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# 6-O-dodecyl-chitosan carbamate-based pH-responsive polymeric micelles for gene delivery

**Huiying Chen,[1,2] Yinan Zhao,[2] Shaohui Cui,[2] Defu Zhi,[2] Shubiao Zhang,[2] Xiaojun Peng[1]**

[1]State Key Laboratory of Fine Chemicals, Dalian University of Technology, 2 Linggong Rd., Hi-Tech Zone, Dalian 116024, People's Republic of China

[2]Key Laboratory of Biotechnology and Bioresources Utilization - The State Ethnic Affairs Commission-Ministry of Education, Dalian Nationalities University, Economic Development Zone, Dalian 116600, People's Republic of China

Correspondence to: S. Zhang (E-mail: zsb@dlnu.edu.cn)

**ABSTRACT:** pH-responsiveness is highly desirable in the stimuli-responsive controlled release because of the distinct advantages of the fast response of pH-triggered release and the available pH-difference between intra- and extra-cells. The present work reported a kind of novel pH-responsive polymeric micelles, which was derived from biopolymer of 6-O-dodecyl-chitosan carbamate (DCC) and evaluated as gene-controlled release vector. The amphiphilic and amino-rich DDC was synthesized through a protection-graft-deprotection method. $^{13}C$ CP/MAS NMR, FTIR, and elemental analysis identified that dodecyls were chemoselectively grafting at 6-hydroxyls of chitosan via the pH-responsive bonds of carbamate, and the substitute degree (SD) was 14%. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) showed that DCC self-assembled into polymeric micelles in aqueous solutions. The DCC polymeric micelles formed complexes with pDNA, which was elucidated by Gel retardation, TEM, and DLS. Transfection and cytotoxicity assays in A549 cells showed that DCC polymeric micelles were suitable for gene delivery. The improved transfection was attributed to the pH-responsiveness and the moderate pDNA-binding affinity, which led to easier release of pDNA intra-cells. The synthesized DCC polymeric micelles might be a promising and safe candidate as nonviral vectors for gene delivery. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42469.

**KEYWORDS:** biomaterials; drug delivery systems; micelles; self-assembly; stimuli-sensitive polymers

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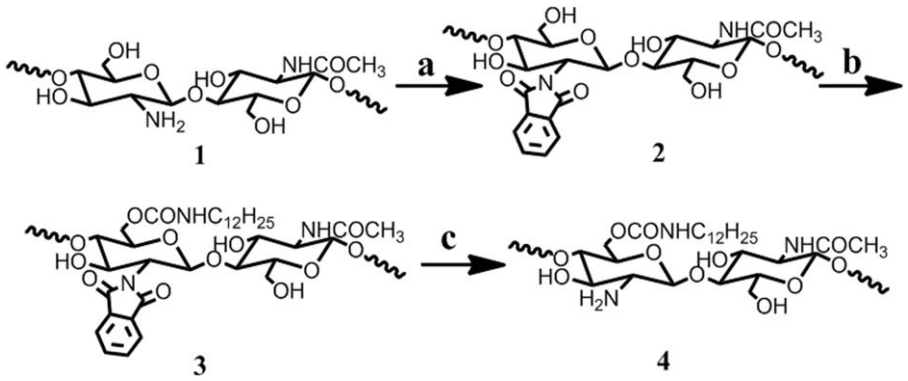
## INTRODUCTION

Cancer remains one of the most incurable diseases associated with high mortality currently.[1] Gene therapy has gained significant attention over the past two decades as a promising method to treat human cancer.[2,3] However, little success has been achieved in its clinical application because of the lack of safe and efficient gene delivery systems.[4,5] Viral vectors are costly and un-targeted, associated with some severe side effects such as immunogenicity and carcinogenicity. Therefore, nonviral vectors, especially nanocarriers, have become attractive alternatives to viral vectors because of their advantages such as ease of synthesis, low immune response, and better safety.[6] Spurred by advances in biomaterial sciences, recent efforts have focused on the development of polymeric micelles for delivery of anticancer drugs.[7,8] Such polymeric micelles have unique properties, including nanoscale size, core-shell structure, high thermodynamic stability, good biocompatibility, prolonged circulation in the bloodstream, and passively accumulation at tumor tissues by the enhanced permeation and retention (EPR) effects.[8] Therefore, synthesis of biocompatible and biodegradable polymers is of great importance for the application of polymeric micelles in gene therapy.

Chitosan (CS) is widely applied in wound dressing, tissue engineering, drug, and gene delivery, thanks to the good biocompatibility and biodegradability. Moreover, CS has the capability to temporarily open the tight intercellular epithelial junctions to facilitate the uptake of drugs.[9–13] CS amphiphiles were first introduced in the late 1990s.[14] They are prepared by conjugating hydrophobic units or sometimes additional hydrophilic units to CS. Polymeric micelles can self-assemble in aqueous media by virtue of their amphiphilicity.[15,16] These polymeric micelles are ideal vectors for the delivery of drugs and genes, because the amphiphile nanoparticles of CS will not be recognized as foreign by the mammalian immune system.[16] CS amphiphiles with positive charges can combine with the phosphate anions of pDNA by electrostatic interactions and therefore can be used as gene delivery vectors. Such amphiphiles include *N*-palmitoyl glycol CS,[15] glycol CS,[17] linoleic acid, and poly(beta-malic acid)




**Scheme 1.** Synthesis of 6-O-dodecyl-chitosan carbamate (DCC): (a) phthalic anhydride, DMF, 120°C, 8 h; (b) *N,N'*-carbonyldiimidazole (CDI), NMP, 50°C, 3 h; then dodecyl amine, NMP, 40°C, 3 h; (c) hydrazine monohydrate, methanol/$H_2O$, 40°C, 18 h.

grafted CS,[18] *N*-α-tocopherol CS,[19] stearic acid–modified CS,[20,21] deoxycholic acid–modified CS,[22] 5 beta-cholanic acid–modified glycol CS,[23,24] and *N*-alkylated CS.[25] In these cases, the hydrophobic units or additional hydrophilic units are mostly grafted to CS's amino groups, which are more chemically active than CS's hydroxyl groups.[26–28] It has been found the amino groups have close correlation to some appealing properties of CS, such as the bioadhesive property, the ability to promote cell proliferation and tissue regeneration, and the electrostatic complexation.[29–31] Therefore, modifying CS at amino groups will deteriorate CS's biological functions. Liberating the amino groups of CS from occupation would contribute to superior performance of CS-based gene delivery systems. In addition, the carbamate bonds are prone to be hydrolyzed in acidic environments.[32–34] The intracellular compartments in cancer cells, i.e., lysosomes and endosomes, have a lower pH than the normal tissues (pH 4–6 vs. pH 7.4).[35] Developing delivery systems responsive to such a pH-decreased environment in intracellular compartments would give another inspiring solution to enhance the efficiency of cancer-targeting therapy.

In this article, we designed and synthesized an amino-rich and pH-sensitive amphiphilic CS derivative, 6-O-dodecyl-chitosan carbamate (DCC). By a three-step synthesis process, the amino groups in CS were well preserved in the final products, and the dodecyl groups were selectively grafted to 6-hydroxyls of CS via carbamate bonds. Its gene delivery performance was investigated, and the improved performance was well consistent with the advantages afforded by the functionalized structures.

## EXPERIMENTAL

CS (viscosity = 40,000 cps, deacetylation degree = 85%) was purchased from Jinan Haidebei Marine Bioengineering Co. Polyethylenimine ($M_w$ = 25000 kDa, PEI-25) was purchased from Sigma-Aldrich Chemical Co. *N,N'*-carbonyldiimidazole (CDI), phthalic anhydride, and dodecyl amine (analytical grade) were supplied by Beijing Chemicals Co. (Beijing, China). Luciferase activity assay kits and the protein assay kits were obtained from Biyuntian Biologic Co. (Nanjing, China). Dulbecco's modified eagle's medium (DMEM) and trypsin were obtained from Gibco BRL (Gaithersberg, MD). Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Co. (Hangzhou, China). The pGFP-N2 and pGL3 plasmids were amplified in *Escherichia coli* cells and purified using the endo-free Qiagen kit (Qiagen, Valencia, CA). The quality of purified pDNA was checked by electrophoresis on a 1.0% agarose gel and the concentration of pDNA was determined by UV absorbance at 260 and 280 nm. All the organic solvents used in synthesis were of analytical grade and dehydrated before use.

### Synthesis of 6-O-Dodecyl-Chitosan Carbamate

As shown in Scheme 1(a), to obtain the target CS derivative DCC, 2-*N*-phthaloyl-chitosan (PC, 2) was first synthesized according to a method developed by Kurita *et al.*[26] In brief, CS (1.0 g, 6.20 mmol) and phthalic anhydride (2.76 g, 18.60 mmol) were suspended in DMF (30 mL, containing 5% water). The mixture was heated to 120°C under magnetic stirring in $N_2$ atmosphere for 8 h. Then, the mixture was cooled to room temperature and poured into ice water. The resulting pale tan precipitate was collected by centrifugation, followed by thorough purification by Soxhlet extraction with ethanol and then drying in vacuum to obtain 0.8 g of PC.

Then, PC (0.5 g, 2 mmol) was dissolved in 20 mL dehydrated *N*-methyl-2-pyrrolidone (NMP) and then dropped slowly into 20 mL NMP solution containing 0.29 g CDI. After stirring at 50°C under $N_2$ atmosphere for 3 h, 20 mL NMP solution containing 0.1 g dodecyl amine was dropped into the reaction mixture and stirred at 40°C for another 3 h. NMP was evaporated in vacuum and the residue was washed with methanol and water. Finally, light yellow powders of 2-*N*-phthaloyl-6-O-dodecyl-chitosan carbamate (PDCC, 3) were got after drying in vacuum (0.6 g) [Scheme 1(b)].

To remove the *N*-phthaloyl group from PDCC (0.2 g), the product was stirred in hydrazine monohydrate and ethanol (v/v = 1/10) at 40°C for 18 h [Scheme 1(c)].[36] Then, the solvent was evaporated under vacuum, and the derived solid residues were suspended in water and dialyzed against water for 3 days and lyophilized. After Soxhlet extraction with ethanol and the followed vacuum drying, 0.1 g light pale powders of 6-O-dodecyl-chitosan carbamate (DCC, 4) product were obtained.

### Characterization of PC, PDCC, and DCC

The composition of the prepared products was analyzed by $^{13}C$-CP/MAS NMR (Bruker AVANCE III, 500 MHz), elemental

analysis (Vario EL, Germany), and FTIR (Shimadzu IR Prestige-21). $^{13}$C-CP/MAS NMR spectra were recorded at 25°C and $^{13}$C radio frequency field strength for cross polarization was set to 68 kHz. FTIR spectra were recorded on KBr pellets in the range from 4000 to 400 $cm^{-1}$ at atmosphere. The substitute degree (SD) of dodecyl group was analyzed by elemental analysis. To evaluate the degradability of DCC in acidic environment, DCC was dissolved in aqueous hydrochloric acid (pH = 5) and gently stirred in 37°C for 12 or 24 h. Then the solution was dialyzed against water, lyophilized, Soxhlet extracted with ethanol and dried in vacuum. The products were analyzed by elemental analysis and the degradation was identified by the SD changes after the hydrolysis.

### Preparation of DCC/pDNA and CS/pDNA Polyplexes

All polyplexes were freshly prepared before use and all the solutions were filtered through a 0.45-$\mu$m aseptic filter before mixing. CS and DCC solutions (0.1 mg/mL) were prepared by first dissolving CS and DCC in 0.1*M* acetic acid and then dialyzed against deionized water for 24 h.[30] The DCC or CS solution was added to pGFPN2 or pGL3 solution with gentle vortex, and then incubated at room temperature for 30 min to prepare polyplex. The weight ratio of polymer to pDNA was defined as N/P ratio.

### Gel Retardation Assay

The performance of DCC copolymer condensing pDNA was investigated by electrophoresis on a 1% agarose gel. Different amounts of DCC were combined with pDNA (0.1 mg of pGL3, N/P ratio varied from 1 to 10). After incubation for 30 min, the polyplexes were loaded on a 1% agarose gel with Tris–acetate (TAE) running buffer and subjected to electrophoresis at 90 V and 50 mA for 60 min. Fluorescence of the intercalated dye (ethidium bromide) was measured using a gel imaging system (Syngene).

### Particle size, Morphology and ζ-Potential Measurement

The particle size and morphology measurement was performed on transmission electron microscopy (TEM, JEM-2100 microscope, Japan), operated at an accelerating voltage of 200 kV. The TEM samples were prepared by depositing a diluted polymer or polyplex suspension on a carbon-coated copper grid, followed with air drying for 10 min and then negatively stained in 2 wt % uranyl acetate solution for 10 s.

The hydrodynamic diameters and ζ-potentials of CS, DCC polymic micelles and their polyplexes were measured on an electrophoretic light scattering spectrophotometer (DLS, Nano ZS 90, Malvern, UK), with 90 scattering angles at 25°C, in triplicate. The CS/pDNA and DCC/pDNA polyplexes were prepared in water at N/P ratio of 8. To evaluate the likelihood of anionic serum proteins complexing with the polyplexes, the hydrodynamic diameters of the polyplexes over time were also investigated in 10% serum by DLS.

### Cell Culture

Human lung adenocarcinoma cell line A549 were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in DMEM medium containing 10% fetal bovine serum and antibiotics (100 U penicillin/ml and 100 mg streptomycin/ml) in 100 mL culture flask at 37°C in a humidified atmosphere containing 5% $CO_2$. The cell confluence reached 80% before use.

### Cell Transfection

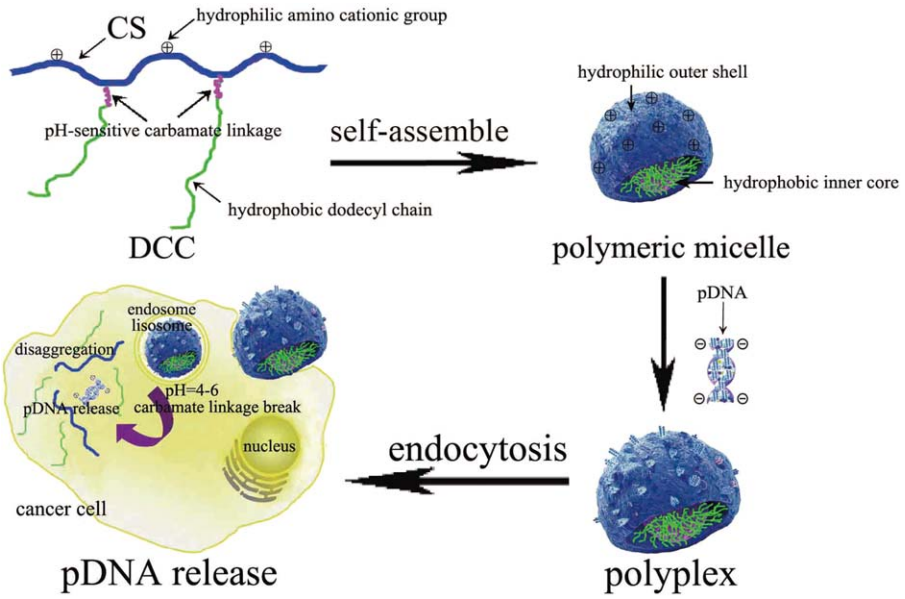
The transfection efficiency was measured by green fluorescence protein (GFP) fluorescent imaging and luciferase assay, pGFP-N2 and pGL3 plasmid pDNA used as reporter gene. One day before transfection, 0.5–2 $\times$ $10^4$ cells were seeded per well in 500 $\mu$L growth medium (DMEM) until the required cell number was obtained (80% confluence) at the time of transfection. All polyplexes were prepared as mentioned above. The weight ratio of DCC to DNA varied from 1 to 10. The polyplexes were added to the 24-well plate and incubated for 5 h in DMEM without serum and antibiotics at 37°C under 5% $CO_2$ atmosphere. Then, the DMEM was replaced with the fresh DMEM with 10% serum. After 48 h, the growth medium was removed from each well, and luciferase activity was measured after the lysis buffer was added into each well of 96-well plate and incubation for 5 min at room temperature. The protein concentrations of cell lysates per well were determined using BCA protein assay reagent, and the transfection efficiency can be obtained as the relative luciferase activity. Data were expressed as relative light units (RLU) per mg protein. PEI-25 was used as control.

### MTT Assay

Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay method. For cell viability assay, A549 cells were seeded into a 96-well plate at a density of 5 $\times$ $10^3$ cells/well. After incubated for 12 h, all polyplexes prepared as mentioned above were added with 100 $\mu$L serum-free DMEM medium to replace the culture medium. After incubation at 37°C under 5% $CO_2$ atmosphere for 24 h, 20 $\mu$L of MTT solution (5 mg/mL) was added to each well and incubated for another 4.5 h. Then, the cultured media was replaced by 150 $\mu$L DMSO and then the optical density (OD) was measured on an enzyme microplate reader (Tecan Sunrise) at 570 nm. The cell viability was calculated as following: cell viability (%) = OD570 (sample)/OD570 (control) $\times$ 100, where OD570 (sample) represented OD measured from the group treated with polyplexes, and OD570 (control) represented OD measured from the group treated with DMEM medium only. All the experiments were performed in triplicate to ascertain the reproducibility.

## RESULTS AND DISCUSSION

A novel pH-sensitive amphiphile (DCC) was synthesized, which could self-assemble into pH-responsive polymeric micelles in aqueous solutions. By taking advantage of the pH-responsiveness of DCC polymeric micelles and the pH difference between the endosomes/lysosomes and the environment in cancer cells, DCC polymeric micelles could combine with pDNA before entering into cancer cells and release it after encapsulating in endosomes/lysosomes. As illustrated in Scheme 2, the pH-sensitive carbamate bonds of DCC broke in the acidic endosomes/lysosomes, resulting in the decomposition of DCC polymeric micelles and the release of pDNA.




**Scheme 2.** Illustrative self-assembled DCC polymeric micelles for pDNA encapsulation and the intracellular pH-responsive release of pDNA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### Synthesis and Characterization of DCC

The synthesis route of DCC from CS was shown in Scheme 1. First, the amino groups of CS were protected by *N*-phthaloylation.[26] Then CDI, an efficient and environment-friendly coupling agent,[32] was added to couple hydroxyls of PC with dodecyl amine via carbamate bonds to form PDCC. Dodecyl group was used as the hydrophobic portion because of its high efficiency in gene delivery when used as a lipid-like modifier of vectors.[25,37] Finally, the phthaloyl group was removed and amino group was recovered by hydrazine reduction to get DCC.[36]

Figure 1 shows the solid-state NMR results of CS and its derivatives by $^{13}$C-CP/MAS NMR method. The carbon backbone of CS was observed at $\delta$ 105.1 (C1), $\delta$ 57.6 (C2), $\delta$ 75.1 (C3, C5), $\delta$ 83.0 (C4), and $\delta$ 60.2 (C6). Two additional $^{13}$C peaks observed at $\delta$ 173.0 and $\delta$ 23.6 were assigned to the carbonyl and methyl groups, which are typically present in CS's skeleton due to uncompleted deacetylation (deacetylation degree = 85%, as mentioned in specification). After modifying CS with phthalic anhydride, PC was obtained [Scheme 1(a)]. The structure of PC could be readily confirmed by the observation of $^{13}$C resonances of the *N*-phthaloyl substituent at $\delta$ 168.2 (C7', C8'), $\delta$ 133.9–122.7 (C1'-C6'), respectively [Figure 1(2)].[36] After coupling PC with dodecyl amine by CDI, the PDCC formation was confirmed by the $^{13}$C peaks of dodecyl at $\delta$ 14.2–40.4 and carbamate groups at $\delta$ 156.0 [Figure 1(3)], respectively. The NMR spectra further identified that PDCC was converted into DCC through dephthaloylation by hydrazine hydrate, as clearly shown

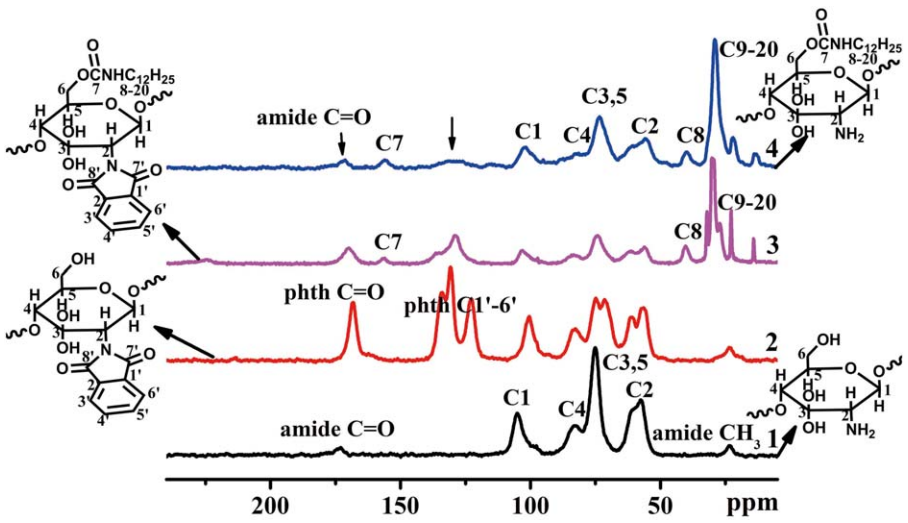



**Figure 1.** $^{13}$C-CP/MAS NMR spectra of (1) chitosan (CS), (2) 2-*N*-phthaloyl-chitosan (PC), (3) 2-*N*-phthaloyl-6-O-dodecyl-chitosan carbamate (PDCC), and (4) 6-O-dodecyl-chitosan carbamate (DCC). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

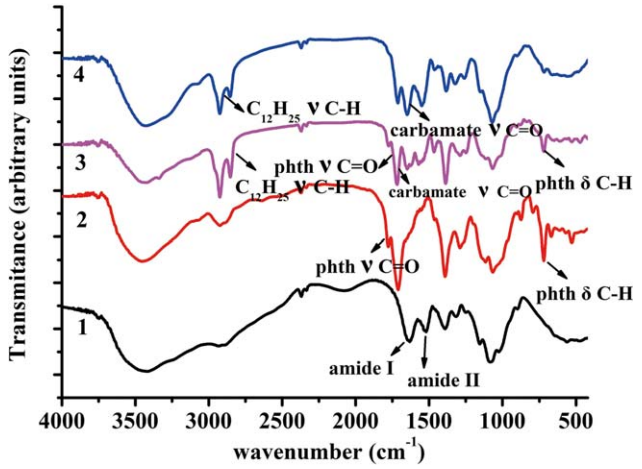



**Figure 2.** FTIR spectra (KBr pellets) of (1) chitosan (CS), (2) 2-*N*-phthaloyl-chitosan (PC), (3) 2-*N*-phthaloyl-6-O-dodecyl-chitosan carbamate (PDCC), and (4) 6-O-dodecyl-chitosan carbamate (DCC). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

by the disappearance of $^{13}C$ signal at $\delta$ 122.7–133.9, which belonged to the typical $^{13}C$ resonances of the *N*-phthaloyl substituent [Figure 1(4)].

FTIR spectra also clarified the structures of PC, PDCC, and DCC. As shown in Figure 2, the broad intense bands at 3450–3200 $cm^{-1}$ were assigned to $v_{OH}$ and $v_{NH}$ vibrations of CS and its derivatives. In Figure 2(1), characteristic IR absorption of amide groups appeared at 1656 $cm^{-1}$ (amide I) and 1597 $cm^{-1}$ (amide II), respectively. The $v_{C\text{-}O}$ of the glucopyranose rings appeared at 1032 $cm^{-1}$. Compared with CS, the IR spectrum of PC [Figure 2(2)] showed two intense additional peaks at 1778 and 1709 $cm^{-1}$, which corresponded to the characteristic $v_{C=O}$ vibrations of the phthalimide groups grafted. IR fingerprint characteristic absorption of aromatic rings at 720 $cm^{-1}$ ($\delta_{CH}$) further confirmed the structure of PC. The observations agreed with the data reported by Kurita *et al.*[26] The IR spectrum of PDCC [Figure 2(3)] exhibited some evidently new peaks at 2923, 2862 ($v_{CH}$ vibrations of aliphatic chain), and 1530 $cm^{-1}$ ($v_{C=O}$ vibrations of carbamate group), which suggested that dodecyls were coupled with PC through carbamate groups. After deprotecting the primary amines by dephthaloylation of PDCC, the peaks at 720 $cm^{-1}$ (IR fingerprint characteristic absorption of aromatic rings), 1778 and 1709 $cm^{-1}$ ($v_{C=O}$ vibrations of phthalimide groups) disappeared in [Figure 2(4)], demonstrating the formation of DCC.

The elemental analysis results are listed in Table I. The SD was calculated by comparing the molar ratio of C to N obtained from elemental analysis in each derivative, referring to the method described in the literature.[38] One molecule of monosaccharide contains one nitrogen atom. Therefore, the increase in the molar ratio of C/N indicates the increasing carbon content in monosaccharide of CS chain. In the case of DCC derivative, dodecyl group contains 13 carbon atoms and 1 nitrogen atom. Accordingly, the SD of dodecyls can be calculated to be 14% according to eq. (1).

**Table I.** Elemental Analyses and the Substitution Degree (SD) of DCC

| | Content % | | | |
|---|---|---|---|---|
| | C | N | C/N[a] | SD (%) |
| CS | 23.64 | 4.08 | 6.27 | - |
| DCC | 28.83 | 4.40 | 7.1 | 14 |
| DCC[b] (12h) | 26.07 | 3.79 | 6.87 | 9.2 |
| DCC[c] (24h) | 25.36 | 3.81 | 6.66 | 5.7 |

[a] The molar ratio of carbon to nitrogen.
[b] Degradation in aqueous hydrochloric acid (pH = 5) for 12 h.
[c] Degradation in aqueous hydrochloric acid (pH = 5) for 24 h.
CS, chitosan; DCC, 6-O-dodecyl-chitosan carbamate.

$$SD = \frac{x - 6.27}{13 - x} \times 100\% \quad (1)$$

Where SD is the substitute degree, 6.27 is the ratio of C to N in CS, 13 is ratio of C to N in $C_{12}H_{25}NHCO$- grafts, and $x$ is the ratio of C to N in DCC.

The degradability of DCC in acidic environment was tested in hydrochloric acid solution (pH = 5), and reflected by the change of SD after the degradation. After degradation in the acidic solution for 12 and 24 h, the SD of DCC decreased from the initial value of 14 to 9.2% and 5.7% (Table I), respectively. This indicates that DCC could dissociate, which should be ascribed to the hydrolysis of carbamate bonds in the acidic environment. Thus, the strategy of controlled release by taking advantage of DCC degradation in the acidic environment of endosomes/lysosomes would be valid.[32,33]

### Characterization of DCC Polymeric Micelles, DCC/pDNA, and CS/pDNA Polyplexes

CS and DCC solutions (0.1 mg/mL) were prepared by dissolving CS and DCC in 0.1*M* acetic acid first, and then dialyzed in deionized water for 24 h. The polyplexes were freshly prepared before use by adding DCC or CS solution to pGFP-N2 or pGL3 solution and then incubating at room temperature for 30 min. The polyplexes were characterized with electrophoretic retardation on a 1% agarose gel. It was known that once the negatively charged pDNA complexed with the positively charged vector, the migration of pDNA in electric field toward the anode will be retarded due to the charge neutralization. Figure 3(a) shows

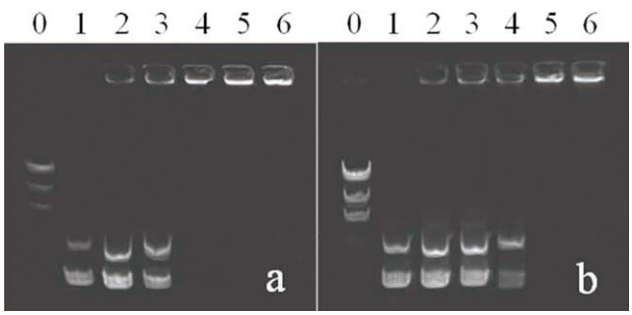



**Figure 3.** Electrophoresis of CS/pDNA (a) and DCC/pDNA (b) polyplexes on agarose gels. Lane 0: Mar k DNA; lanes 1–6: N/P = 1, 2, 4, 6, 8, 10, respectively.

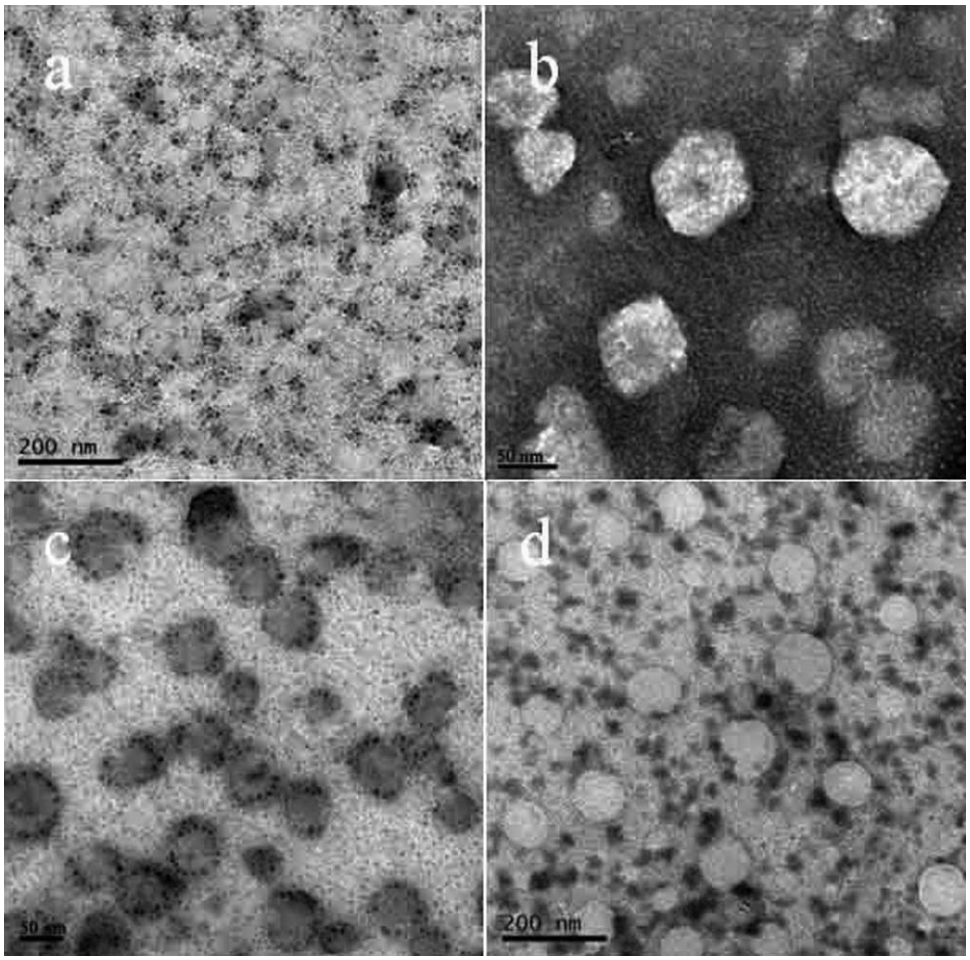
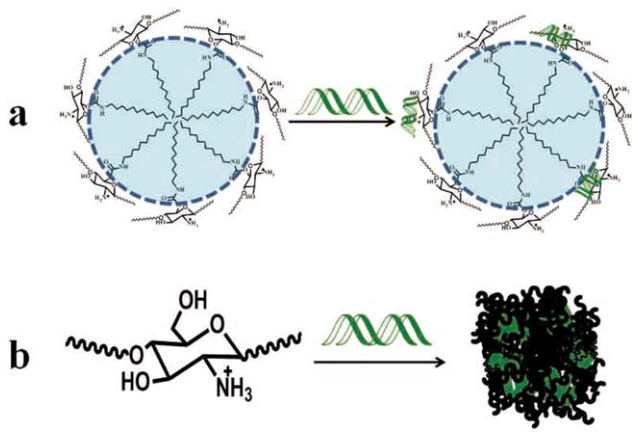



**Figure 4.** TEM images of polyplexes: CS (a), CS/pDNA (N/P = 8/1) (b), DCC (c), and DCC/pDNA (N/P = 8/1) (d).

that the complete retardation occurred at the N/P ratio of 6 for the CS/pDNA polyplex. For DCC, the polyplex formed at a higher N/P ratio of 8 [Figure 3(b)]. This indicates that DCC had a weaker binding affinity to pDNA than CS.[39] It was reported that the too strong binding affinity between CS and pDNA hindered the intracellular release of pDNA and resulted in low transfection efficiency.[38] The O-hydrophobic modification to CS weakened the binding affinity to pDNA and would benefit the transfection.

TEM images of CS and DCC are shown in Figure 4(a–c), respectively. There were no self-assembled particles of CS in the TEM image.[40] In contrast, DCC self-assembled into polymeric micelles as shown in Figure 4(c). Figure 4(b–d) shows the morphology of CS/pDNA and DCC/pDNA polyplexes, respectively. Both of the polyplexes appeared in quasi-spherical shape. The nanoparticles of CS/pDNA polyplexes were formed by electrostatic interaction and entanglement,[41,42] as shown in Scheme 3(b). However, DCC self-assembled into polymeric micelles in the size of about 50 nm.[21,22] When DCC polymeric micelles complexed with pDNA, the particle size increased to about 100 nm, which was remarkably larger than the size of 60 nm of CS/pDNA. As elucidated in Scheme 3(a), DCC polymeric micelles complexed with pDNA by only the electrostatic interaction, due to opposite charges on their surface.[30] Therefore, the binding affinity between DCC and pDNA was weaker




**Scheme 3.** Proposed complexation scheme of DCC/pDNA (a) and CS/pDNA (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Table II.** Hydro-sizes and Zeta-potentials of CS/pDNA, DCC/pDNA, CS, and DCC Measured by Dynamic Light Scattering (DLS) Method

| | Hydro-sizes (nm) | Zeta potential (mV) | PDI |
|---|---|---|---|
| CS | — | — | — |
| DCC | 113 | 42.6 | 0.427 |
| CS/pDNA[a] | 176 | 26.2 | 1.115 |
| DCC/pDNA[a] | 237 | 31.7 | 0.643 |

[a] The N/P ratio of polyplex equal to 8.
CS, chitosan; DCC, 6-O-dodecyl-chitosan carbamate; PDI, polydispersity index.

than that of CS and pDNA, resulting in a larger particle size of DCC/pDNA than that of CS/pDNA.

The sizes and ζ-potentials of DCC polymeric micelles and the polyplexes were measured by DLS (Table II). The results of DLS further confirmed the formation of self-assembled DCC polymeric micelles, in contrast to the absence of polymeric micelles of CS. The sizes of DCC polymeric micelles, CS/pDNA and DCC/pDNA were 113, 176, and 237 nm, respectively. The hydrodynamic diameter increased in an order: DCC < CS/pDNA < DCC/pDNA, which was consistent with the results of TEM. The larger size of DCC/pDNA indicated a weaker binding force between DCC and pDNA than that of CS/pDNA, which would benefit the transfection. The ζ-potential of DCC polymeric micelles was 42.6 mV (Table II) and arising from the positive amino groups which headed out of polymeric micelles of DCC (as shown in Scheme 3(a)).[21,43] When the positive DCC polymeric micelles bound negative pDNA, the ζ-potential of DCC/pDNA polyplexes was decreased to 31.7 mV due to the electrical neutralization. For CS, the electrical attraction and the entanglement effects were both involved in the formation of CS/pDNA polyplexes [Scheme 3(b)], resulting in a smaller size and a lower ζ-potential.

The stability of the polyplexes in 10% serum was investigated by DLS over a time period of 60 min, as shown in Figure 5. The results showed that the hydro-sizes of the DCC/pDNA polyplexes slightly increased by 7% in the first 10 min, then slowly increased by 10% in the following 50 min. The hydro-sizes of the CS/pDNA polyplexes nearly unchanged over a 60-min tested time period. It could be concluded that there was no significant aggregation of both the polyplexes in 10% serum in the tested 60 min and that DCC polymeric micelles were suitable for gene transfection in the presence of serum.

### Cellular Delivery of pDNA by DCC Polymeric Micelles

pGFP-N2 and pGL3 were used as report genes to evaluate the gene delivery efficiency of DCC polymeric micelles. Figure 6

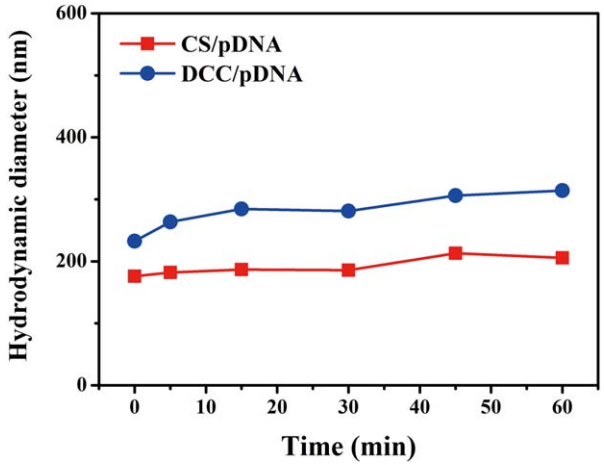



**Figure 5.** Time-dependent hydro-sizes of DCC/pDNA and CS/pDNA polyplexes in 10% serum. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

shows the GFP fluorescent images of A549 cells transfected for 24 h by CS/pDNA and DCC/pDNA polyplexes at various N/P ratios, using PEI-25 as control (N/P = 10). The fluorescence density and intensity of cells transfected by DCC polymeric micelles were notably stronger than those of cells transfected by CS, and comparable to that of PEI-25. The results demonstrated that the modification of CS by our synthesis strategy effectively improved the gene delivery ability.

Figure 7 presents the pGL3 transfection results of DCC/pDNA and CS/pDNA polyplexes in A549 cells at different N/P ratios, with PEI-25 as control. In the N/P ratio range of 1–10, the transfection efficiency of DCC/pDNA polyplex consistently surpassed that of CS/pDNA polyplex. The transfection efficiency increased with the N/P ratio from 1 : 1 to 8 : 1, and leveled off at a higher N/P ratio of 10. At N/P ratio of 8, the transfection efficiency of DCC/pDNA polyplex reached 45,000 RLU/mg, which is 2.5 folds of that of CS/pDNA at the same N/P ratio and comparable to that of PEI-25/pDNA polyplex (N/P = 10). For CS/pDNA, the difficulty in endosomal escape and pDNA release resulted in low transfection efficiency of CS.[25,40] In contrast, after the modification, the dodecyl chains of DCC enhanced the membrane disturbance and thus promoted the endocytosis. Concurrently, the well reserved amino groups of DCC benefited the penetration and also promoted the endocytosis of the polyplex. Once the DCC/pDNA polyplex entering into the cell, the pH-sensitive carbamate bonds of DCC would be broken in the acidic environment of endosomes and lysosomes. The partially degraded DCC polymeric micelles were more readily to release pDNA, which was regarded as the rate

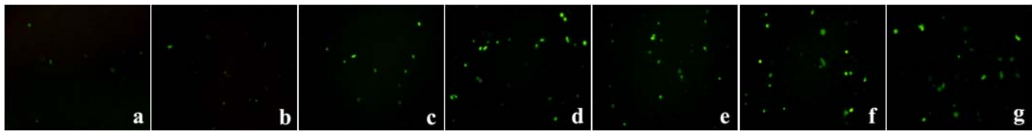



**Figure 6.** Representative fluorescence images of A549 cells exposed to CS/pDNA at N/P = 6 (a), CS/pDNA at N/P = 8 (b), CS/pDNA at N/P = 10 (c), DCC/pDNA at N/P = 6 (d), DCC/pDNA at N/P = 8 (e), DCC/pDNA at N/P = 10 (f), and PEI-25/pDNA at N/P = 10 (g) polyplexes after transfection of 24 h, pGFP-N2 as report gene. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

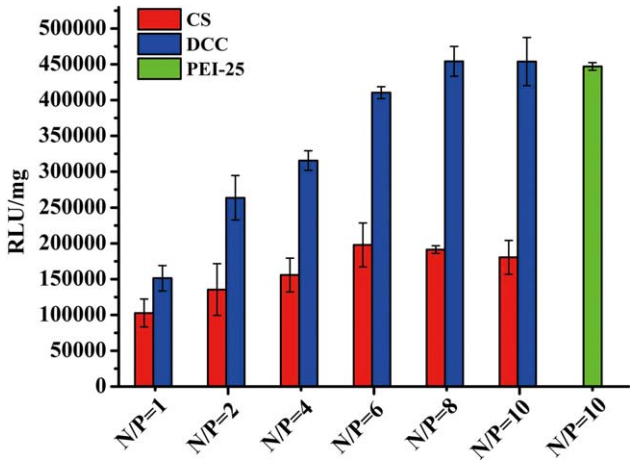



**Figure 7.** pGL3 expression of CS/pDNA and DCC/pDNA polyplexes in A549 cells, PEI-25 as control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

determining step in the CS-mediated gene delivery.[44] The unoccupied amino groups of DCC could effectively condense pDNA and afford the proton sponge effects for the osmotic bursting of endosomes, which benefited the transfection efficiency. The binding affinity between DCC polymeric micelles and pDNA was weaker than that of CS and pDNA, as clarified by the electrophoresis on agarose gels. This could promote the cellular pDNA release from the polyplexes. The multiple advantages afforded by the amino-rich, pH-sensitive carbamate bonds and dodecyl moiety jointly contributed to the transfection efficiency of DCC, which was much higher than that of CS.

### Cytotoxicity of DCC/pDNA Polyplex

The results of cytotoxicity for DCC and CS polyplexes at different N/P ratios are shown in Figure 8. It is well known that CS has good biocompatibility and low cytotoxicity. Even the N/P ratio was as high as 10, the cell viability of CS/pDNA polyplex was still above 80%. After the modification, the DDC presented low cytotoxicity similar to that of CS, with the cell viability of DCC/pDNA polyplex close to that of CS/pDNA polyplex when N/P ratios varied from 1 to 10. In contrast, the cell viability of PEI-25/pDNA polyplex was only 33% at the N/P ratio of 1 and further decreased to 15% at the N/P ratio of 10. Evidently, both DCC and CS had much lower cytotoxicity than PEI-25. The dodecyl moiety of DCC had nearly no negative effect on the biocompatibility of DCC. It can be concluded that as a derivative of CS, DCC is a biocompatible vector with low cytotoxicity and could serve as a safe candidate for the gene delivery.

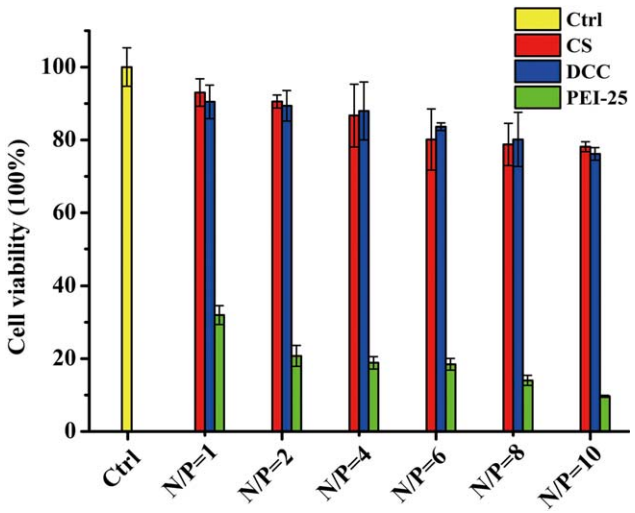



**Figure 8.** Cytotoxicity of CS/pDNA and DCC/pDNA polyplexes in A549 cells by MTT methods, PEI-25/pDNA as positive control. Untransfected cells as control (Ctrl) and the cell viability value set as 100%. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

## CONCLUSIONS

A novel pH-responsive and amino-rich gene vector DCC was synthesized and tested for gene delivery. By grafting dodecyl onto the primary hydroxyl groups of CS in a controlled way, amino groups were well retained in DCC, which was confirmed by the characterizations of $^{13}$C-CP/MAS NMR, FTIR and elemental analysis. DCC self-assembled into polymeric micelles and bonded pDNA to form DCC/pDNA polyplex by the electrostatic interaction. The weak binding force between DCC and pDNA benefited the intracellular release of pDNA and enhanced the gene transfection. The carbamate bonds linking dodecyl and hydroxyl of CS can be degraded in acidic environment of endosomes/lysosomes, endorsing DCC the pH-responsive property and accordingly benefiting the intracellular pDNA release and transfection. The dodecyl modification, the pH-sensitive carbamate and the unoccupied amino groups in DCC were all proposed to be responsible for the excellent performance in gene transfection. Therefore, the gene transfection efficiency of DCC was much higher than that of CS and comparable to that of PEI-25. The cytotoxicity of DCC/pDNA polyplex was as low as that of CS/pDNA polyplex and much lower than that of PEI-25, suggesting that DCC could be a promising nonviral gene vector for clinical applications in the future.

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