J Mater Sci: Mater Med (2010) 21:223–229
DOI 10.1007/s10856-009-3849-3

Hydrophilic/lipophilic *N*-methylene phosphonic chitosan as a promising non-viral vector for gene delivery

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Received: 22 May 2009 / Accepted: 4 August 2009 / Published online: 14 August 2009

Abstract Cationic amphiphilic drugs have recently been shown to inhibit receptor recycling by disrupting the assembly–disassembly of clathrin at the plasma membrane and endosomes. It is therefore proposed that amphiphilic and cationic polysaccharide macromolecule, when used as gene delivery vectors, may have potential ability to direct the disassembly process of cell membrane organization, and penetrate across the cell membrane into cell and nucleus. In the current study, *N*-methylene phosphonic chitosan (NMPCS), an amphiphilic macromolecule, was synthesized by incorporating the methylene phosphonic group into the amino groups of chitosan (CS) using formaldehyde as the coupling agent, and characterized with a FTIR spectrometer. NMPCS/DNA or CS/DNA complexes were prepared using a complex coacervation method, and characterized by agarose gel electrophoresis retardation assay and dynamic light scattering (DLS). MTT assay was employed to evaluate the cytotoxicity of the polymers and pGL3-control luciferase plasmid was utilized as a reporter gene to assess the transgenic efficacy of the polymers. It was demonstrated that NMPCS was able to fully entrap the DNA at N/P ratio of 2:1, whereas CS entrapped the DNA completely at N/P ratio of 1:1. DLS showed that the NMPCS/DNA or CS/DNA complexes were of mean diameters ranging from 110 to 180 nm. Neither NMPCS nor CS induced significant loss of cell viability at the concentrations ranging from 1 to 50 μg/ml, whereas PEI at 5 μg/ml started to result in significantly decreased cell viability. The expression of transgene mediated by NMPCS was much higher (more than 100-folds) than that mediated by CS, indicating that NMPCS was a more efficacious gene ferrying vector than CS.

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1 Introduction

Gene therapy is to introduce therapeutic genes into specific cells for the purpose of correcting the dysfunction of specific genes or over-expressing therapeutically useful proteins so as to take curative effects for human diseases or provide immunologic protection [1, 2]. Potential clinical application of gene therapy depends greatly on the development of safe and efficient gene delivery systems, to which the most distinct drawback is the lack of effective gene ferrying vectors. Non-viral vectors have been attracting increasing research efforts owing to the consideration of safety and feasibility for manufacturing. Cationic polymers are among the most attractive non-viral gene carriers, which include polyethylenimine (PEI), polyallylamine (PAA), poly-L-lysine (PLL), and chitosan (CS).

Chitosan is a naturally occurring linear polysaccharide consisting of β-(1, 4) linked D-glucosamine and N-acetyl-D-glucosamine and produced by deacetylation of chitin [3, 4]. It is a nontoxic, positively charged biodegradable and biocompatible polymer, which is able to condense and protect DNA against nuclease degradation [5]. However, the application of chitosan as a gene carrier is limited by its relatively low transgenic efficacy. Several chitosan derivatives, such as quaternized chitosan [6], galactosylated chitosan [7], water-soluble chitosan (WSC)-urocanic acid (UA) [8], and folate-chitosan [9], methoxy poly(ethylene glycol)-modified galactosylated chitosan [10], methylated

N-(4-pyridinylmethyl) chitosan [11], have demonstrated improved water solubility, gene delivering efficacy, and specificity to target cells.

Self-assembly is ubiquitous in the body of creatures [12, 13], and the characteristic of biological self-assembly is the variety and complexity of the functions it produces. Living cells and organisms are dynamic self-assembly systems since many biomacromolecules such as proteins, polysaccharides, nucleic acids and lipid molecules, are able to generate complicated and ordered structures with different functions through self-assembly. Hydrophilic/lipophilic, electrostatic interactions, van der Waals, hydrogen and coordination bonds are noncovalent or weak covalent interactions molecular self-assembly involves [12]. The biomembranes are generally self-directed assemblies of amphiphilic molecules with a dual hydrophilic–hydrophobic character, and thus central to cell functions [14]. Cationic amphiphilic drugs have recently been shown to inhibit receptor recycling by disrupting the assembly–disassembly of clathrin at the plasma membrane and endosomes [15]. It is therefore proposed that amphiphilic and cationic polysaccharide macromolecule, when used as gene delivery vectors, may have potential ability to direct the disassembly process of cell membrane organization via perturbing or disrupting the hydrophilic/lipophilic, electrostatic, and other noncovalent interactions, and penetrate across the cell membrane into cell and nucleus.

In the present study, the feasibility of the *N*-methylene phosphonic chitosan (NMPCS) as a gene carrier was investigated and the results demonstrated that NMPCS mediated a significantly higher transgene expression in comparison to CS.

2 Materials and methods

2.1 Materials

CS (Mw 50 kDa, deacetylation degree 97%) was purchased from Qingdao Hecreat Bio-tech Company Ltd (Qingdao, China), Polyethylenimine (PEI) (branched, Mw 25 kDa), Kanamycin and 3- (4,5-dimethylthiazol-2-yl)–2,5-diphenyl-tetrazoium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 liquid media were provided by Hyclone (Logan, UT, USA). Penicillin/streptomycin, trypsin-EDTA, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). pGL3-control plasmid and luciferase assay system (Cat. No. E1501) were purchased from Promega (Madison, WI, USA). Endofree Plasmid Mega Kits were purchased from Qiagen GmbH (Hilden, Germany). BCA Protein Assay Kit (Cat. No. 23225) was purchased from Pierce (Rockford, IL, USA). All other chemicals were of biological grade.

2.2 Cell line and cell culture

The human cervical carcinoma epithelioid cell line HeLa was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured in RPMI 1640 medium supplemented with 10%FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were maintained at 37°C in a humidified 5% CO_2 incubator. Cells were detached by trypsinization and harvested by low speed centrifugation, and enumerated using a hemocytometer.

2.3 Reporter gene

pGL3-control plasmid encoding the luciferase was employed to assess the efficiency of gene transfection. The plasmid was propagated in *E. coli* and purified using Endofree Plasmid Mega Kits according to the manufacturer's instructions. The quality and quantity of DNA were determined by measuring UV absorbance at 260 and 280 nm.

2.4 Synthesis and characterization of NMPCS

NMPCS was synthesized according to the method described by Agulló et al. [16]. Briefly, 4 ml of phosphorous acid (PA) solution (50% W/V) was added drop-wise to 100 ml of 2% CS solution in 1% glacial acetic acid with continuous stirring at room temperature for 1 h. The temperature of the reaction vessel was subsequently raised to 70°C and 5 ml of 36.5% (W/W) formaldehyde solution was added drop-wise to the reaction mixture over 1 h with reflux. Heating was protracted for 6 h at the same temperature. The resulting solution was dialyzed for 3 days using Cellu SepH1 membrane (MWCO 3000) against deionized water, and lyophilized for product collection. The FTIR spectra of CS and NMPCS were recorded in KBr pellets using a FTIR spectrometer (FTS3000, Bio-rad, USA) in the range between 4000 and 400 cm^{-1}. The substitution degree of *N*-methylene phosphonic group in NMPCS was characterized by X-ray photoelectron spectroscope (PHI 1600 ESCA, Perkin Elmer, USA).

2.5 Preparation and characterization of NMPCS/DNA particles

NMPCS/DNA or CS/DNA particles at various charge ratios (N/P, ±) were prepared by the complex coacervation method [17] with minor modifications. In brief, 0.02% NMPCS in sterilized 50 mM NaAc/HAc buffer (pH 5.3) and DNA (20 μg/ml) in sterilized 5 mM sodium chloride

solution were separately preheated at 55°C for 10 min. To prepare NMPCS/DNA or CS/DNA nanoparticles at various N/P ratios, equal volume of the DNA solution and the NMPSC or CS solution of varied concentrations were mixed and votexed for 40 s with a CAT ×120 homogenizer (Ingenieurburo CAT, Staufen, Germany), and subsequently left 30 min for the nanoparticles to completely form. Complex formation was confirmed by agarose gel electrophoresis using ethidium bromide as fluorescent dye. The size and distribution of NMPCS/DNA at different N/P ratios were examined with dynamic light scattering (DLS, BI-90 Plus, USA). PEI/DNA complexes (N/P ratio 4:1) were prepared according to the method previously described by Intra et al. [18].

2.6 MTT assay for cell viability

HeLa cells were seeded at 1.5×10^4 cells/well on 96-well plates and grown for 24 h. The medium was removed and the cells were rinsed twice with PBS immediately prior to addition of 200 µl of the culture medium containing various amount of NMPCS or CS or PEI ranging from 1 to 50 µg/ml, with fresh medium utilized as negative control. 24 h post exposure, the medium was removed and the cells were rinsed with PBS,followed by addition of 100 µl of RPMI 1640 medium containing 500 µg/ml MTT to each well. After 5 h incubation at 37°C, the medium was replaced with 100 µl of DMSO and the plates were shaken at 240 rpm for 10 min, followed by measuring the absorbance at 570 nm with a microplate reader (SpectraMax plus 384, MD, USA). The cell viability was presented as the percentage of the absorbance of the polymer treated cells to that of the untreated cells.

2.7 Cell transfection

7.5×10^4 of HeLa cells were seeded in each well of the 24-well plates and incubated for 24 h. After removal of the medium, the cells were rinsed twice with PBS, followed by addition of 500 µl of serum-free media with different pH values (6.5, 7.0, and 7.5, respectively). 50 µl of nanoparticle suspension containing 2 µg of DNA was added to each well. Five hours after nanoparticle exposure, the media were removed and the cells were rinsed with PBS, followed by addition of fresh media with 10% FBS. Forty eight hours later, the media were removed and 200 µl of lysis buffer (Promega, Madison, IL, USA) was added to each well. The cells were gently scraped off the plate with a cell-scraper and the resulting cell lysates were centrifuged at 12,000×g for 1 min. The supernatant was collected and the amount of proteins wherein was measured using BCA assay. Twenty five micro liters of the supernatant was mixed with 100 µl of luciferase substrates for the determination of luciferase activity by measuring light emission using a luminometer ($TD20/20^n$, Turner BioSystems, Sunnyvale, CA, USA) according to the manufacturer's instructions. The luminescence units were standardized based on the amount of proteins in the supernatant and the results were expressed as the relative light units (RLU)/mg protein.

2.8 Statistical analysis

Data were presented as the mean of six individual observations with standard deviation. The statistical analysis was performed using the ANOVA (a one-way analysis of variance), followed by Bonferroni *t*-test for comparison with the control group. Statistical significance was determined at $P < 0.05$.

3 Results

3.1 Synthesis and characterization of *N*-methylene phosphonic chitosan

NMPCS was synthesized by incorporating the methylene phosphonic group from phosphorous acid into the amino groups of chitosan using formaldehyde as the coupling agent, as previously described by Heras et al. [16, 19]. Figure 1 showed the synthesis scheme of NMPCS.

As shown in Fig. 2, significant shift around 1070, 944, and 519 cm^{-1} corresponding to the stretching vibration or bending vibration peak of v P–OH and a shift at 1538.06 cm^{-1} corresponding to the δ NH were observed in NMPCS, while the peak located in 2500–3500 cm^{-1} ran weakened and got wider, indicating the substitution of

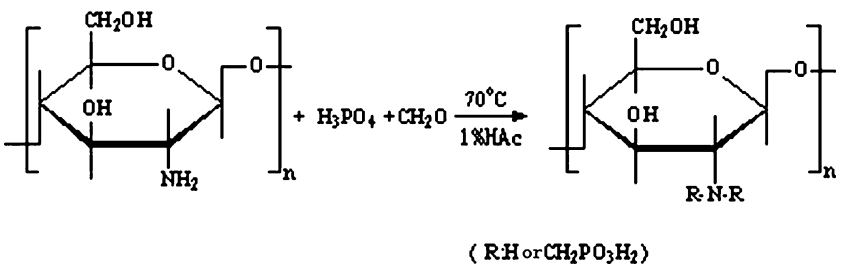

Fig. 1 Synthesis scheme of NMPCS

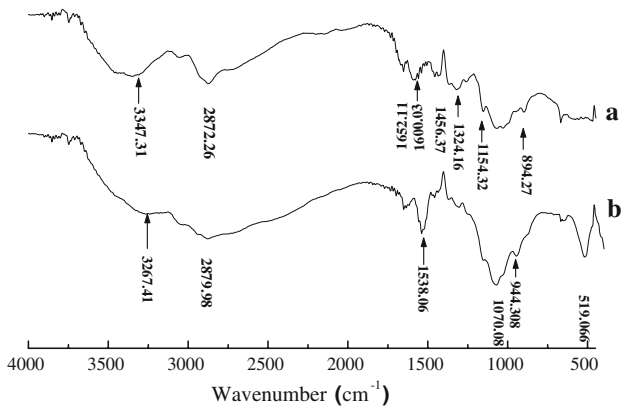

Fig. 2 FTIR spectra of CS (*a*) and NMPCS (*b*)

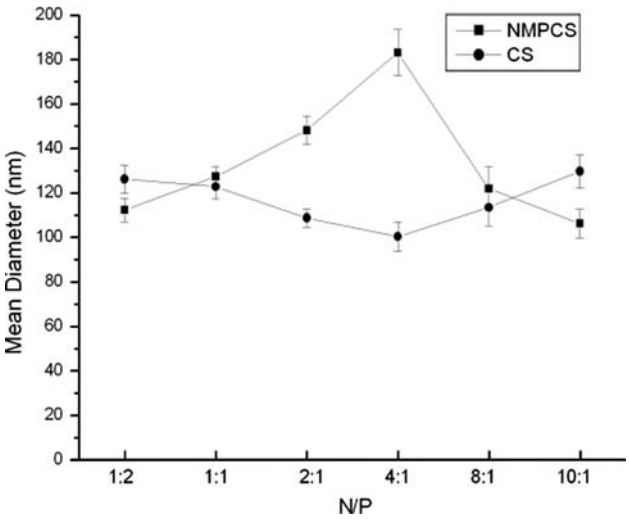

Fig. 4 Mean diameters of CS/DNA complexes and NMPCS/DNA complexes

$-CH_2PO_3H_2$ to H atoms in amino groups. The substitution degree of *N*-methylene phosphonic group estimated by X-ray photoelectron spectroscope was about 0.28.

3.2 Characterization of NMPCS/DNA complexes

Agarose gel electrophoretic retardation assay was employed to investigate the interaction between the polymers and DNA. The results showed that the DNA was entirely entrapped by NMPCS at the N/P ratio of 2:1 and beyond, whereas CS was able to fully entrap the DNA starting at the N/P ratio of 1:1, as shown in Fig. 3, suggesting that substitution of $-CH_2PO_3H_2$ to the $-NH_2$ led to the decrease of positive charge and hence weakening power of NMPCS for condensing DNA.

Dynamic light scattering analysis indicated that the diameters of NMPCS/DNA nanoparticles ranged from 110 to 180 nm, whereas the mean diameters of CS/DNA nanoparticles ranged from 100 to 130 nm (Fig. 4). It was demonstrated that, as the N/P ratio ranged from 1:2 to 4:1, the mean diameters of NMPCS/DNA complexes increased with the increase of N/P ratio, and then decreased with further increase of N/P ratio. In contrast to NMPCS/DNA nanoparticles, the mean diameters of CS/DNA nanoparticles decreased with the increase of N/P ratio until 4:1 and then increased with further increase of N/P ratio. Previous investigations suggested that the major force complexing CS and DNA together was the electrostatic interaction between negatively charged DNA and positively charged chitosan and thus the N/P ratio was cited as an important factor influencing the size of the chitosan/DNA nanoparticles. At lower N/P ratio range, the size of CS/DNA would decrease with the increase of N/P ratio, since the electrostatic interaction between CS and DNA increases with the increase of N/P ratio. However, as the N/P ratio gets even higher, the intermolecular repulsion between CS, which is a function of the excessive amount of polycations, would become another factor influencing the size of the nanoparticles and lead to the increase in particle size. This should provide the explanation for the size pattern of CS/DNA nanoparticles observed in the current study. Since NMPCS has a lower density of positive charge in comparison to CS, it is not unexpected that NMPCS/DNA nanoparticles would display a different size pattern with changes in N/P ratio. However, the underlying mechanism is still not clear and needs to be further elucidated.

3.3 Cytotoxicity evaluation

MTT assay was employed to evaluate the cytotoxicity of NMPCS and CS against HeLa cells. It was demonstrated that neither NMPCS nor CS induced significant loss of cell

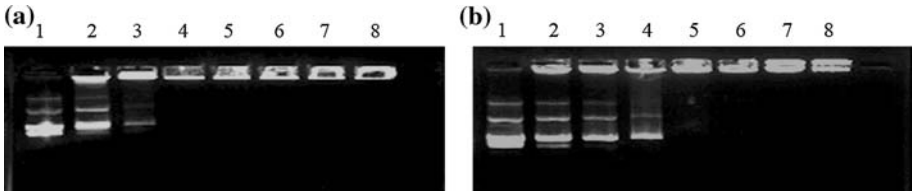

Fig. 3 Agarose gel electrophoresis for analysis of CS/DNA (**a**) or NMPCS/DNA (**b**) complexes. Lane 1: Free DNA pGL3-control, Lane 2: N/P = 1:4, Lane 3: N/P = 1:2, Lane 4: N/P = 1:1, Lane 5: N/P = 2:1, Lane 6: N/P = 4:1, Lane 7: N/P = 8:1, Lane 8: N/P = 10:1

viability at the concentrations from 1 to 50 μg/ml, whereas PEI started to show significant cytotoxicity at 5 μg/ml, as shown in Fig. 5.

3.4 Transgenic efficacy of NMPCS/DNA particles

The transgenic efficacy of NMPCS/DNA nanoparticles was compared with that of CS/DNA nanoparticles under different pH values (Fig. 6). PEI/DNA polyplexes at a N/P ratio of 4:1 were employed as an experimental control because they were reported to be efficacious for gene transfer and have a mean diameter similar to that of CS/DNA complexes [20]. It was revealed that the transgene expression mediated by NMPCS, even though a little bit lower than that mediated by PEI, was much higher (more than 100 folds) than that mediated by CS, indicating that NMPCS was a more efficient gene ferrying vector than CS. The results in the present study also demonstrated a reversed correlation between the level of transgene expression and the pH value of the medium at the pH ranging from 6.5 to 7.5.

4 Discussion

Cytotoxicity is one of the major drawbacks to the medical application of polymeric gene carriers [21]. In the current study, MTT was employed to assess the toxicity of NMPCS against HeLa cells and it was demonstrated that neither NMPCS nor CS induced significant loss of cell viability at the concentrations from 1 to 50 μg/ml, whereas PEI started to show significant cytotoxicity at 5 μg/ml,

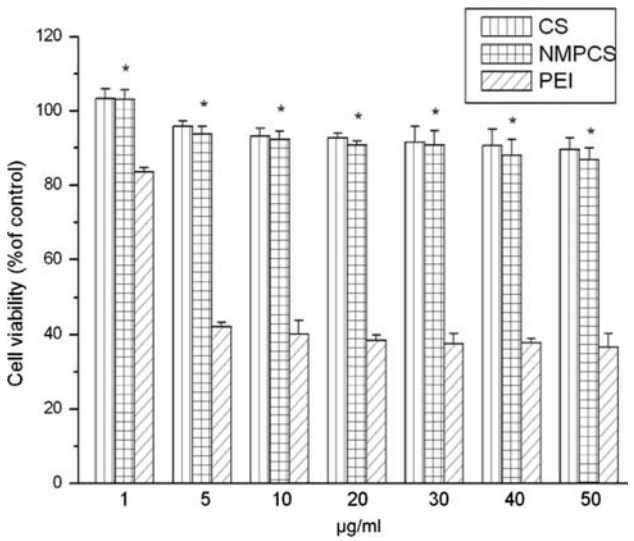

Fig. 5 Viability of HeLa cells after 24 h of co-incubation with CS, NMPCS and PEI at the concentrations ranging from 1 to 50 μg/ml, respectively. Cell viability is presented as the percentage of the OD value of experimental groups to that of the negative control. * $P < 0.05$ in comparison to PEI ($n = 6$)

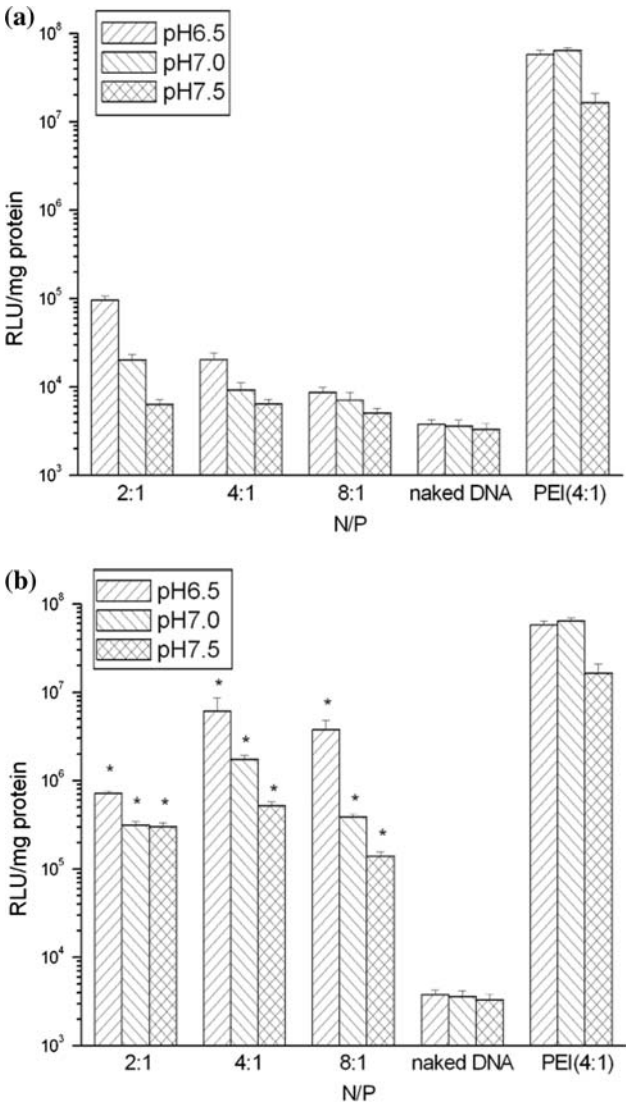

Fig. 6 Gene expression in HeLa cells mediated by CS/DNA complexes (**a**) or NMPCS/DNA complexes (**b**) at various N/P ratios and under various pH values. The results are expressed as the mean values of 6 tests. * $P < 0.05$ in comparison to CS/DNA nanoparticles under the same condition ($n = 6$)

suggesting that the incorporation of methylene phosphonic group into CS did not have a significant impact on its cytotoxicity. Since previous investigations demonstrated that the cytotoxicity of polyplexes formed by DNA and chitosan or its derivatives was mainly determined by the polymers, it is reasonable for us to propose that nanoparticle containing the respective amount of NMPCS would not be cytotoxic [9, 22].

In order to be expressed in the cells, a transgene needs to get through several biological barriers. It should efficiently pass across the cell membranes at first, escape as quickly as possible from the endosomes, and finally get into the nucleus. The higher transgenic efficacy of NMPCS in comparison with CS may be associated with its hydrophilic/

lipophilic property, although the effect of structure-function relationship on the transfection activity is still poorly understood to date [23]. As previously described by Thomas and Klibanov, the self-assembly mediated by amphiphilic interactions led to more efficient DNA condensation, enhanced endocytosis of the complexes by cells, and in turn, improved transgene expression [24]. The disassembly process of cell membrane may also be directed by hydrophilic/lipophilic, electrostatic, and other noncovalent interactions via activating or tuning cell signal transduction pathway [25]. The interaction of NMPCS with the amphiphilic components on plasma membranes, such as lipid bilayers, is able to make a perturbation or disruption on membrane organization [15], and possibly creates a specific room or space for successfully transporting DNA into cells and the nucleus. The ratio of NMPCS to DNA affects the stability of the complex formed, which in turn leads to varied cell penetration, DNA release in the cytoplasm, and hence the transgene expression. This could provide a possible explanation for the observation in the current study that the highest level of transgene expression was achieved with the NMPCS/DNA nanoparticles at the N/P ratio of 4:1, whereas the nanoprticles at N/P ratio of 2:1 or 8:1 delivered a lower transgene expression.

The present study also observed a reversed correlation between the level of transgene expression and the pH value of the medium at the pH ranging from 6.5 to 7.5, in line with the previous reports that the reduction of pH value increased the number of protonated amines on the CS backbone, leading to enhanced interaction with cell membranes, further disruption on membrane organization, and quicker escape from the endosomes [26–28].

5 Conclusions

Taken together, the hydrophilic/lipophilic NMPCS was demonstrated to be an efficacious gene carrier and relatively nontoxic, which would find potential medical applications. However, more detailed investigations are needed for deeper understanding the mechanism underlying the interactions between NMPCS and the cells in order to further elevate its transgenic efficacy.

Acknowledgments This research was jointly supported by the Ministry of Science and Technology of China (Grant No: 2005DIB1J094, 2006CB933203) and the National Natural Science Foundation of China (Grant No: 50233020, 90406024).

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