# Improving Transfection of Human Pulmonary Epithelial Cells by Doping LMW-PEI-*g*-Chitosan with β-Estradiol

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**ABSTRACT:** PEI-grafted chitosan (PEI-CMCS) copolymer was synthesized through EDC-mediated amidation reaction between carboxymethyl chitosan and low-molecular-weight polyethyleneimine (LMW PEI). PEI-CMCS and CS/DNA complexes were characterized. Compared with pristine chitosan, the PEI-CMCS exhibited an enhanced ability to condense DNA. Incorporation of LMW PEI to chitosan was found to achieve higher transfection efficiency and much lower cytotoxicity than PEI25K at an optimum weight ratio of 15 in COS-7 cells. However, PEI-CMCS was incompetent to transfer refractory pulmonary epithelial cells. It was demonstrated that doping complex medium or treating cells with a steroid hormone—β-estradiol significantly improved the transfection efficiency of 16HBE and A549 cells. This study suggests that steroid hormone may become an additive for other cationic polymers to facilitate gene transfection in pulmonary cells. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 874–882, 2011

**Key words:** chitosan; polyethyleneimine; gene delivery; pulmonary epithelial cell; β-estradiol

## INTRODUCTION

The lung is an attractive target for gene therapy since it is easily accessible and represents the organ where lethal congenital and acquired diseases occur.[1] Clinical pulmonary disorders, such as cystic fibrosis (CF), are suitable candidates for gene therapy.[2] The growing interest in nonviral vector systems has spurred researchers to develop advanced materials for delivery of DNA and siRNA to lung cells with high efficiency and low toxicity.

Of the materials utilized as nonviral vectors, polyethyleneimine (PEI), a synthetic polycation, is the most widely investigated because of its strong ability to condense DNA and transfect a broad range of cell lines with high efficiency.[3] The transfection efficiency of PEI is closely related to its structure and molecular weight. Generally, the PEI with high molecular weight shows high efficiency, but has high cytotoxicity.[4,5] Although low-molecular-weight PEIs have lower cytotoxicity, their efficiency deteriorates with the drop of molecular weight.

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Chitosan, comprised of β-(1-4) linked 2-amino-2-deoxy-D-glucose, has been proposed as one of the potential nonviral vectors for gene transfer, benefiting from its cationic character, biodegradability, and biocompatibility.[6] However, the clinical use of chitosan is limited by the low-transfection efficiency.[7] To improve transfection efficiency of chitosan polyplexes, numerous modifications to the polymer structure have been made. Several strategies were utilized to improve the cationic property of chitosan.[8–10] To increase the buffering capacity of chitosan, two important modifications were investigated. One attempt was to conjugate chitosan with varying ratios of urocanic acid. These imidazole-containing derivatives showed reduced cytotoxicity and significantly enhanced transfection efficiency due to the increased buffering capability.[11] The same buffering capacity was also achieved by conjugating chitosan with polyethyleneimine. In particular, grafting low-molecular-weight PEI to chitosan could achieve comparable or even superior transfection efficiency to PEI25K, but exhibited much lower cytotoxicity.[12–14] Nonetheless, to the best of our knowledge, no work has been reported on exploring PEI-chitosan as a gene delivery system for refractory human pulmonary epithelial cells thus far.

In this work, aiming at designing a low cytotoxic nonviral vector for airway gene delivery, we synthesized PEI-grafted chitosan (PEI-CMCS) copolymer

through an amidation reaction between carboxymethyl chitosan and low-molecular-weight PEI. The physicochemical properties of PEI-CMCS/DNA complexes were examined. The cytotoxicity and transfection efficiency of the PEI-CMCS/DNA complexes were first investigated *in vitro* using COS-7 cells. On the basis of the observation that β-estradiol can increase liposome-mediated gene uptake and promote nuclear accumulation of the transgene,[15] we will incorporate β-estradiol into PEI-CMCS to improve gene delivery to human bronchial epithelial cell line (16HBE) and human alveolar epithelia cell line (A549).

## EXPERIMENTAL

### Materials

Chitosan (CS, molecular weight 50 kDa, deacetylation degree 92%) was supplied by AK Biotech (Shandong, China). 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), branched polyethyleneimine (PEI, 25K and 800 Da), ethidium bromide (EB, 95%), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT, 98%) were purchased from Sigma-Aldrich (Shanghai, China). Chloroacetic acid was supplied by Alfa Aesar Co. Plasmid pGL3-control with SV40 promoter and enhancer sequences encoding luciferase was obtained from Promega, Madison, WI. pEGFP-C1 encoding a red-shifted variant of wild-type green fluorescent protein (GFP) was purchased from Clontech, Mountain View, CA. The plasmids were amplified in *Escherichia coli* and purified by the differential precipitation method. All other reagents used were of analytical grade.

### Synthesis and characterization of PEI-CMCS

Carboxymethyl chitosan (CMCS) was prepared by alkalization and carboxymethylation reactions. Briefly, chitosan (5 g) was suspended in 40% aqueous NaOH solution (50 mL) overnight. After the excessive alkali solution was extracted, alkali-treated chitosan was reacted with monochloroacetic acid (20 g) in isopropyl alcohol at 65°C for 5 h according to the reported method.[16] The product was dissolved in water and the remaining precipitate was removed by centrifugation. Excess anhydrous ethanol was added to the solution to precipitate out CMCS, which was redissolved in distilled water and reprecipitated twice more and then dried under vacuum.

### Synthesis of PEI-CMCS

PEI was grafted onto the CMCS using EDC as a coupling reagent. In brief, CMCS (220 mg) and branched PEI800 (3.2 g) were dissolved in 30 mL of distilled water; then, the pH of the mixture solution was adjusted to 5.5 by 1M HCl solution. After the solution was stirred at room temperature for 30 min, 10 mL aqueous solution containing 192 mg of EDC and 115 mg NHS were added dropwise to the mixture. The solution was kept stirring at room temperature for 24 h. Subsequently, the solution was dialyzed (Spectra/Por® membrane: MWCO = 7000) against deionized water and lyophilized.

### Chemical characterization

The compositions of the prepared CMCS and PEI-CMCS were estimated by $^1$H-NMR measurement (UNITY plus-500, Varian, USA) using $D_2O$ as a solvent. The $^1$H-NMR of pristine chitosan was measured as a comparison in $CD_3COOD/D_2O$ solution. The substitution degree of the PEI-CMCS was determined by elemental analysis. The elemental analysis (C, N, and H) of samples was performed on a Flash Elemental Analyzer 1112 (ELEMENTAR Vanio EL, Germany).

### Preparation of PEI-CMCS/DNA complexes

PEI-CMCS was dissolved in distilled water to a concentration of 2 mg/mL. The solutions were sterilized by extruding through 0.22 μm filter. PEI-CMCS/pDNA complexes at different weight ratios were formulated by adding different volume of PEI-CMCS solution to an equal volume of a defined pDNA solution, vortexed for 15 s, and then incubated for 30 min at room temperature.

### Gel retardation assay

The electrophoresis was performed to confirm the ability of PEI-CMCS to condense DNA. PEI-CMCS/pDNA complexes with various weight ratios from 0.1 to 4 were prepared as described earlier. The final pDNA concentration was controlled to be 0.1 mg/well. The complexes were incubated at room temperature for 30 min. The complex solutions were loaded onto 1.0 wt % agarose gel with EB staining and Tris-acetate (TAE) running buffer at 100 V for 40 min.

### Transmission electron microscopy (TEM)

The morphology and size of polymer/pDNA complexes with different ratios were observed using TEM(JEOL JEM-100CXII). Briefly, Polymer/pDNA complexes were formed to a concentration of 10 μg/mL. A drop of the complex solution was deposited on a carbon-coated grid. After 5 min, 1.5 wt % phosphotungstic acid (PTA) was added to negatively stain the complexes, and then, the complexes were recorded on films with transmission electron microscopy (TEM). The particle sizes were estimated from the measurement of about tens of particles found in a randomly chosen area in TEM pictures.

### Measurement of particle size and zeta potential

The particle sizes and zeta potentials of PEI-CMCS/pDNA complexes were measured using a Zeta-PALS/Zeta Potential Analyzer (Brookhaven, Austria) at 25°C. The PEI-CMCS/DNA complexes were prepared by adding appropriate volume of copolymer solution to 1 μg of DNA solution at weight ratios ranging from 1 to 20. Then, the solutions containing complexes were diluted by distilled water for particle size and zeta-potential measurement.

### Cell culture

African green monkey kidney cells (COS-7), human bronchial epithelial cells (16HBE), and human alveolar epithelial cells (A549) were purchased from Peking Union Medical College (Beijing, China). All the above cell lines were grown in essential medium, respectively, (DMEM, MEM, F-12) supplemented with 10% fetal calf serum at 37°C in a 5% $CO_2$ humidified air atmosphere.

### Cytotoxicity assay

The cytotoxicity of PEI-CMCS was evaluated by MTT assay using COS-7 cells. Briefly, $1 \times 10^4$ cells/well were seeded in 96-well plates and incubated for 24 h at 37°C in 5% $CO_2$ humidified atmosphere. PEI-CMCS at an increasing concentration from 0 to 200 μg/mL was added to each well and incubated for 24 h before refreshing the medium with fresh complete medium (200 μL/well). After additional incubation for 24 h, 20 μL/well MTT (5 mg/mL in PBS) was added to each well, and the plate was further incubated for 4 h. Then, all media were removed and 150 μL/well DMSO was added. The plate was gently shaken for 10 min. The absorbance of each well was measured at 570 nm on a $\sum$960 plate-reader (Metertech) with pure DMSO as a blank. Nontreated cells were used as a control and the relative cell viability (mean% ± SD, $n = 3$) was expressed as $Abs_{sample}/Abs_{control} \times 100\%$.

### Transfection of COS-7 cells

COS-7 cells were seeded at a density of $4 \times 10^4$ cells/well in 24-well plates and incubated for 24 h at 37°C in 5% $CO_2$ humidified atmosphere. Before transfection, the culture medium was removed and replaced with DMEM supplemented with 10% FBS without antibiotics (450 μL/well). Various complexes at different weight ratios were formed based on the protocol in section "Preparation of PEI-CMCS/DNA complexes" and added to the 24-well plates (50 μL in water, containing 1 μg DNA/well). After incubating at 37°C in 5% $CO_2$ for 24 h, the medium containing complex solution was then replaced with 500 μL of fresh complete medium and the cells were incubated for an additional 24 h. Transfection tests were performed in triplicate. Following incubation, the medium was removed, and the cells were washed with PBS twice. The cells in each well were treated with 150 μL of reporter lysis buffer (RLB, Promega) followed by freeze-thaw cycles to ensure complete lysis. The lysate was centrifuged for 4 min at 13,000r/min at room temperature and the supernatant was collected for luminescence measurements. The luminescence of each sample was measured by 1420 Multilabel counter (Wallac, USA) using Bright-GloTM luciferase assay system (Promega, USA) according to the manufacturer's protocol. The results were expressed as relative light units (RLU) per milligram of cell protein, and the protein concentration of each well was measured by a BCA protein assay (Pierce, Rockford, IL). Branched polyethylenimine (PEI) 25 kDa was used as a positive control with PEI/DNA = 15/1(w/w).

### Transfection of 16HBE and A549 cells doped with β-estradiol

β-estradiol was dissolved in 95% ethanol to a concentration of 10 mg/mL. 16HBE cells and A549 cells were seeded and incubated following the above procedure. The vector/DNA complexes were prepared under an optimum ratio. The complex media were mixed with estradiol solution over a range of doses before adding into cell plates. Other operations were performed following the protocol in the above section.

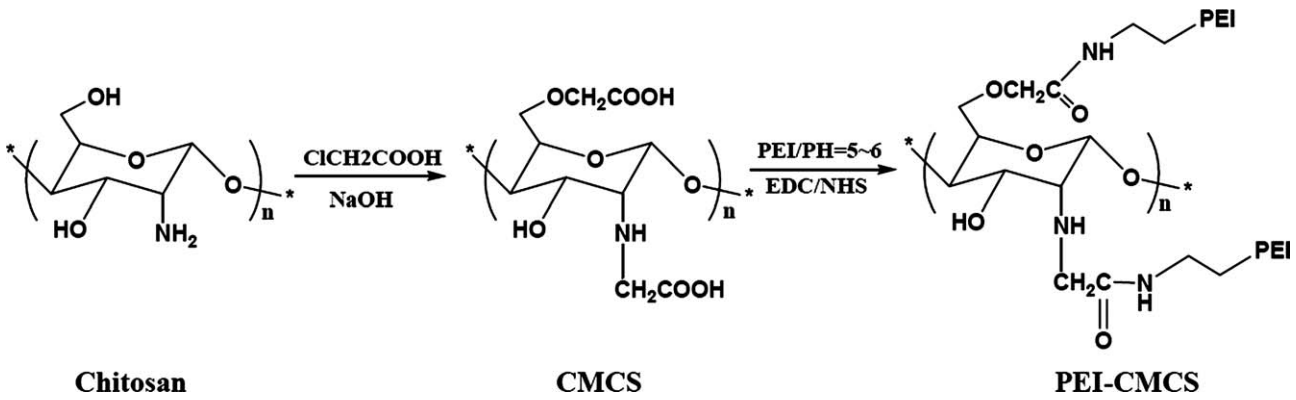
### GFP transfections

To directly visualize the infected cells expressing GFP, pEGFP-C1 plasmid was transferred to different cell lines with PEI-CMCS vectors in terms of the aforementioned method. The GFP-expressing cells were observed by fluorescence microscope (DMI4000B, Leica, Germany).

## RESULTS AND DISCUSSION

### Characterization of PEI-CMCS

In this work, 50 kDa chitosan was used taking into account its ability to condense DNA as well as the low cytotoxicity. The synthesis of PEI-CMCS copolymer involved two steps as illustrated in Scheme 1. Carboxyl group was introduced to the chain of CS in the first step, and then, PEI with low molecular weight was linked to CMCS through EDC-mediated amidation (Scheme 1). During the coupling reaction, a large excess of PEI800 was added to ensure that no intra- or intermolecular crosslinking reaction occurred.




**Scheme 1** Synthesis of PEI-CMCS.

The structures of CS, CMCS, and PEI-CMCS are confirmed by $^1$H-NMR as shown in Figure 1. The $^1$H-NMR spectra of CS in $CD_3COOD/D_2O$ show the feature peak of $CH_3$ in *N*-acetyl residue at 1.8 ppm and multiplets from 2.9 to 3.7 ppm corresponding to the ring methine protons (H3, H4, H5, and H6) [Fig. 1(A)]. For CMCS, new peaks locating at 3.2 and 4.3 ppm are assigned to the protons of —$CH_2$—COO— substituted on the $C_2$ amino group and the $C_6$ hydroxyl group, respectively, [Fig. 1(B)].[17] The stronger peak at 4.3 ppm verifies that the substitutions mainly occur on the $C_6$ hydroxyl group. In Figure 1(C), we can observe several new spectral peaks spanning from 2.2 to 3.0 ppm, which are attributed to the methylene of branched PEI800, confirming the successful grafting of PEI800.[18] The chemical shifts of PEI in the PEI-CMCS are in agreement with those of branched PEI itself.

The degree of substitution (DS) of carboxymethyl on CMSC and that of branched PEI800 on PEI-CMCS are calculated based on elemental analysis data. The DS values of carboxymethyl and PEI are estimated as 1.26 and 0.27, respectively. The number of carboxymethyl groups or PEI coupled to the anhydroglucose units of chitosan was defined as the DS.

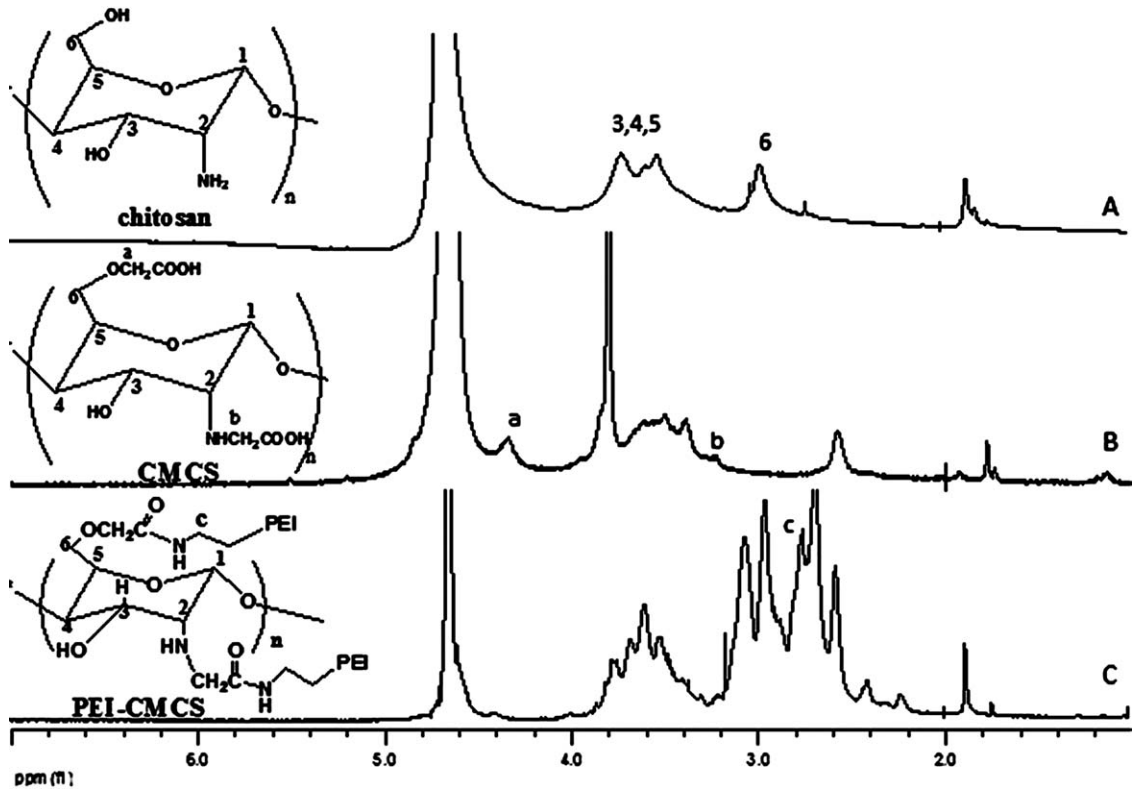



**Figure 1** $^1$H-NMR spectra of (A) CS ($D_2O$ as a solvent); (B) CMCS ($CD_3COOD/D_2O$ as a solvent); and (C) PEI-CMCS ($CD_3COOD/D_2O$ as a solvent).

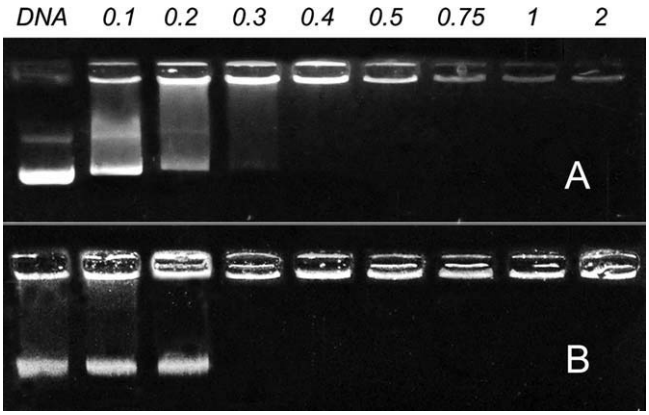



**Figure 2** Electrophoretic analysis of pDNA mobility in the complexes with (A) CS and (B) PEI-CMCS.

### Gel retardation assay

Formation of stable complexes between cationic polymers and DNA is an important prerequisite for efficient gene delivery. For gel retardation assay, polymer/DNA complexes were prepared at various weight ratios ranging from 1 : 10 to 2 : 1. As presented in Figure 2, CS and PEI-CMCS can completely retard DNA above the weight ratios of 0.4 : 1. This indicates that both chitosan and PEI-CMCS are capable of condensing DNA efficiently. On the other hand, the critical complexing ratio of PEI-CMCS decreases compared with that of unmodified chitosan, which reveals that incorporation of PEI into chitosan increases the density of positive charges, thereby contributing to a stronger binding strength with DNA. In our experiment, we found that CMCS could not completely stop the electrophoretic mobility of DNA even if the weight ratio was raised up to 50 : 1. It is evident that negatively charged carboxymethyl groups considerably dampen the ability of chitosan to condense DNA.

### TEM observation

TEM was used to observe the morphology of the complexes. PEI-CMCS/DNA complexes were formed at weight ratios of 1 : 1, 5 : 1, and 15 : 1. As shown in Figure 3, PEI-CMCS condenses DNA into spherical nanosized particles with diameters ranging from 30 to 200 nm. The particle sizes of complexes decrease as the weight ratio increases due to the enhanced condensation ability. At the weight ratio of 15 : 1, PEI-CMCS complexes were condensed to nanoparticles with size less than 50 nm.

### Particle size and zeta potential of PEI-CMCS/DNA complexes

We have used a laser particle size analyzer to study the change of particle sizes of PEI-CMCS/DNA complexes with different weight ratios (Table I). Generally, the average sizes exhibit a dropping trend upon increasing weight ratio. To examine the surface charges of PEI-CMCS/DNA complexes, the zeta potentials were measured with varied weight ratios ranging from 1 to 20 (Table I). The zeta potentials of PEI-CMCS/pDNA complexes are increased along with the increment of weight ratios, indicating much more PEI-CMCS binds to DNA molecules. The larger sized observed with laser particle size analyzer relative to TEM is due to the hydration of nanoparticles in solution.

### *In vitro* transfection of PEI-CMCS

The transfection efficiency of PEI-CMCS was assessed first in COS-7 cells using luciferase plasmid reporter gene. PEI25K with an optimal ratio was studied as the positive control. With the weight ratio increasing from 1 to 30, the transfection efficiency is increased and reaches the maximum value at the weight ratio of 15 : 1 (Fig. 4). The efficiency slightly decreases and levels off while the complexing ratio is above 15. A rational explanation is that when the weight ratio is increased, the PEI-CMCS tends to condense DNA into more compact nanocomplexes, offering a better protection of the DNA from degradation. On the other hand, too tight condensates may limit gene unpacking and higher complexing ratio could also result in the increased cytotoxicity, accordingly reducing gene expression level. At the optimal ratio, the efficiency of PEI-CMCS is superior to that of the commercially available PEI25K.

### Cytotoxicity of PEI-CMCS

In this study, incorporating low-molecular-weight PEI to chitosan was to achieve a potent nonviral vector for gene delivery with low cytotoxicity. To assess the cytotoxicity of this vector, we evaluated the relative cell viability of PEI-CMCS at various concentrations comparing with that of PEI25K by MTT assay method. It is evident from Figure 5 that the PEI-CMCS exhibits rather low cytotoxicity over a wide range of doses. Even at 200 μg/mL, 80% cells still remain alive. For PEI25K, along with increasing concentration, the cell viability declines significantly. This result indicates that grafting PEI800 onto chitosan can produce a low-cytotoxic nonviral vector.

### Doping β-estradiol to enhance the transfection efficiency of PEI-CMCS

In spite of high transfection level achieved by PEI-CMCS in COS-7 cells, the efficiency declined dramatically when it was applied to pulmonary epithelial cells, such as 16HBE and A549 cells. This

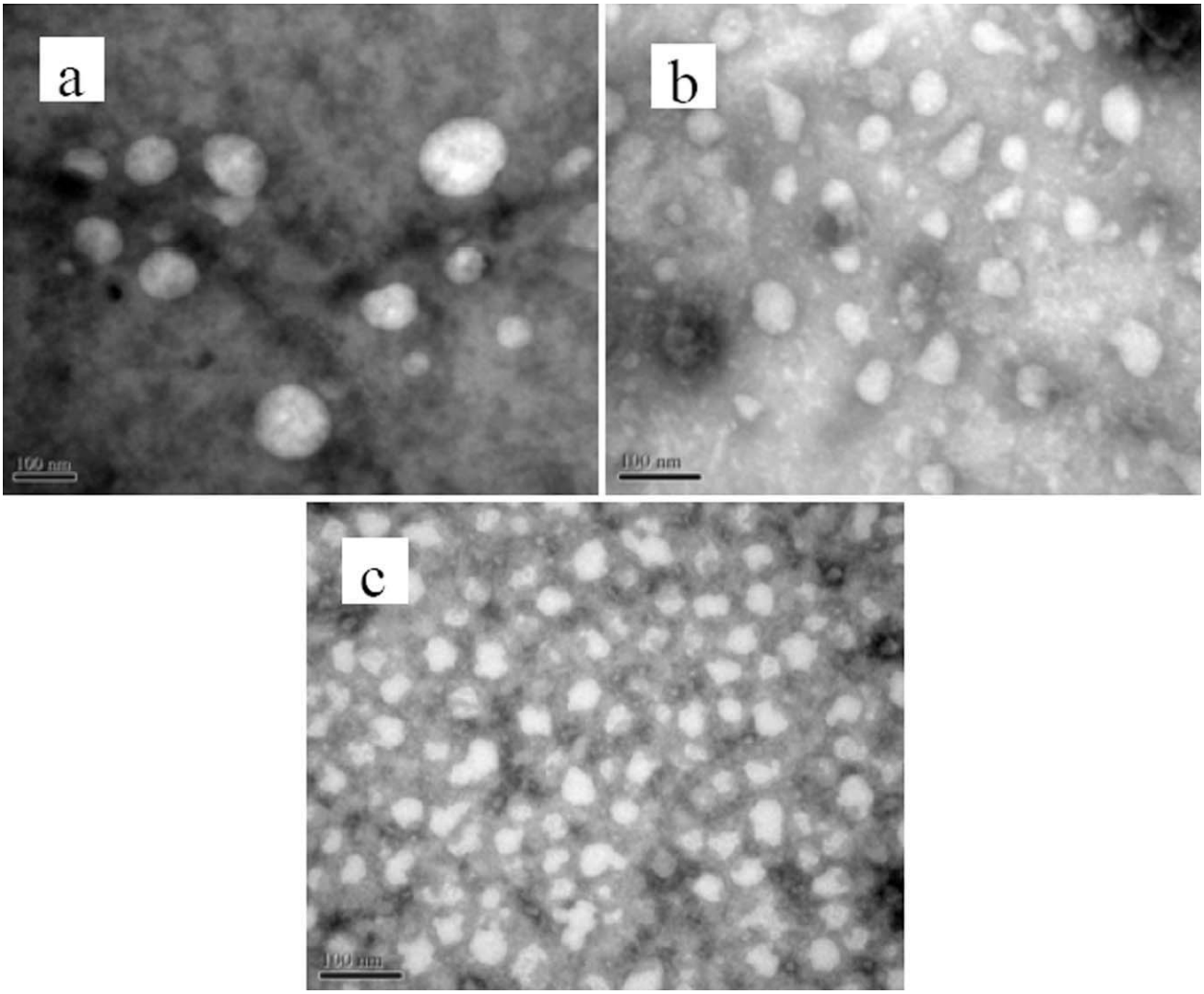



**Figure 3** TEM images of PEI-CMCS/DNA complexes at weight ratios of 1 : 1 (a), 5 : 1 (b), and 15 : 1 (c).

poses a great obstacle to its application in gene therapy of respiratory disease. Cellular uptake, endosomal escape, cytoplasmic mobility, and nuclear entry are deemed to be the four main barriers for polymer-mediated transfection *in vitro*.[19] Estradiol, a steroid hormone, has been reported to increase liposome-mediated gene uptake *in vitro* and promote nuclear accumulation of the transgene.[15] To examine

**TABLE I**
**Particle Sizes and Zeta Potentials of PEI-CMCS/DNA Complexes**

| Vector : DNA (w/w) | Particle size (nm) | Zeta potential (mV) |
|---|---|---|
| 1 : 1 | 565 ± 20.1 | 7.05 ± 1.5 |
| 5 : 1 | 371 ± 15.7 | 13.86 ± 0.8 |
| 10 : 1 | 365 ± 20.1 | 15.12 ± 1.4 |
| 15 : 1 | 285.7 ± 4.2 | 16.83 ± 0.7 |
| 20 : 1 | 290.6 ± 7.1 | 17.09 ± 1.1 |

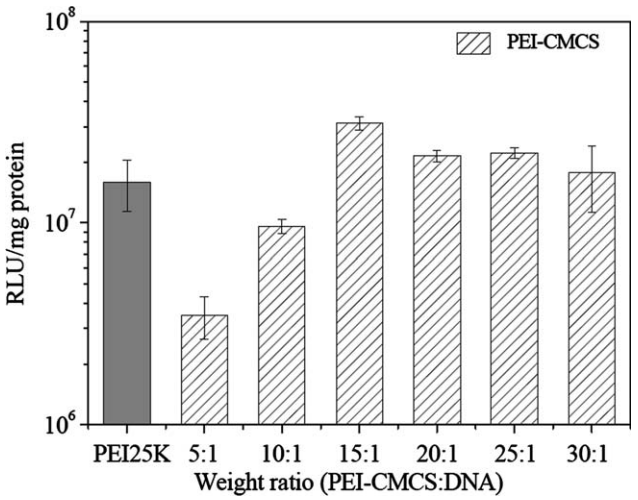



**Figure 4** *In vitro* transfection efficiency of PEI-CMCS/DNA complexes by luciferase assay in COS-7 cell line at various weight ratios (mean ± SD, $n = 3$).

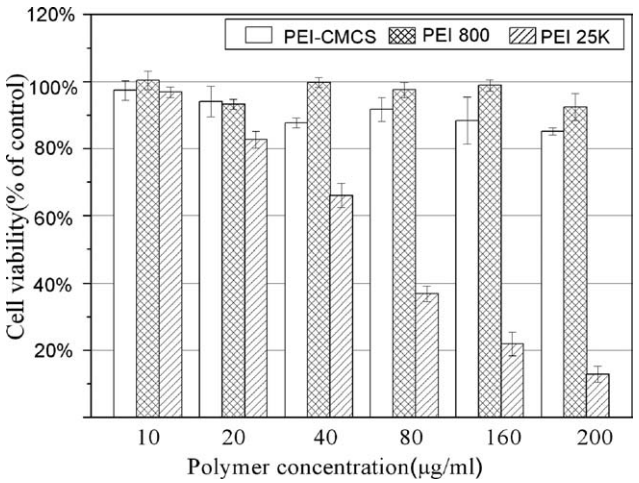



**Figure 5** Viability of COS-7 cells versus the concentrations of PEI-CMCS, PEI 800, and PEI25K.

the influence of β-estradiol on the transfection efficiency of PEI-CMCS, we performed the transfection experiments with increasing concentrations of β-estradiol in 16HBE and A549 cells. Figure 6(A,B) show the transfection efficiency of PEI-CMCS/DNA complexes at a weight ratio: 15 : 1 under different β-estradiol concentrations. Figure 6(A) shows that β-estradiol substantially enhances the transfection efficiency of PEI-CMCS in 16HBE cells at an appropriate concentration. A 25-fold enhancement is seen at the optimum concentration of 100 μ*M* compared with the polyplexes in the absence of β-estradiol. Further increase in the concentration leads to a decrease in efficiency. A possible reason is that high dose of estradiol is detrimental to the cells. A 12-fold enhancement of efficiency for A549 is observed under a concentration of 400 μ*M* [Fig. 6(B)], suggesting a lower sensitivity to estradiol. From GFP assay experiment, the improvement using β-estradiol is also apparent (Fig. 7). The above results have confirmed the enhancement of transfection efficiency by using steroid polymers for pulmonary epithelial cells.

Since β-estradiol may affect cellular membrane stability as well as subcellular processes in cytoplasmic and nuclear, it is possible that the observed enhanced efficiency in the presence of estradiol represents the addictive effects both on uptake phase and nuclear transportation phase.[20,21] To discern the two different contributions of estradiol to the transfection efficiency, we evaluated the effects of doping mode of estradiol on gene expression during different stages (Fig. 8). Pretreatment with estradiol before transfection does not show an evident improvement of transfection efficiency, which implies that the cellular internalization of complexes is only marginally influenced by doping estradiol. In contrast, an obvious additive effects of including β-estradiol in the culture medium after transfection is observed over a wide range of feeding time. We found a significant increase of efficiency when cells were post-treated with estradiol 4 h after transfection. Since the medium of cell was refreshed, it is reasonable to reckon that no cellular uptake occurred afterward. Thus, it can be considered that the increment of efficiency is mainly due to the intracellular process affected by estradiol. This increment could still be observed when cells were post-treated with estradiol 24 h after transfection. The results demonstrate that cytoplasmic mobility and nuclear location may be a main rate-limiting step for transfection of breath epithelial cells.

Methyl-prednisolone was reported to enhance liposome-mediated transfection in 16HBE cells.[21] Several other glucocorticoids have also been incorporated onto cationic polymers to facilitate nuclear localization aiming to improve transfection efficiency.[22,23] We have also tested the influence of methyl-prednisolone corticosterone, cortisone, and dexamethasone on PEI-CMCS-mediated transfection in pulmonary epithelial

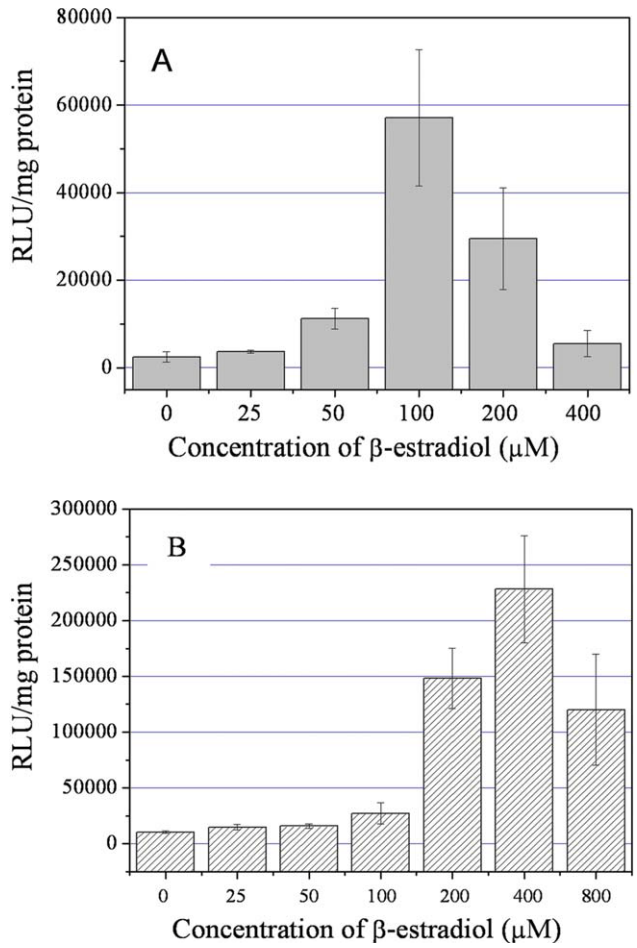



**Figure 6** Luciferase expression mediated by PEI-CMCS under different β-estradiol concentrations in 16HBE cells (A) and A549 cells (B).

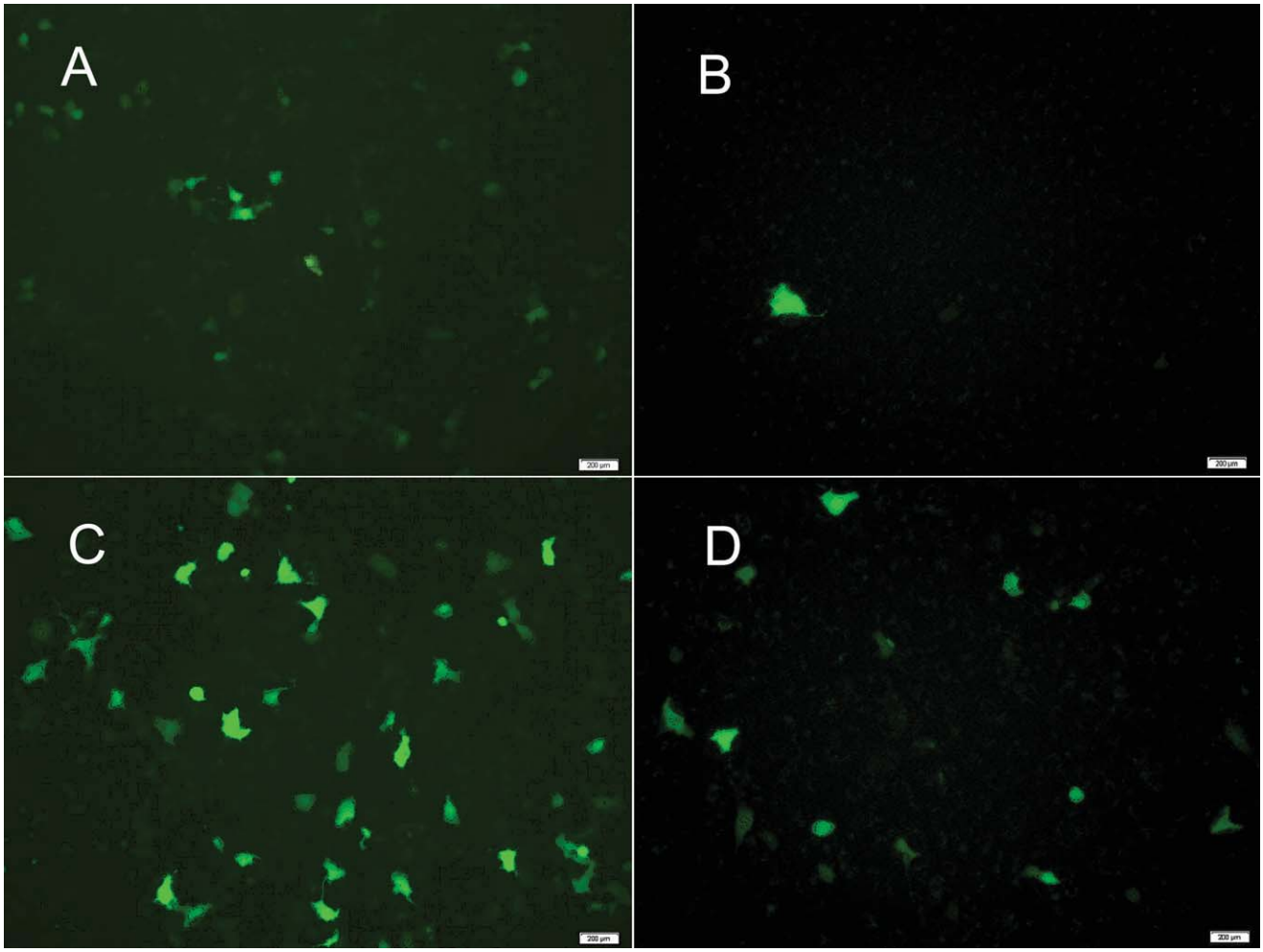



**Figure 7** GFP transfection in 16HBE cells and in A549 cells. Transfection of 16HBE (A) and A549 (B) in the absence of β-estradiol; transfection of 16HBE (C) and A549 (D) in the presence of β-estradiol. Scale bar = 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

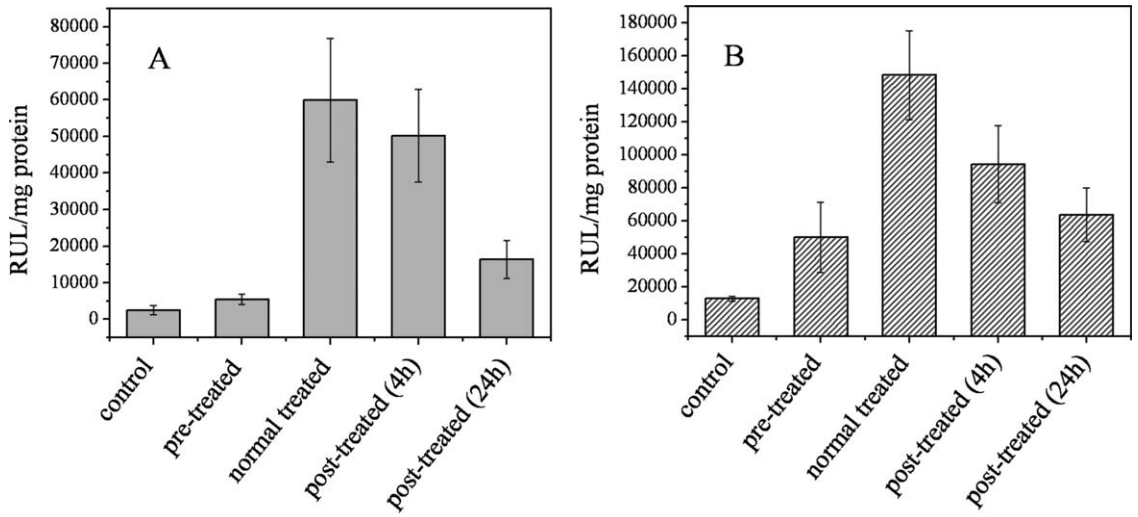



**Figure 8** Gene expression achieved with exposure to β-estradiol at different feeding time. PEI-CMCS polyplex in the absence of estradion serves as a negative control. The pretreated group denotes that cells were exposed to estradiol 1 h before transfection; normal treated group denotes that vector/DNA complexes were mixed with β-estradiol before transfection, as depicted in Figure 6. The post-treated group denotes that cells were exposed to estradiol 4 and 24 h after transfection. (A: 16HBE cell; B: A549 cell).

cells but did not lead to any noticeable improvement. It should be pointed out that high dose of β-estradiol might cause potential side effect.

## CONCLUSIONS

In this work, low-molecular-weight polyethyleneimine was grafted to chitosan via EDC chemistry to form a copolymer vector (PEI-CMCS). The PEI-CMCS showed an increased ability to condense DNA to nanocomplexes suitable for gene delivery. Compared with PEI25K, this copolymer showed lower cytotoxicity and enhanced transfection efficiency in COS-7 cells. However, PEI-CMCS was shown to be impotent in mediating efficient gene delivery to pulmonary epithelial cells, such as 16HBE and A549 cells. A salient increment in transfection efficiency of 16HBE and A549 cells could be achieved by doping culture medium or treating cells with a steroid hormone-β-estradiol. Steroid hormone is a promising accelerator for cationic polymer-mediated gene transfection in pulmonary cells.

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