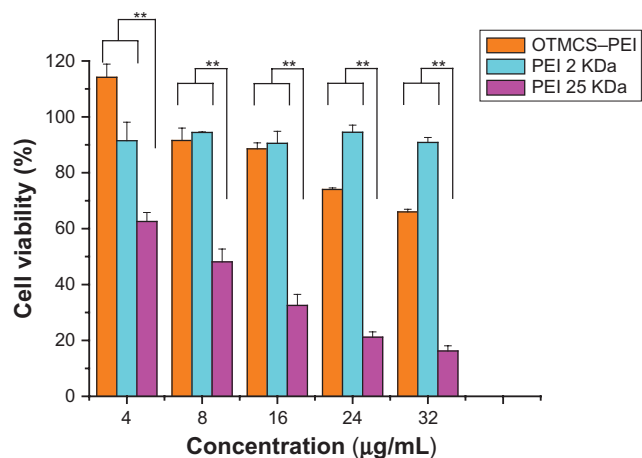


**Figure 6** (A) Agarose gel electrophoresis of the OTMCS-PEI/DNA complex at various w/w ratios. Protection of OTMCS-PEI on plasmid DNA. (B) Protection of plasmid DNA from degradation by DNase I at different concentrations of 0, 0.375, 0.75, 1, 1.5, 2.25, and 3 U DNase I/ $\mu$ g DNA. (C) Protection of plasmid DNA from dissociation by serum at varying concentrations of 10%, 25%, and 50%. The lanes 10%, 25%, and 50% without "+" refer to the presence of naked DNA with 10%, 25%, and 50% serum; the lanes 10%, 25%, and 50% with "+" refer to the presence of OTMCS-PEI/DNA complex at w/w ratio 20 besides 10%, 25%, and 50% serum. (D) Protection of plasmid DNA from dissociation by sodium heparin at varying concentrations of 700, 800, 900, 1000, 1100, and 1200  $\mu$ g/mL.

**Abbreviation:** OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine.

As shown in Figure 8A, bright green fluorescent segments demonstrated that the pEGFP reporter gene was effectively transfected into HeLa cells by the synthesized OTMCS-PEI polymer. With the increase of w/w ratios, more green fluorescence positive cells were observed. In Figure 8B,





**Figure 7** Cytotoxicity of OTMCS-PEI at various concentrations in HeLa cell lines using the MTT assay.

**Notes:** The data were expressed as mean values ( $\pm$ standard deviations,  $n = 3$ );  $**P < 0.01$ .

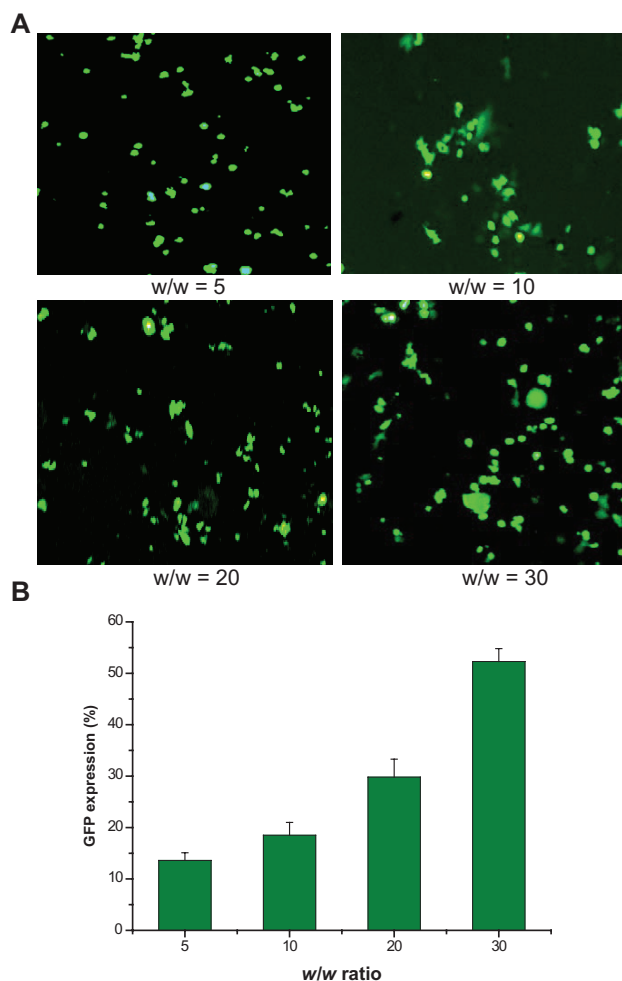
**Abbreviations:** OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

the percentage of green-fluorescent-protein-expressed cells was counted by a flow cytometer.<sup>27</sup> As w/w ratios increased, the percentage of green-fluorescent-protein-expressed cells increased. This was in accordance with the fluorescent images.

Figure 9A shows the results of the gene transfection efficiency of OTMCS-PEI/pGL3-Control complexes in HeLa cells in comparison with other controlled complexes. PEI 25 KDa showed the best transfection efficiency at w/w of 10. As the w/w ratios increased, its gene transfection decreased, which mainly resulted from high cytotoxicity. OTMCS-PEI showed much higher gene transfer ability compared with PEI 25 KDa at the designated w/w ratios. It exhibited the maximal transfection efficiency at the weight ratio of 30. The highest luciferase expression level obtained was more than 320 times higher compared with PEI 2 KDa, even about 10 times higher than PEI 25 KDa at optimal conditions.

### In vivo transfection efficiency

In order to evaluate the DNA delivery efficiency of OTMCS-PEI, pGL3-Control gene transfection in vivo was performed using a tail-vein injection of pDNA complexed with OTMCS-PEI. It was found that the transfection efficiency of OTMCS-PEI was obviously higher than PEI 25 KDa in every tissue (Figure 9B). This was in line with the experimental results in vitro. The reporter gene expression of OTMCS-PEI/DNA complexes was highest in lung, followed by the subcutaneous transplanted



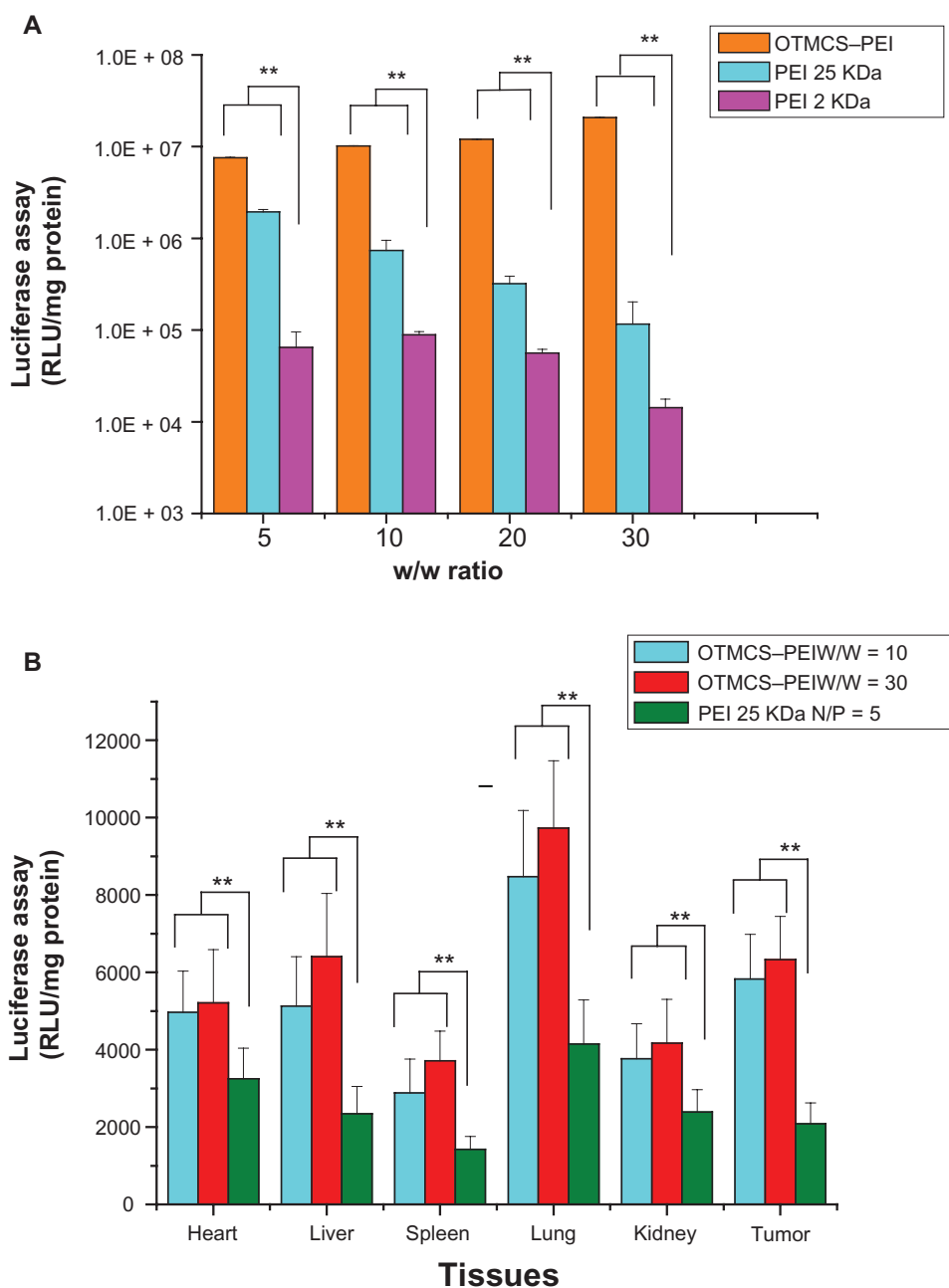
**Figure 8** GFP gene transfection in HeLa cells by OTMCS-PEI at w/w ratios of 5, 10, 20, and 30. (A) Percentage of GFP gene transfection in HeLa cells by flow cytometry analysis. (B) Representative fluorescence images for gene transfection in HeLa cells using OTMCS-PEI at various w/w ratios.

**Note:** \*The mean  $\pm$  standard deviation,  $n = 6$ .

**Abbreviations:** GFP, green fluorescent protein; OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine.

tumor and liver. In contrast, the gene expression of PEI 25 KDa/DNA in tumor was lower than in lung and heart. In the case of the pulmonary accumulation of the complexes, on the one hand, because of abundant capillaries in the lung, the complexes with larger sizes were easily entrapped mechanically. At the same time, the complexes with surface positive charges were easily taken up by the lung due to electrostatic interaction.<sup>28-31</sup> Therefore, in our future work, we intend to modify a tumor-targeted group to improve cell selection.

The transfection efficiency assay in vitro and in vivo showed that the micelle-like structure based on amphiphilic chitosan and low-molecular weight (LMW) PEI enhanced its stability in body fluid and transfection efficiency, compared with PEI 25 KDa.



**Figure 9 (A)** Transfection efficiency of OTMCS-PEI/DNA complex in HeLa cell line at w/w ratios of 5, 10, 20, and 30.\* **(B)** Transfection efficiency of pGL3-Control as a reporter gene in mice.

**Notes:** BALB/c athymic mice were inoculated with B16 cells. Luciferase gene expression was determined after administration of OTMCS-PEI/DNA complex (w/w = 10) OTMCS-PEI/DNA complex (w/w = 30), and PEI 25 KDa/DNA complex (N/P = 5);<sup>†</sup> each data point represents the mean  $\pm$  standard deviation (n = 6); \*\*P < 0.01; <sup>†</sup> results were expressed in RLU/mg protein.

**Abbreviations:** OTMCS-PEI, amphiphilic chitosan cross-linked with low-molecular weight polyethylenimine; RLU, relative light units.

## Conclusion

We developed a new degradable gene vector OTMCS-PEI by cross-linking low-molecular weight PEI with amphiphilic chitosan. Through various physicochemical methods, we confirmed that OTMCS-PEI had great ability to form complexes with DNA, and suitable physicochemical properties for gene delivery. The vector showed controlled degradation.

The degradation profile was suitable to a zero-order model. The half-life was about 30 hours. In the meantime, the OTMCS-PEI could deliver DNA to the nucleus, and then degraded into micromolecules. The polymer showed favorable buffering capacity. The particle sizes of OTMCS-PEI/DNA complexes were around 150–200 nm and the zeta potentials ranged from 10 mV to 30 mV, which was proper

for a gene vector. The OTMCS-PEI polymer was able to condense DNA completely at the weight ratio of 0.6. The polymer could protect plasmid DNA from being digested by DNase I at a concentration of 2.25 U DNase I/ $\mu$ g DNA. At the same time, they were able to resist dissociation induced by 50% FBS and 1100  $\mu$ g/mL sodium heparin. Additionally, the new gene vector showed much lower cytotoxicity and higher gene transfection efficiency in the HeLa cell lines, compared with PEI 25 KDa. The transfection efficiency of the polymer in vivo was also higher than that of PEI 25 KDa. Except for the accumulation in lung, more DNA transported by OTMCS-PEI polymer acted on transplanted tumors. In general, this new polymer could be a potential candidate in gene delivery, with high transfection efficiency and low cytotoxicity. However, it is worth improving its tumor targeting. Moreover, both the delivery of therapeutic gene transferred by this gene vector and the therapeutic efficiency need to be studied. We are currently conducting more comprehensive studies with the aim of answering these questions, to be presented in another manuscript.

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## Disclosure

The authors report no conflicts of interest in this work.

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