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Synthesis and preliminary cellular evaluation of phosphonium chitosan derivatives as novel non-viral vector

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ABSTRACT

In this study, *N*-phosphonium chitosans (NPCSs) with two degrees of substitution were synthesized in a homogeneous system as nonviral gene vectors. Grafted polymer/DNA complexes at various charge ratios were formulated and characterized. Particle sizes of NPCS/DNA complexes were between 110 and 160 nm as determined by dynamic light scattering. Accordingly, scanning electron microscopy photo of NPCS/DNA complexes exhibited a compact morphology. Zeta potentials of these complexes changed as the charge ratio and pH varied. The cytotoxicity assay showed that NPCS polymers were less toxic than branched PEI-25K. Furthermore, gene transfection efficiencies of NPCS/DNA complexes showed that the gene transfection ability of the grafted polymer was much better than chitosan and NPCS with the degree of substitution of 21.5% had comparative gene transfection efficiency to branched PEI-25K. Together, these results suggest that the low toxic NPCS grafted polymers could be used as effective gene delivery vectors.

1. Introduction

Gene therapy is a promising way for curing various genetic diseases (De Luca, Pellegrini, & Mavilio, 2009; Kohn & Candotti, 2009) as well as cancers (Brannon-Peppas & Blanchette, 2012; Gomez-Navarro, Curiel, & Douglas, 1999; Kochanek & Gansbacher, 2010) by delivering genes to specific cells or organs. Successful clinical application of gene therapy requires safe and high efficient gene delivery vectors (Midoux, Pichon, Yaouanc, & Jaffrès, 2009; Sun & Zhang, 2010). Non-viral gene vectors have gained increasing attention as alternatives to viral vectors, since they offer advantages of minimal immune response, stable in storage and ease of large-scale production (Park, Jeong, & Kim, 2006; Srinivas, Samanta, & Chaudhuri, 2009). Among numerous non-viral systems, cationic polymers are widely accepted because of their high ability to form polyelectrolyte complexes with plasmid DNA through charge interaction and self-assembling (Pack, Hoffman, Pun, & Stayton, 2005). To date, cationic gene delivery polymers, including poly(ethylenimine) (PEI) (Zhou et al., 2011), poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) (Mathew, Cao, Collin, Wang, & Pandit, 2012), gelatin (Zorzi, Párraga, Seijo, & Sánchez, 2011) and poly(L-lysine) (PLL) (Zhang, Ma, Su, & Benkirane-Jessel, 2011), have been broadly studied. However, their high cytotoxicity and low biodegradability lead to a risk that these polymers might be accumulated in cells or body, particularly, after repeated administration (Gao, Kim, & Liu, 2007).

Chitosan, a natural linear cationic polysaccharide derived from chitin, is considered as a good candidate for gene delivery vector due to its biocompatibility, biodegradability, low toxicity and high potential ability to complexate DNA (Chopra et al., 2006; Di Martino, Sittinger, & Risbud, 2005). However, in spite of these excellent characteristics, the poor water-solubility and low gene transfection efficiency of chitosan limited its application. It has been suggested that the low transfection efficiency was attributed to the strong interactions between chitosan and DNA (Köping-Höggård et al., 2001; Ross, 2001), resulting in highly stable particles, thereby preventing dissociation within the cell and ultimately precluding translation of the DNA. To improve gene transfection efficiency, a number of chemically modified chitosan derivatives were synthesized in order to obtain desirable physicochemical characteristics (Kievit et al., 2009; Strand et al., 2010; Wang, He, Tang, & Yin, 2011). Although the results showed that the transfection efficiency of the modified chitosan based vectors exhibited superior properties to that of chitosan, further investigations on improved gene transfection efficiency and the structure–behavior relations are still required for the practical application.

In this study, quaternary phosphonium chitosan derivatives with two different *DS* were synthesized and characterized using ^{1}H NMR and FT-IR. A series of polymer/DNA complexes with different charge ratios were prepared and their physicochemical properties such as particle sizes, zeta potentials and plasmid DNA-binding abilities were investigated. Morphology of the lyophilized complex was investigated with SEM. And cytotoxicity of polymers was

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evaluated using MTT assay. A number of variables influencing in vitro gene transfection efficiency such as polymer/DNA charge ratio, degree of substituted phosphonium, pH of culture medium were also investigated.

2. Materials and methods

2.1. Materials

Chitosan with a viscosity-average molecular weight of 150 kDa and 93% degree of deacetylation (DD) was purchased from Zhejiang Yuhuan Ocean Biochemistry Co., Ltd. (5-Carboxypentyl) triphenylphosphonium bromide (CTPB) was purchased from Gracia Chemical Technology Co., Ltd. (Chengdu, China) with a purity of 98%. 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) and 1-hydroxybenzotrizole (HOBt) were purchased from Shanghai Medpep Co., Ltd. Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Beijing Solarbio Science & Technology Co., Ltd. DMEM was obtained from GIBCO (Grand Island, NY, USA). Other chemicals used were of analytical grade. All chemicals were used without further purification. The pEGFP-N1 plasmid DNAs were a kind gift from Professor Tuo Jin, and then were extracted from *E. coli* and purified with a NucleoBond® Xtra Maxi kit.

2.2. Methods

2.2.1. Synthesis and characterization of N-phosphonium chitosan (NPCS)

NPCS was synthesized according to our previous study (Wang, Xu, Guo, Peng, & Tang, 2011) with a little modification. Briefly, chitosan (0.4 g, 2.44 mmol of glycosyl units) was stirred with two equivalents of HOBt (0.664 g, 4.88 mmol) in 30 mL of H_2O/DMSO (v/v = 2/1) mixture overnight at 15 °C. A certain amount of CTPB in H_2O/DMSO (v/v = 2/1) was added to the chitosan solution followed by the addition of EDC·HCl in DMSO (0.705 g, 4.88 mmol). The reaction was carried out at 15 for 24 h. The mixture was precipitated in diethyl ether/acetone (v/v = 1/2), followed by dialyzing (MWCO = 3500) and lyophilizing to obtain the product NPCS. The degree of substitution (*DS*) of CTPB to monosaccharide residue of chitosan was calculated based on ^{1}H NMR spectra, which was obtained using a MERCURY plus 400 spectrometer. Fourier-transformed infrared (FT-IR) spectra of chitosan and NPCS were recorded on EOUINOX55 spectrophotometer (Bruker, Germany). Samples were prepared as KBr pellet and were scanned against a blank KBr pellet background at wavelength range of 4000–400 cm^{-1} with resolution of 4.0 cm^{-1}.

2.2.2. Preparation of polymer/DNA complexes

Chitosan/DNA, NPCS/DNA complexes were prepared at various charge ratios (N/P), which were expressed as the molar ratio of the amine groups in chitosan or its derivatives to the phosphate groups in DNA molecules. First, DNA solution of 20 μg/mL was prepared in deionized water. NPCS was dissolved in 15 mM sodium acetate buffer (pH 5.5). Chitosan was first dissolved in 0.5% acetic acid, and then adjusted to pH 5.5 with NaOH. Equal volumes of DNA and polymer solutions were mixed with the final N/P from 1/1 to 16/1. The mixtures were vortexed for 30 s then incubated at 37 °C for 60 min prior to further analyses.

2.2.3. Complex particle size and zeta potential measurements

The hydrodynamic diameters of complexes were measured by dynamic laser scattering (DLS), using a Malvern Zetasizer Nano-S with scattering angle of 173° at 25 °C. The zeta potential of complexes were measured by Particle Size Analyzer (90 Plus Model, Brookhaven Instruments) at 25 °C. All the analyses were run in triplicate.

2.2.4. Morphology study

The polymer/DNA complexes were prepared according to the conditions described above. 100 μL of complex suspension was deposited onto a silicon slide. The lyophilized samples were coated with gold for 1 min before observing the morphology of complexes with a scanning electron microscope (SEM, JEOL, Tokyo, Japan).

2.2.5. Gel electrophoresis assay

20 μL polymer/DNA complexes were prepared as described above before the adding of loading buffer. Then, the samples were applied to a 1% agarose gel in TAE buffer (40 mM Tris/HCl, 1 mM EDTA, pH 7.4) containing 0.6 μg/mL of ethidium bromide at 90 V for 25 min. DNA retardation was observed by irradiation with UV light (Tanon 2500, Tanon Science and Technology Co., Ltd., Shanghai, China).

2.2.6. MTT assay for polymer cytotoxicity

HEK 293 and HeLa cells were seeded in the 96-well plate at a density of 8000 cell/well and grown in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. The growth medium was replaced with 200 L complete DMEM culture medium that contained desired amount of the test polymers. Incubations were made for 4 h before removal of media containing polymers and then MTT solution (0.5 mg/mL final concentration) in serum-free DMEM medium was added to each well and incubated for 4 h under normal growing conditions. Afterwards, the medium was removed and 200 L DMSO was added. The plate was mildly shaken for 10 min to ensure the complete dissolution of formazan. The absorbance was measured at 570 nm using an ELISA plate reader (Varioskan Flash). The relative cell viability was calculated as: cell viability (%) = $(OD_{sample}/OD_{control}) \times 100$, where untreated cells were considered as control. Each value was averaged from 4 independent experiments.

2.2.7. In vitro gene transfection

HEK293 and HeLa cells were seeded in 24-well plates at a density of 5×10^4 cells/well in 1 mL of complete medium and incubated for 24 h at 37 °C in 5% CO_2. When the cells were grown to the 70–80% confluence, the culture medium was replaced with 1 mL medium containing 200 μL of complexes (different N/P ratios of polymer/DNA complexes). EGFP-N1 was used as the reporter plasmid to assay the transfection efficiency. After incubation for 4 h, the medium was replaced with fresh complete medium and the cells were incubated for another 48–72 h (post-transfection time). The cells were then analyzed for green fluorescence protein expression with a fluorescence microscope (Ti-U, Nikon, Japan). Finally, cells were collected and resuspended in PBS (pH 7.4). The EGFP expression levels were quantified by using the flow cytometer (FACSCalibur, BD, USA) in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm and analyzed with CellQuest software. A total of 10,000 events were collected for each sample.

3. Results and discussion

3.1. Synthesis and characterization of NPCS

Chitosan is an important biodegradable material for a variety of biological applications, but the poor water-solubility and low gene transfection efficiency of chitosan limited its application. To improve the transfection efficiency of chitosan, we established a procedure to synthesize phosphonium grafted chitosan polymers under homogeneous condition. By varying the feed ratio of CTPB

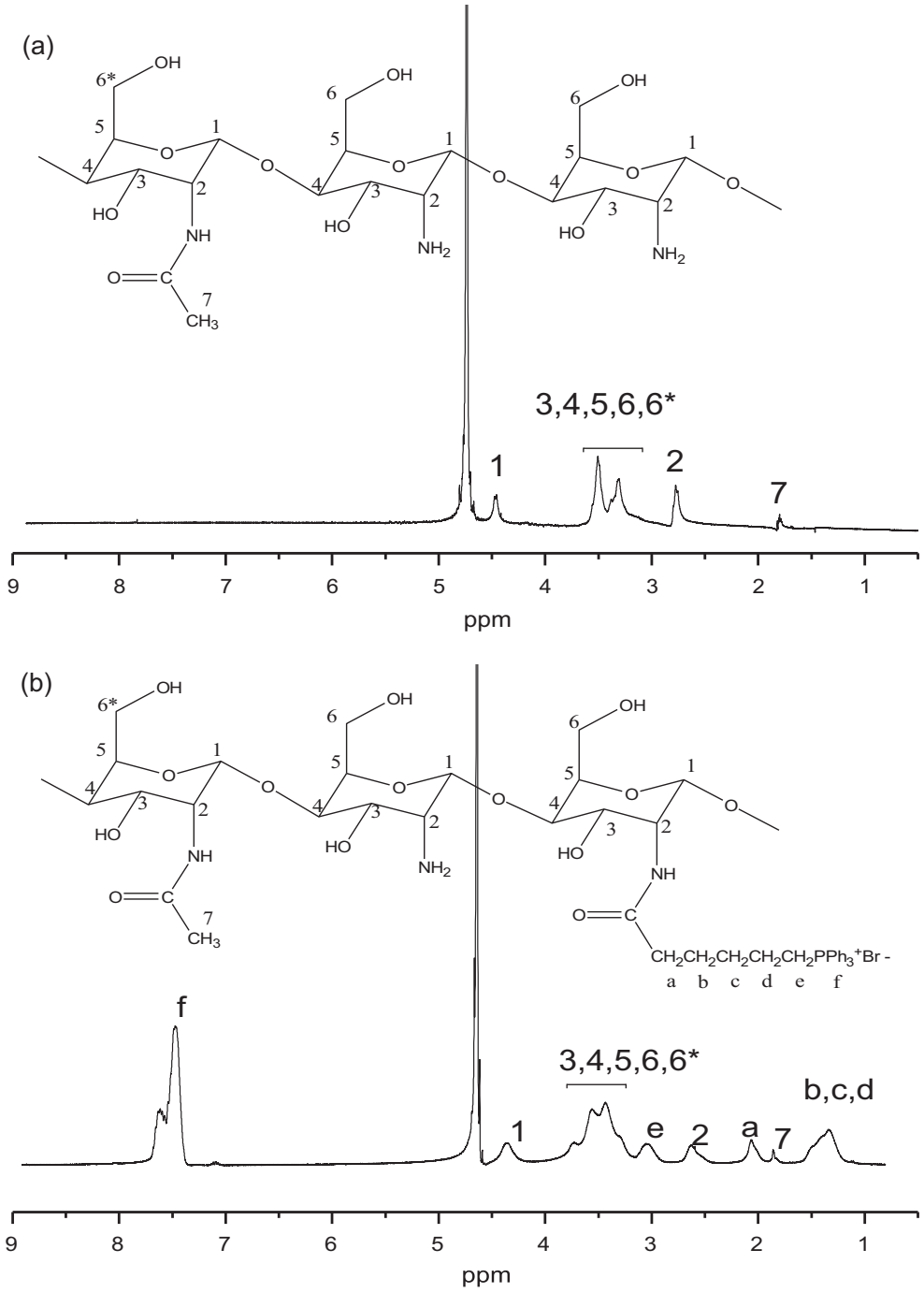

Fig. 1. ^{1}H NMR spectra of (a) chitosan, (b) NPCS (*DS* = 21.5%).

to chitosan in the reaction solution, we synthesized grafted polymers with different degrees of substitution. The chemical structure of the prepared NPCS polymers was examined by ^{1}H NMR spectra. As shown in Fig. 1, all the peaks appeared in the ^{1}H NMR spectra were consistent with the target polymer structure (Wang, Xu, et al., 2011). The phosphonium grafting ratios were determined by comparing the integration areas of peak f (assigned to benzene rings of CTPB, 15 protons) and peak 7 (assigned to the methyl group of *N*-acetyl-glucosamine unit of chitosan, 3 protons). Table 1 shows that as the feed molar ratio of CTPB to chitosan increased, higher molar degree of substitution (*DS*) of NPCS were synthesized, but it was not linearly dependent. However, a higher *DS* of more than 20% was hard to obtain (data not shown), which might be related to the introduction of triphenylphosphonium group that has three large benzene groups with very strong steric hindrance. As it can be seen in FT-IR measurement (Fig. 2), there is an increase of transmittance intensity at 1642 cm^{-1} and 1387 cm^{-1} (belonging to the benzene

Table 1
Degrees of substitution (*DS*) of N-phosphonium chitosan (NPCS).

Sample	Feed molar ratio (chitosan/CTPB)[a]	*DS*[b]
NPCS1	1/0.5	12.1%
NPCS2	1/1	21.5%

[a] Molar ratio was defined as the glycosyl units of chitosan to CTPBs.
[b] *DS* was calculated from ^{1}H NMR data.

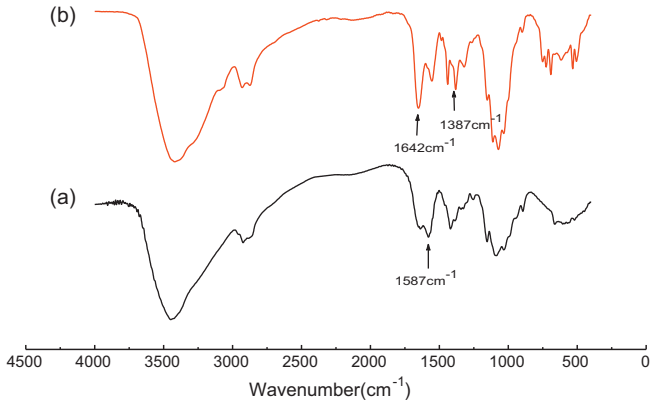

Fig. 2. FT-IR spectra of (a) chitosan, (b) NPCS (*DS* = 21.5%).

rings in CTPB) and decrease of transmittance intensity at 1587 cm^{-1} (belonging to the N-H bending vibration of primary amines in chitosan backbone). In addition, there were no absorption bands of ester groups around 1740 cm^{-1} for NPCS. These indicated that CTPB were successfully coupled to chitosan and the phosphonium groups were mainly introduced to the amino groups of chitosan.

3.2. Particle size, zeta potential and morphology investigations

In order to transfer plasmid DNA efficiently into the target cells with polycationic vectors, it is of great importance to prepare complexes with suitable particle size, surface charge and DNA condensation ability. Particle size was determined by means of dynamic light scattering (DLS) and shown in Table 2. It was found that complexes prepared from chitosan, NPCS1, NPCS2 were all smaller than 200 nm, and the particle sizes tended to decrease with the increase of N/P ratio. The NPCS complexes showed particle size ranging from 110 to 160 nm, which were smaller than that of chitosan at certain N/P ratios. As the medium pH changed from 6.5 to 7.4, all particle sizes increased more or less, however, the increase of chitosan complexes were bigger than that of NPCS complexes. PEI-25K produced the smallest complexes with diameters around 80 nm due to its strong DNA binding and compacting ability and showed negligible increase in diameters as the medium pH increased from 6.5 to 7.4. Positively charged surface could benefit the cell membrane binding and intracellular uptake of complexes, as cells in general are characterized by slightly negative charge. Zeta-potential measurement was applied to determine the surface charge of the complexes. As shown in Table 2, all complexes were positively charged, and the absolute values were above 25 mV at pH 6.5. The zeta potential values changed few as the N/P varied. As the pH was adjusted to 7.4, only chitosan/DNA, NPCS/DNA at N/P of 16/1, NPCS2/NDA at N/P of 8/1 and PEI/DNA at N/P of 10/1 had the zeta potential values above 25 mV, other complexes at low N/P showed a comparatively big decrease in zeta potential values. This might be because cationic polymers had a higher degree of protonation at low pH, thus the zeta potential values decreased as the pH increased. And complexes that showed less variation as the pH changed may be due to their possession of more amino groups which results in more positively charged groups and less decrease in zeta potential values. The morphology of the polymer/DNA complexes was observed by scanning electron microscopy (SEM). Fig. 3 is a typical image of complexes, and the diameter of the formed complexes was about 25 nm, which was smaller than that determined by DLS measurement, probably due to the SEM photo was taken as the complex in a dry state while the DLS measurement was taken in solution as the macromolecule in the hydration form (Sergeev et al., 2011).

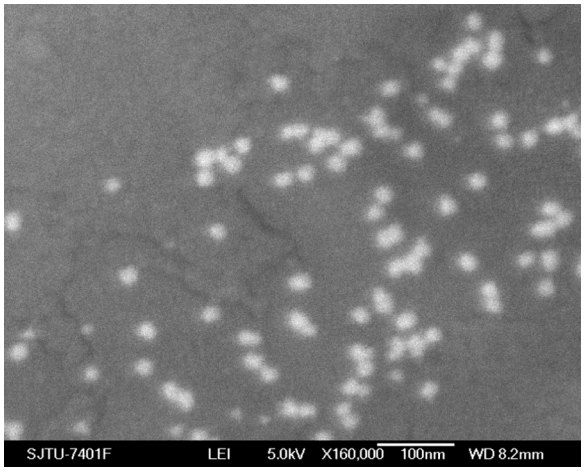

Fig. 3. SEM micrograph of NPCS2/DNA complex at the N/P ratio of 16/1.

3.3. Plasmid DNA-binding ability of the grafted polymers

As an essential characteristic of a gene delivery vector, plasmid DNA-binding ability of the grafted polymers was determined by agarose gel electrophoresis method. The complexes of NPCS/DNA at N/P ratios ranging from 0.25/1 to 24/1 and PEI/DNA at the N/P ratio of 10/1 were electrophoresed separately in agarose gel. Naked DNA was used as a control. As can be seen in Fig. 4., free DNA fractions released from the complexes decreased gradually as the N/P ratios changed from 0.25/1 to 24/1. Complete retardation of DNA was achieved when the N/P ratio was above 4/1 at pH 6.5. At the pH of 7.4, part of the DNA in the corresponding channel was released when the N/P ratio was below 8/1. PEI complex showed a good DNA binding ability. These were in consistent with the zeta potential measurements.

3.4. Cell viability

The in vitro cytotoxicity of the polymer samples was evaluated in the HEK 293 and HeLa cell lines by the MTT assay. Untreated cells were used as control (i.e. 100% viability). Comparisons between the polymers were made at equivalent weight concentrations. The results (see Fig. 5) showed that chitosan exhibited non-toxic in both cell lines, and the cytotoxicity of NPCS polymers and branched PEI-25K was concentration dependent. NPCS polymers have higher cytotoxicity to both cells in a concentration range of 1–100 μg/mL than chitosan but have lower cytotoxicity than PEI-25K. At the concentration of 20, 100, 200 μg/mL, there are significant difference between PEI-25K and NPCS2 at 95% confidence interval and no significant difference between NPCS1 and NPCS2 at the same confidence interval using t test. It is well-known that the cytotoxicity of PEI arises from its amine groups, which lead it to high positively charged in aqueous solutions. Although chitosan also has amine groups present on its monosaccharide rings, its cytotoxicity is much lower. In our case, the grafting of CTPB onto chitosan introduced positively charged groups. Therefore, NPCS polymers showed some cell cytotoxicity to an extent.

3.5. In vitro gene transfection

The gene transfection efficiency of the NPCS polymers was evaluated by in vitro gene transfection experiments. The transfected cells that expressed green fluorescent proteins were directly observed by a reverse fluorescent microscope. NPCS/DNA complexes were formulated with various N/P ratios in order to investigate the optimal conditions for gene transfection. Fig. A1

Table 2
Hydrodynamic diameters and zeta potential of polymer/DNA complexes with different N/P ratios.

Complex	N/P	Particle size (nm)[a]	Zeta potential (mV)[a]	Particle size (nm)[b]	Zeta potential (mV)[b]
CS/DNA	16/1	137.5 ± 0.7	36.8 ± 2.6	147.2 ± 6.7	29.8 ± 2.6
CS/DNA	8/1	151.2 ± 1.2	35.9 ± 0.7	159.7 ± 3.9	12.6 ± 1.7
CS/DNA	4/1	191.8 ± 2.4	36.9 ± 0.4	201.0 ± 5.5	10.9 ± 2.3
NPCS1/DNA	16/1	115.1 ± 5.1	36.6 ± 0.8	119.4 ± 4.3	30.0 ± 1.6
NPCS1/DNA	8/1	128.4 ± 7.2	34.2 ± 1.7	133.1 ± 6.1	12.2 ± 1.5
NPCS1/DNA	4/1	162.0 ± 2.6	30.3 ± 0.9	160.6 ± 5.8	9.6 ± 0.9
NPCS2/DNA	16/1	121.0 ± 1.8	30.5 ± 1.1	118.8 ± 2.2	30.6 ± 1.0
NPCS2/DNA	8/1	135.6 ± 3.5	30.1 ± 1.8	139.9 ± 4.0	26.5 ± 1.9
NPCS2/DNA	4/1	151.5 ± 1.3	28.3 ± 0.6	158.6 ± 5.6	13.1 ± 2.9
PEI/DNA	10/1	77.4 ± 5.1	33.8 ± 1.9	78.5 ± 2.4	30.4 ± 0.4

[a] Particle size and zeta potential were detected as the complex solutions adjusted to pH 6.5.
[b] Particle size and zeta potential were detected as the complex solutions adjusted to pH 7.4.

(shown in the appendix) displays the typical fluorescence images of the transfected HEK293 cells. It can be seen that NPCS polymers possess reasonable gene transfection ability compared with chitosan, but lower transfection efficiency as compared with branched PEI-25K. NPCS2 showed better transfection efficiency than NPCS1 at a given N/P ratio. These observations are confirmed by the EGFP fluorescence intensity data. As is shown in Fig. 6(a), mean fluorescence intensities of cells treated with NPCS2 are much higher than those treated with NPCS1. This might be NPCS2 with a higher degree of substitution contains more secondary amine groups, which means stronger buffering ability and favorable for endosomal escape. The low transfection of NPCS2 complex at the N/P of 8/1 may due to its incomplete compact of DNA as confirmed by gel electrophoresis assay in Fig. 4. Similar results can also be obtained from the transfected HeLa cells (Fig. A2). We also investigated the influence of medium pH on gene transfection efficiency, since the microenvironment of tumor is about at pH 6.0–6.5. The transfection efficiency was significantly enhanced as the medium pH was adjusted to 6.5, as displayed in Fig. 6(b). Mean fluorescence intensities of cells treated with NPCS2 at N/P ratio of 8/1 increased by 10–15 times as the transfection medium pH adjusted to 6.5. Furthermore, NPCS2/DNA at N/P ratio of 16/1 exhibits a higher EGFP

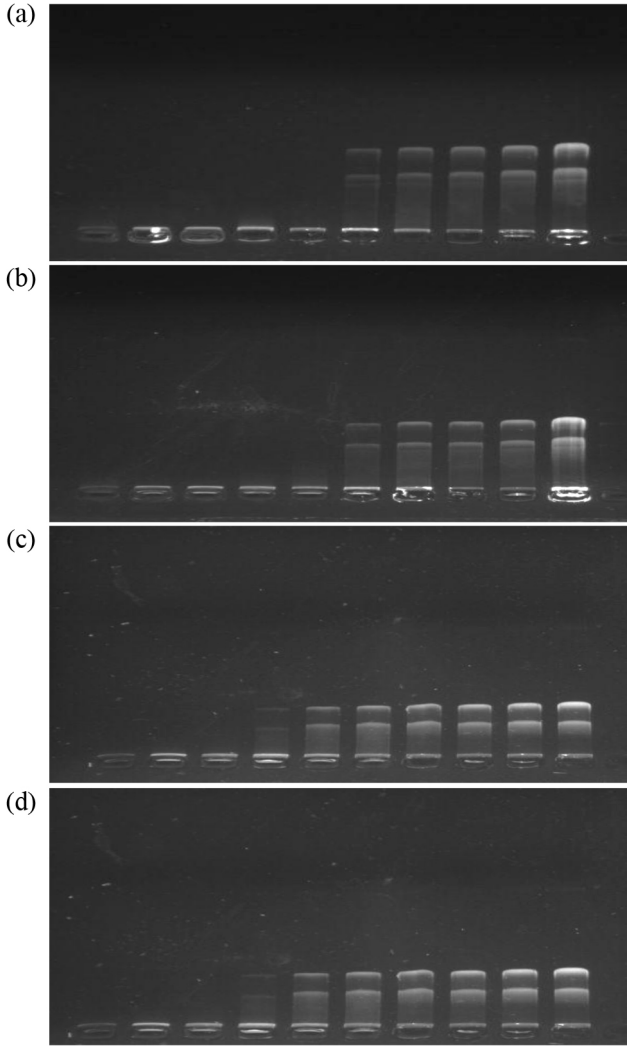

Fig. 4. Gel electrophoresis shift assays for the DNA binding ability of the polymers with various N/P ratios at pH 6.0 of NPCS1/DNA (a) and NPCS2/DNA (b), at pH 7.2 of NPCS1/DNA (c) and NPCS2/DNA (d). The N/P ratio of PEI/DNA is 10/1.

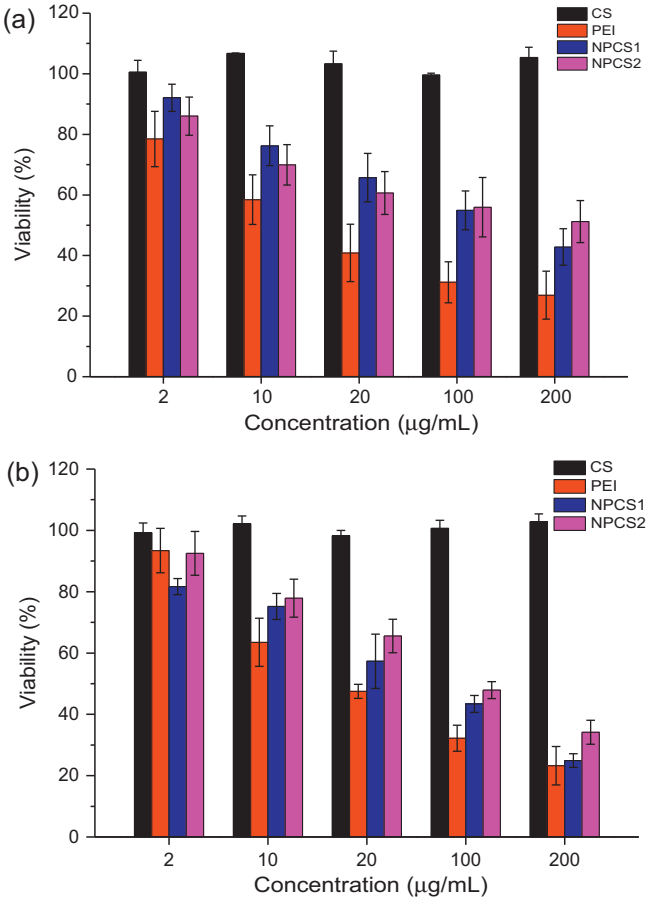

Fig. 5. Cell viability of HEK 293 cells (a) and HeLa cells (b) after incubation with polymers at different concentration at 4 h.

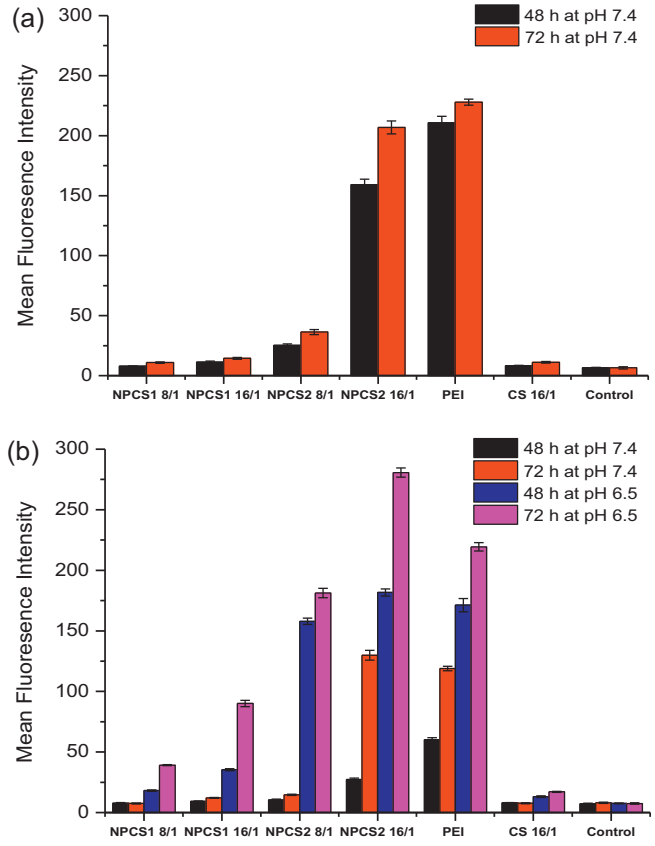

Fig. 6. Fluorescence assays for EGFP expression in complexes treated HEK 293T cells (a) and HeLa cells (b). pH 6.5 means cell culture medium was adjusted to 6.5 during the transfection. The fluorescence intensity was measured at 48 h and 72 h after transfection, untreated cells were used as control ($n = 3$).

expression than PEI/DNA, indicating NPCS2 can be a promising candidate for gene delivery.

4. Conclusion

In this study, phosphonium grafted chitosan polymers with two degrees of substitutions were successfully synthesized. NPCS polymers could formulate compact complexes with plasmid DNA and the surface of complexes was positively charged. NPCS polymers had much lower cytotoxicity than PEI-25K in HEK 293 and HeLa Cells. NPCS polymers had much higher gene transfection abilities than chitosan and NPCS2 showed a comparative gene transfection ability to branch PEI-25K on HeLa cells at pH 6.5. All those indicate that NPCS is a promising candidate for low-toxic and high efficient gene vector. However, further studies in vivo are needed to fully exploit the potential of the NPCS grafted polymers for systematic gene delivery.

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Appendix A.

See Figs. A1–A3.

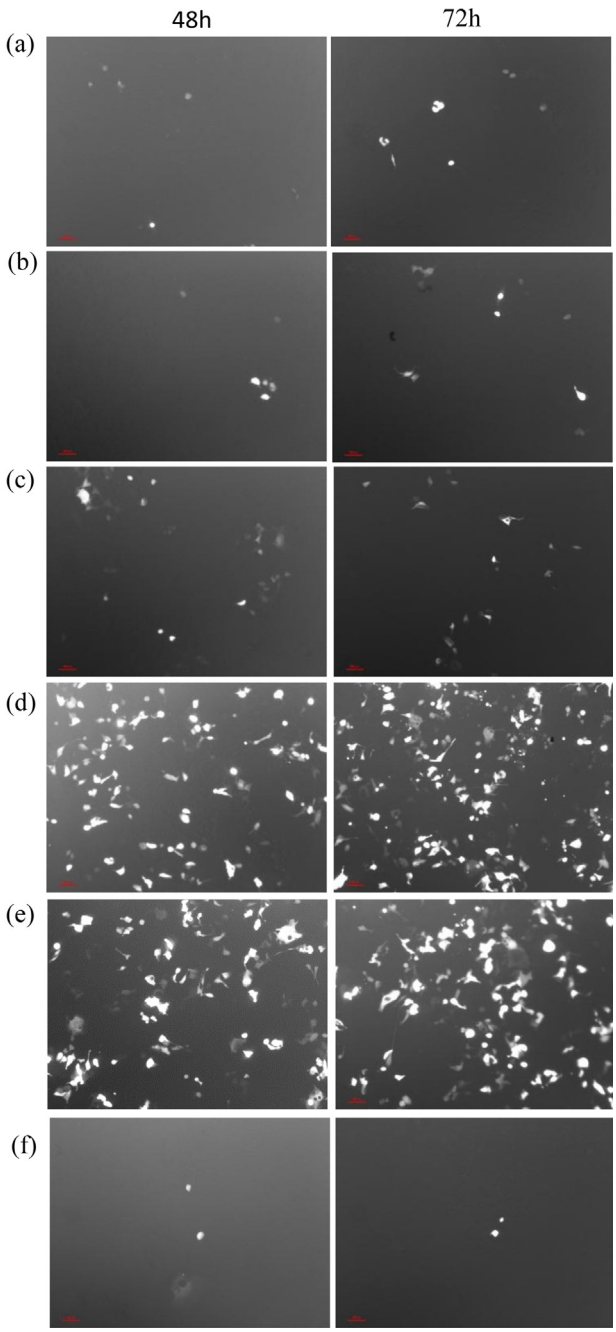

Fig. A1. Representative fluorescent images of 293 cells at 48 h and 72 h after treatment with NPCS1/DNA at N/P ratio of 8/1 (a) and 16/1 (b), NPCS2/DNA at N/P ratio of 8/1 (c) and 16/1 (d), PEI/DNA at N/P ratio of 10/1 (e), CS/DNA at the ratio of 16/1 (f), respectively.

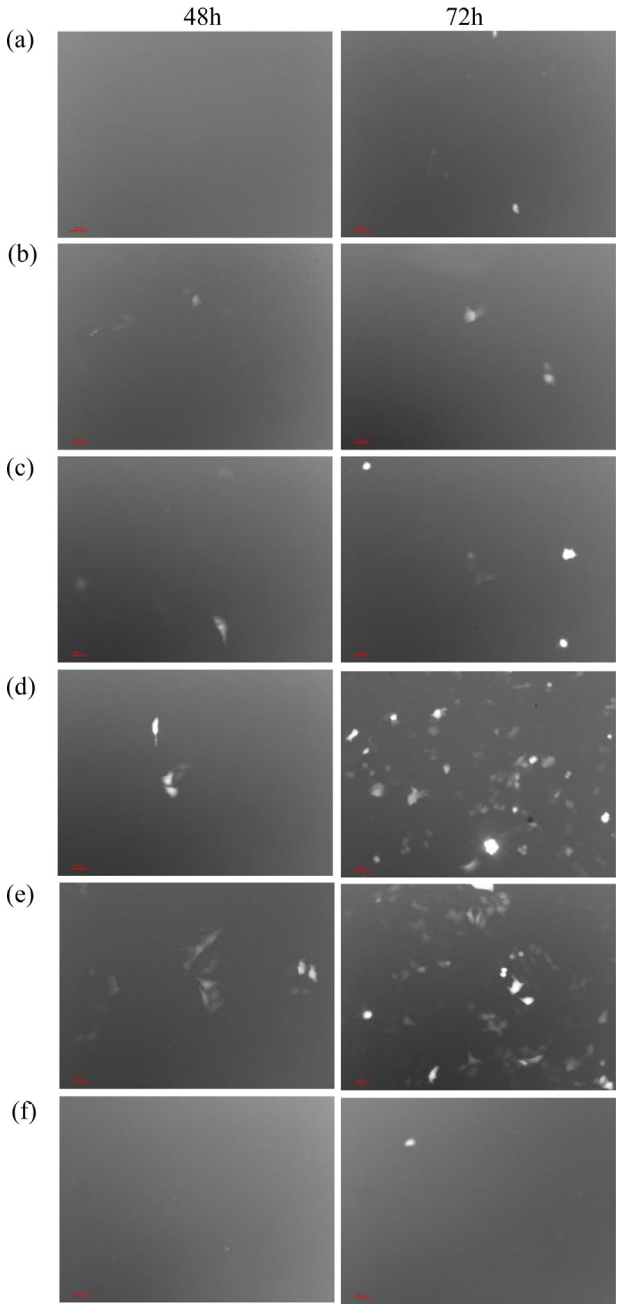

Fig. A2. Representative fluorescent images of HeLa cells at 48 h and 72 h after treatment with NPCS1/DNA at N/P ratio of 8/1 (a) and 16/1 (b), NPCS2/DNA at N/P ratio of 8/1 (c) and 16/1 (d), PEI/DNA at N/P ratio of 10/1 (e), CS/DNA at the ratio of 16/1 (f), respectively.

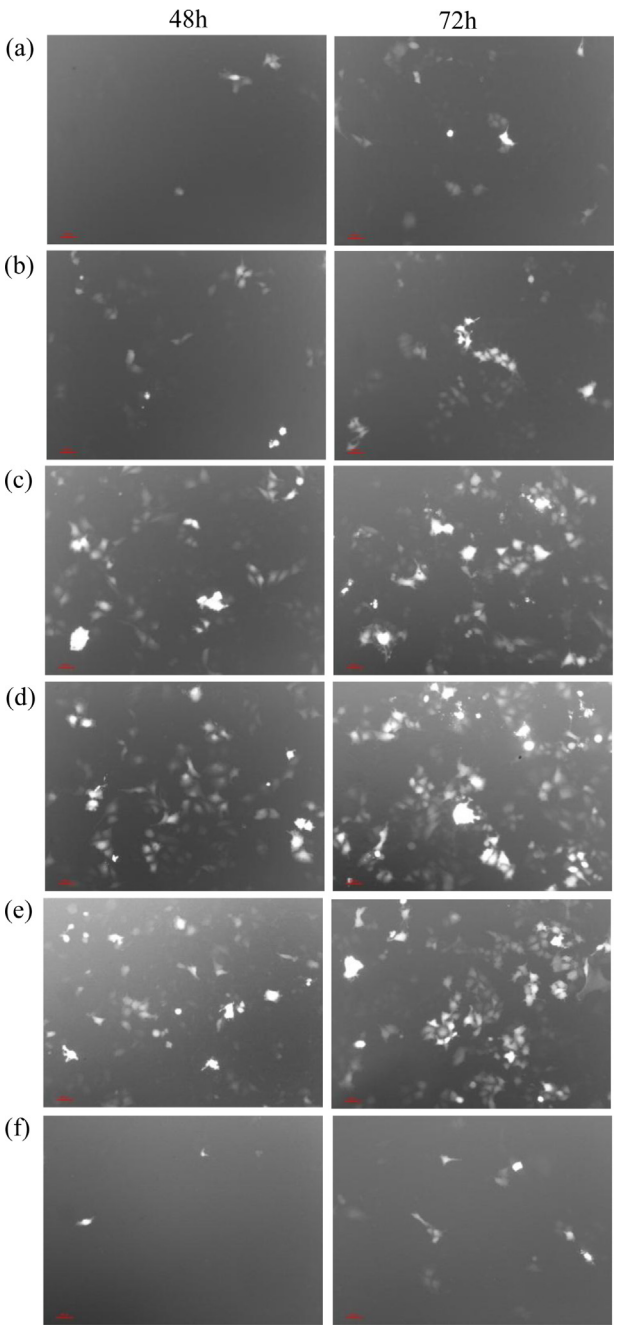

Fig. A3. Representative fluorescent images of HeLa cells at 48 h and 72 h after treatment with NPCS1/DNA at N/P ratio of 8/1 (a) and 16/1 (b), NPCS2/DNA at N/P ratio of 8/1 (c) and 16/1 (d), PEI/DNA at N/P ratio of 10/1 (e), CS/DNA at the ratio of 16/1 (f), respectively. Cell culture medium was adjusted to pH 6.5.

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