A Novel Potential Cationic Polymeric Gene Vector Containing Hydroxy Groups and All Grades of Amino Groups

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ABSTRACT: A novel potential cationic polymeric gene vector, PCT, was synthesized by copolymerizing 3-chloro-1,2-epoxypropane (CEP) and tetraethylene pentamine (TEPA). The protonizable nitrogen content of PCT is 17.7 mmol/g at 1 : 1 of CEP to TEPA, lower than 23.3 mmol/g of polyethylenimine (PEI) but higher than 7.8 mmol/g of polylysine, which indicates PCT is a candidate of polycation with high-content but discrete charge after protonation. PCT has many hydrophilic quaternized amino and hydroxy groups contributing to hydrophilicity. Acid base titration showed that PCT was protonated in both pH 7.4–11 and pH 5.0–7.4 and had ''proton sponge effect.'' In aqueous solution, PCT complexed with weak anionic polymer, poly(acrylic acid) (PAA), into nanoparticles. Fourier transform infrared spectroscopy proved the formation of complex. Particle size is first increased and then decreased as increasing mass ratio θ of PCT to PAA from 0.1 to 1.7, as shown by dynamic light scattering. At θ values of more than 1.7 or less than 0.2, complex particles with mean diameter less than 200 nm were obtained. At θ of 0.5, the UV-Vis absorbance of complex solution at first day was obviously higher than that at 7 day, while at θ of 1.7, two absorbance curves at these two time points had slightly smaller change, indicating that small complex particles were more stable than large ones because of electrostatic repulsion. J Appl Polym Sci 105: 1533–1542, 2007

Key words: gene vector; nanoparticle; complex; cationic polymer; poly(acrylic acid)

INTRODUCTION

Gene therapy through the delivery of therapeutic genetic constructs to cell nucleus is emerging as a revolutionary and promising treatment way for serious human diseases.[1] The nucleic acids are prone to hydrolyze in biological fluids and show low cellular uptake efficiency because of electrostatic repulsion, so direct transfection using naked DNA is difficult to get obvious curing effect.[2] Lack of effective and secure gene vector becomes a bottleneck limiting gene therapy into extensive clinical use. To bring exogenetic gene into cell, many kinds of viral and nonviral vectors have been developed. Although viral vectors have higher transfection efficiency than nonviral ones, they have serious security issues such as endogenous recombination, oncogenic effects, and immunological reactions leading to potentially serious complications.[3] So nonviral vectors are potential alternative to viral ones.[4]

Cationic liposome and polymer are two typical candidates of nonviral gene delivery vectors.[5] Although a number of amphiphilic molecules containing amine have been developed for gene transfer, instability in serum and toxicity remain a limit of their use.[6] Among the nonviral gene vectors currently developed, polycations have attracted the most extensive attention because of its ability to form stable polyelectrolyte complexes (PECs) with DNA via strong electrostatic adsorption, low immune response as well as the ease of synthesis, modification, and scaling up.[7,8] Many polycations can transfer genes *in vitro* or *in vivo*, including poly-L-lysine,[9] polyallyamine,[10] polyamidoamine dendrimers,[11] polyethylenimine (PEI),[12] and poly((2-dimethylamino)ethyl methacrylate).[13]

PEI is one of the most effective nonviral gene vectors because of its strong complex ability and proton sponge effect.[14] PEI is able to deliver large DNA molecules such as 2.3 Mb yeast artificial chromosomes (YACs) as well as plasmids or small oligonucleotides into mammalian cells *in vitro* and *in vivo*.[15] However, PEI bears acute cytotoxicity and short blood circulation time because of its high charge density, which limits its *in vivo* application. Introducing hydrophilic groups to shield and stabilize complex was common way of solving this question, for example using poly(ethylene glycol) (PEG) as hydrophilic group to modify PEI. Erbacher et al. reported that steric stabilization is achieved by creating a brush corona layer of PEG on the surface of polyplexes formed by PEI and

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DNA, thereby leading to an increased blood circulation time. However, the transfection efficiency of PEGylated PEI is significantly lower than that of the corresponding nonmodified PEI because of PEG's charge masking properties.[16,17]

Present polycation gene vector has difficulty in realizing effect clinical gene therapy, so new cationic polymer with optimized structure and constitution is awaited to be introduced into gene delivery field. In this article, we conveniently synthesized a polycation PCT with high nitrogen content and proton sponge effect. Via introducing comonomer, PCT is easy to be modified. Compared to PEI, PCT has slightly lower charge density after protonation, hydrophilic hydroxy and quaternized amino groups with neglectable charge shield due to their small volumes. The protonizing characteristic of PCT was studied using acid base titration. The complexing ability of PCT was verified by choosing poly(acrylic acid) (PAA) as a model polyanion. PAA has wide applications as biomaterials and was commonly used as precursors to study interpolymer complex formation.[18–22] We suppose if PCT can complex PAA, it will complex more easily stronger polyacids, for example, poly(sulfonic acid) and poly(phosphorous acid).

EXPERIMENTAL

Materials

Acrylic acid was purchased from Chengdu Kelong Chemical Reagent Company and was distilled under vacuum over cuprous chloride prior to use. 3-chloro-1,2-epoxypropane (CEP; Kelong, China) was distilled following drying with 4A molecular sieves. Tetraethylene pentamine (TEPA; Kelong, China) was distilled under vacuum following drying with KOH. Chloroform, ethanol, and ethyl ether were analytical pure agents and were used as received.

Synthesis of PCT

TEPA 5 g, 1.2*M* equivalent CEP, and 20 mL $CHCl_3$ were added into 100 mL round-bottom flask following N_2 substitution, reacted within 90°C oil bath for 8 h. After 20 mL ethanol was added, reaction mixture turned clear quickly. Reaction went on at 100°C for 36 h after most of $CHCl_3$ was separated by refluxing. The mixture was concentrated and added with 30 mL ethyl ether. PCT was precipitated during acute stirring. Above liquid was decantated and remnants gummy solid was washed with ethyl ether for three times with acute stirring. Yellow gummy solid was dried under vacuum at 85°C overnight with yield of 100%. The limiting viscosity number of PCT in deionized water was 21.2 by measurement with an Ubbelohde viscometer at 298 ± 0.1 K. The viscosity average molecular weight (M_η) was estimated as 1.7×10^4 at 0.64 of α and 1.4×10^4 of K.

Synthesis of PAA

Acrylic acid (10.09 g, 0.14 mol), potassium peroxydisulfate (0.11 g, 0.00042 mol) and 100 mL deionized water were added to 250-mL mono-neck round-bottom flask equipped with a water reflux condenser and a Teflon stir bar. The mixture was stirred to homogeneous phase at room temperature, then reacted at 60°C for 7 h and 100°C for 6 h. After the mixture was cooled to room temperature, water separator was equipped and 40 mL cyclohexane was added to flask as water-carrying reagent. Water separation was carried out at 110°C under refluxing until no water was separated. After cyclohexane was distilled, 50 mL absolute alcohol was added to flask and distilled at 110°C to remove remnant water. Concentrated alcohol solution of PAA was precipitated with ethyl ether. The resultant PAA was dried under vacuum at 85°C for 24 h. The viscosity average molecular weight (M_η) was estimated as 1.1×10^5 from intrinsic viscosity of the polymer in 2*M* NaOH aqueous solution at a constant temperature of 298 ± 0.1 K, using the Mark-Houwink-Saknrada equation.[23]

Preparation of stock solutions

Certain amounts of PCT and PAA were dissolved in 1 L deionized water respectively, to obtain their stock solutions. Here, nitrogen concentration was 1.05 m*M* for PCT stock solution and carboxylic group concentration was 6 m*M* for PAA stock solution.

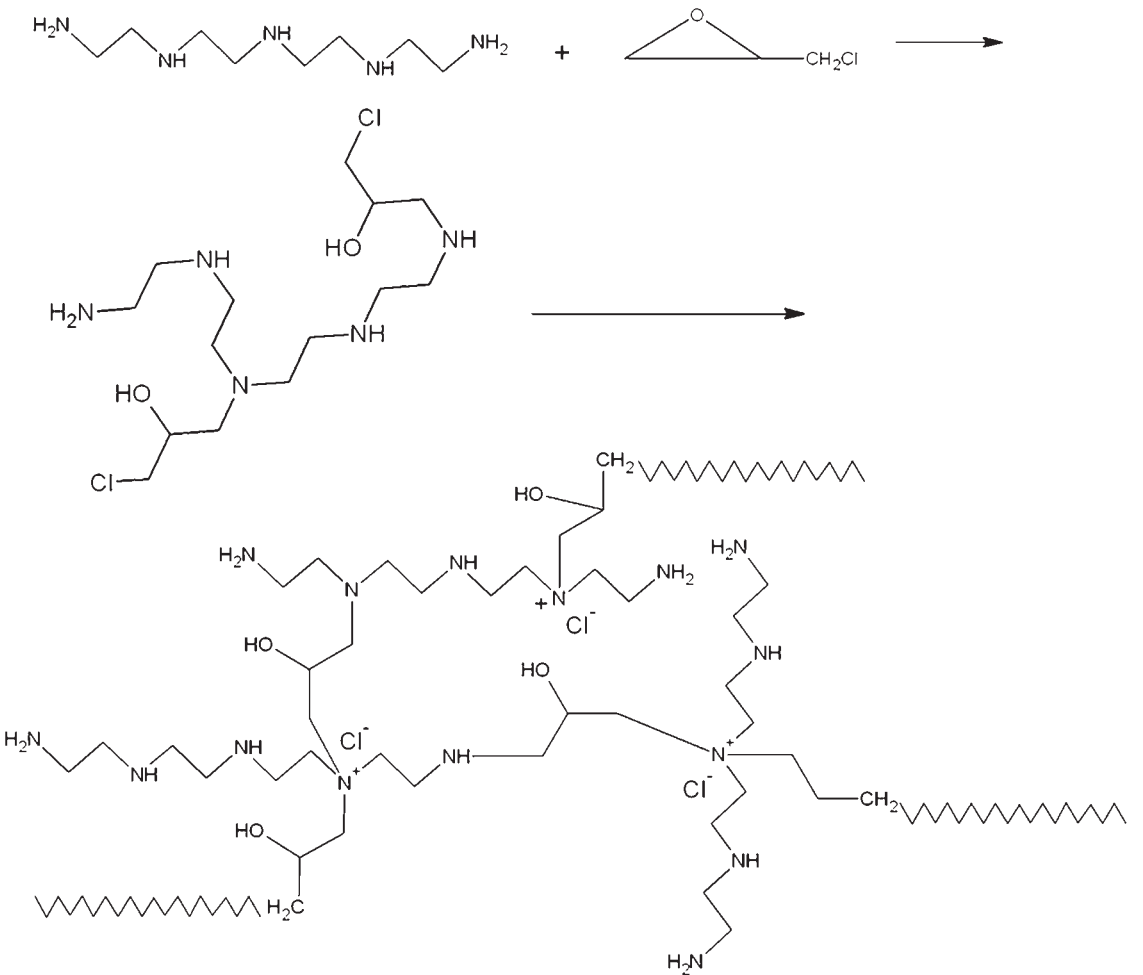
Self-assembly of PCT with PAA

Varied amounts of PCT solution were added slowly to 30 mL PAA solution under gentle vortexing. After addition, the mixture was vortexed for other 5 min. Resultant solutions were analyzed using UV-Vis photospectrometer and particle size analyzer. The mass ratio of PCT to PAA was represented as θ. The θ values studied here were 0.1, 0.2, 0.5, 0.8, and 1.7. Complex solutions were left at room temperature and at certain intervals aliquots of supernatant liquid were withdrawn to determine complex stability by UV-Vis spectroscopy.

Instrument and measurement

^1H NMR spectra were recorded on Brucker Avance DPX 300 NMR spectrometer (Brucker, Germany) using D_2O as solvent for TEPA and PCT.

0.42 g PCT containing 6.97 mmol nitrogen atom was dissolved in 10 mL 150 mmol NaCl solution, added with 0.58 mL concentrated HCl and were titrated with 0.3 mol/L NaOH solution separately. The pH value was measured with Leici pH meter (Shanghai, China).



Scheme 1 Polymerization of CEP with TEPA.

When PTE and PAA solutions were mixed, their complex nanoparticles formed immediately. The sizes of complex particles were measured by dynamic light scattering at room temperature on Malvern MASTERSIZER 2000 (Malvern Instruments, Malvern, UK). Samples were inhaled with Hydro 2000MU and deionized water was selected as dispersion medium. Between two measures, the instrument was washed three times with deionized water.

Complex solutions at varied θ values had different transparences. The UV-Vis spectroscopy was used to characterize turbidity on UV-2401PC photospectrometer (Shimadzu, Japan). Absorbances of supernatant complex solutions were monitored to study the stability of complex nanoparticles.

Fourier transform infrared spectroscopy (FTIR) was carried out on Nicolet MX-1E FTIR Spectrometer. Anhydrous samples of PAA, PCT and their complex were ground to fine power, then mixed with KBr and pressed into thin pieces for measurement.

Morphology of complex nanoparticle was observed by transmission electron microscopy on JEM-100CX (JEOL, Japan).

RESULTS AND DISCUSSION

Reaction between CEP and TEPA

Reaction between CEP and TEPA included two mechanisms. On one hand, amino groups of TEPA open the ternary ring of CEP to produce hydroxyl and tertiary amine groups. On the other hand, chloromethyl groups of CEP quaternized tertiary amines of TEPA unit resulting from first step of addition. We found that when molar ratio of CEP to TEPA was 1.5:1, crosslinking would occur, while at ratio of 1.2 crosslinking did not occur. Both kind of amines in TEPA possibly react with CEP, so hybranched polymer will form during polymerization (Scheme 1). To facilitate epoxy groups completely react with amino groups of TEPA, reaction was first carried out in solution of solvent $CHCl_3$ until precipitate produced. Then ethanol was added to make reaction mixture homogeneous and reaction went on at elevated temperature. ^1H NMR spectra of TEPA and PCT (in D_2O) were shown in Figure 1. Peak at 4.0 ppm is attributed to protons near quaternized amines and peak at 3.6 ppm is characteristic of alkyl protons containing hydroxy

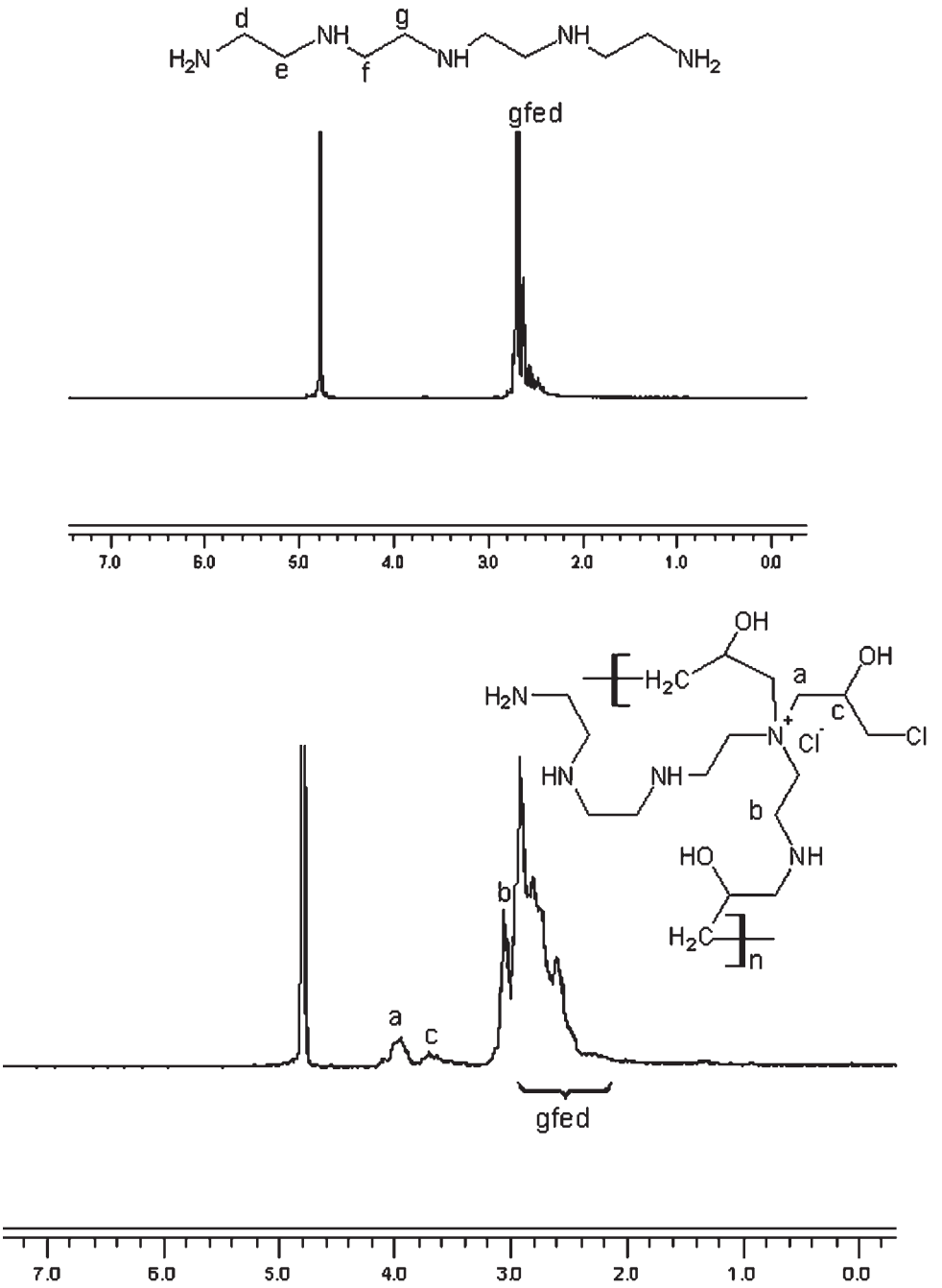


Figure 1 ^{1}H NMR spectra of TEPA and PCT (in D_2O).

groups. Occurrence of these two peaks indicated polymerization was realized by ring-opening and quaternization.

Protonization characteristic of PCT

The complexation of cationic polymer with DNA has relation with the protonization of polymer because protonized polymer shows positive electricity and interacts with ionized negative DNA via electrostatic adsorption. Protonization at endosomal acidic environments promotes endosomal disruption to release complex into cytosol favorable to transfer in cell. To check the protonization characteristic of PCT, acid-base titration was performed (Fig. 2). In the range of pH 7.4–11, protonization of PCT consumed about 0.33 mol NaOH, which is lower than 0.42 mol of TEPA. On one hand it indicates that PCT inherits protonization characteristics of TEPA. On the other hand, it shows that PCT's protonizing amino groups decreases due to quaternization during polymerization. In pH 5.0–7.4 protonizations of PCT and TEPA consumed equivalent HCl indicating PCT has similar proton sponge effect to TEPA.

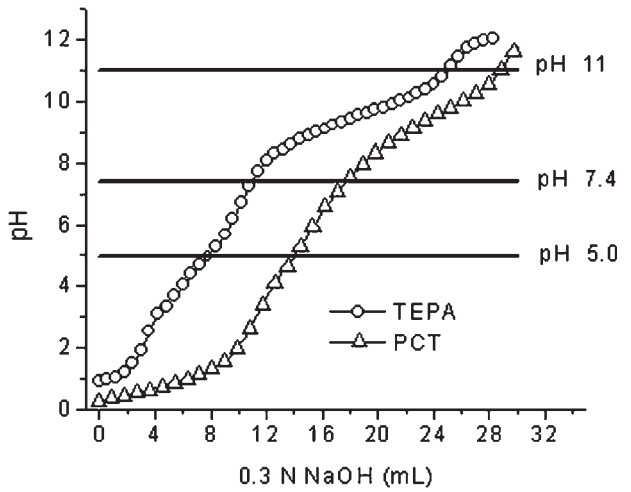


Figure 2 Titrations of PCT and TEPA with 0.3*N* NaOH.

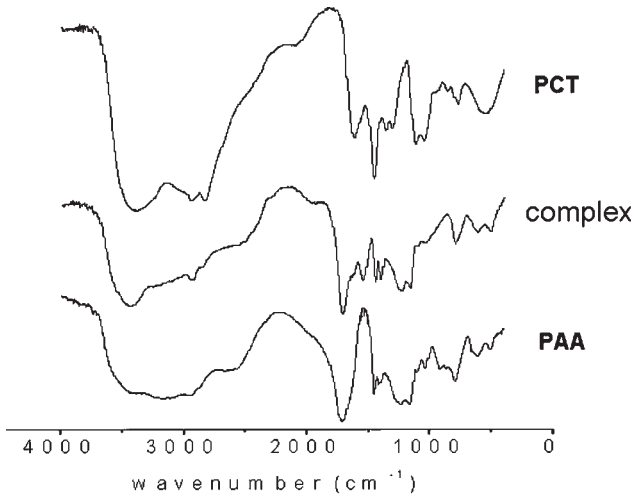


Figure 4 FTIR spectra of PAA, PTE and their complex.

A branched PEI was thought to be a prerequisite for proton sponge.[14] That is to say proton sponge effect mainly results from tertiary amino groups, because in branched PEI there are a lot of tertiary amino groups which do not exist in linear PEI. But it does not explain linear PEI is another gene vector with high efficiency. To explore cause of proton sponge effect, triethylamine only containing tertiary amine and hexamethylenediamine only containing primary amine were also studied by acid-base titration (Fig. 3). Both of them show no buffering capability between pH 5.0 and pH 7.4, which indicates that primary and tertiary amines possibly do not contribute to proton sponge effect. However, TEPA containing two primary amines and three secondary amines protonizes in both pH 5.0–7.4 and pH 7.4–11, and its protonizing nitrogen mole number in pH 7.4–11 is more than that in pH 5.0–7.4. Based on above comparison, it is concluded that proton sponge effect comes possibly from secondary amines and secondary amines contribute to not only proton sponge effect but also combining DNA. In the synthesis of PCT, part of primary amines of TEPA was consumed, which possibly was the cause of PCT's lower protonization ability than TEPA. But quaternized amines resulting from second step reaction would make up the loss of complexing ability to some extent. The first step of reaction not only transferred part of primary amines into secondary amines but also transferred part of second amines into tertiary amines, so secondary amines did not markedly change, which made PCT has similar proton sponge effect to TEPA.

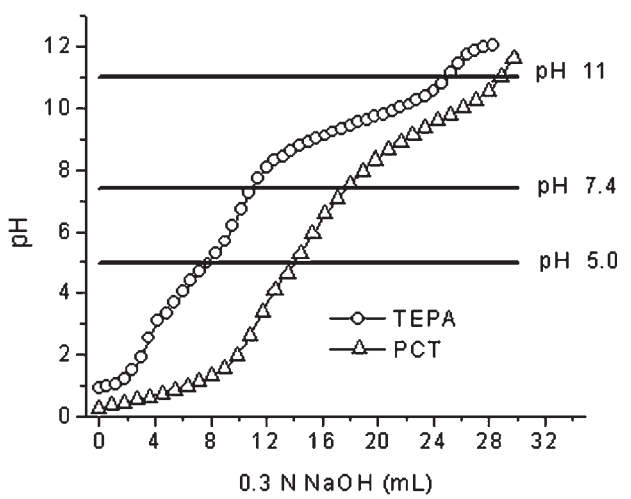


Figure 3 Titrations of triethylamine and hexamethylenediamine with 0.3*N* NaOH.

Complexation of PCT and PAA

Based on charge density, protonization traits, and molecular structure, it can be forecast that PCT has good complexation character with polyanion. To examine this supposition, we studied the complexation of PCT with PAA by FTIR spectroscopy, dynamic light scattering, and UV-Vis spectroscopy. Stable and small complex microparticle is an advantageous condition for gene therapy because small

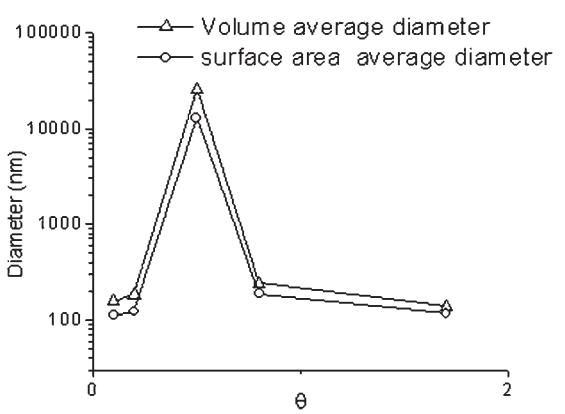


Figure 5 Complex diameters at different θ.

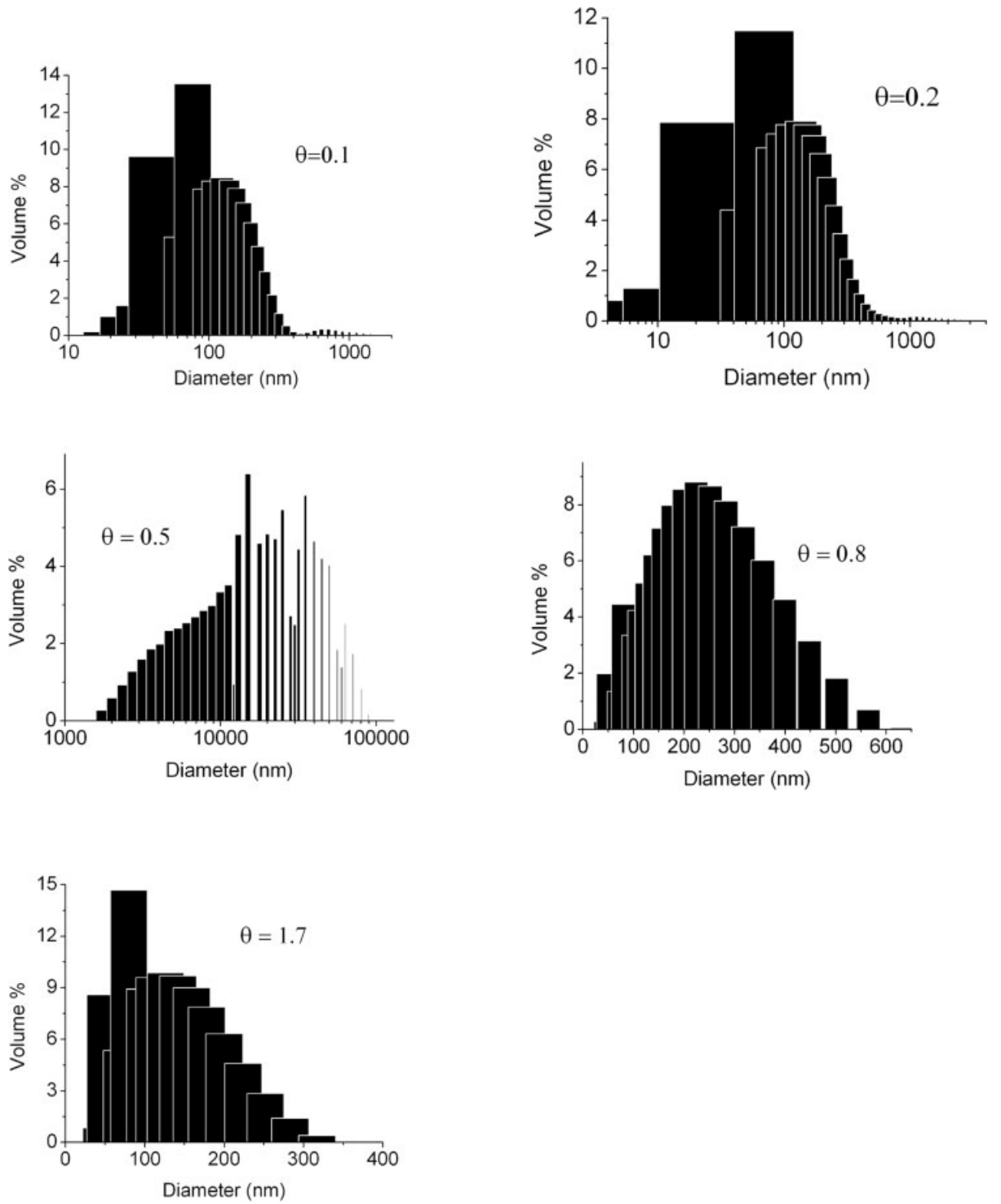


Figure 6 Particle diameter volume distributions of complex at varied θ values.

particles are easily transferred in blood circulation and into disease tissue, and then endocytosed into cell.[24]

FTIR spectroscopy of PCT, PAA and their complex

FTIR spectra of PAA, PCT and their complex are shown in Figure 4. In spectrum of PAA, a broad peak of COO—H stretching vibration is observed to overlap with CH_2 stretching band because of association of carboxylic acid group. Carbonyl group stretching vibration appears at 1714.5 cm^{-1}. Peaks at 1454.1 cm^{-1} and 1416.2 cm^{-1} are attributed to CH and CH_2 bending vibration. Peak at 1247.2 cm^{-1} is attributed to C—O stretching vibration. Peak at 1163.6 cm^{-1} is attributed to C—C stretching vibration. In spectrum of

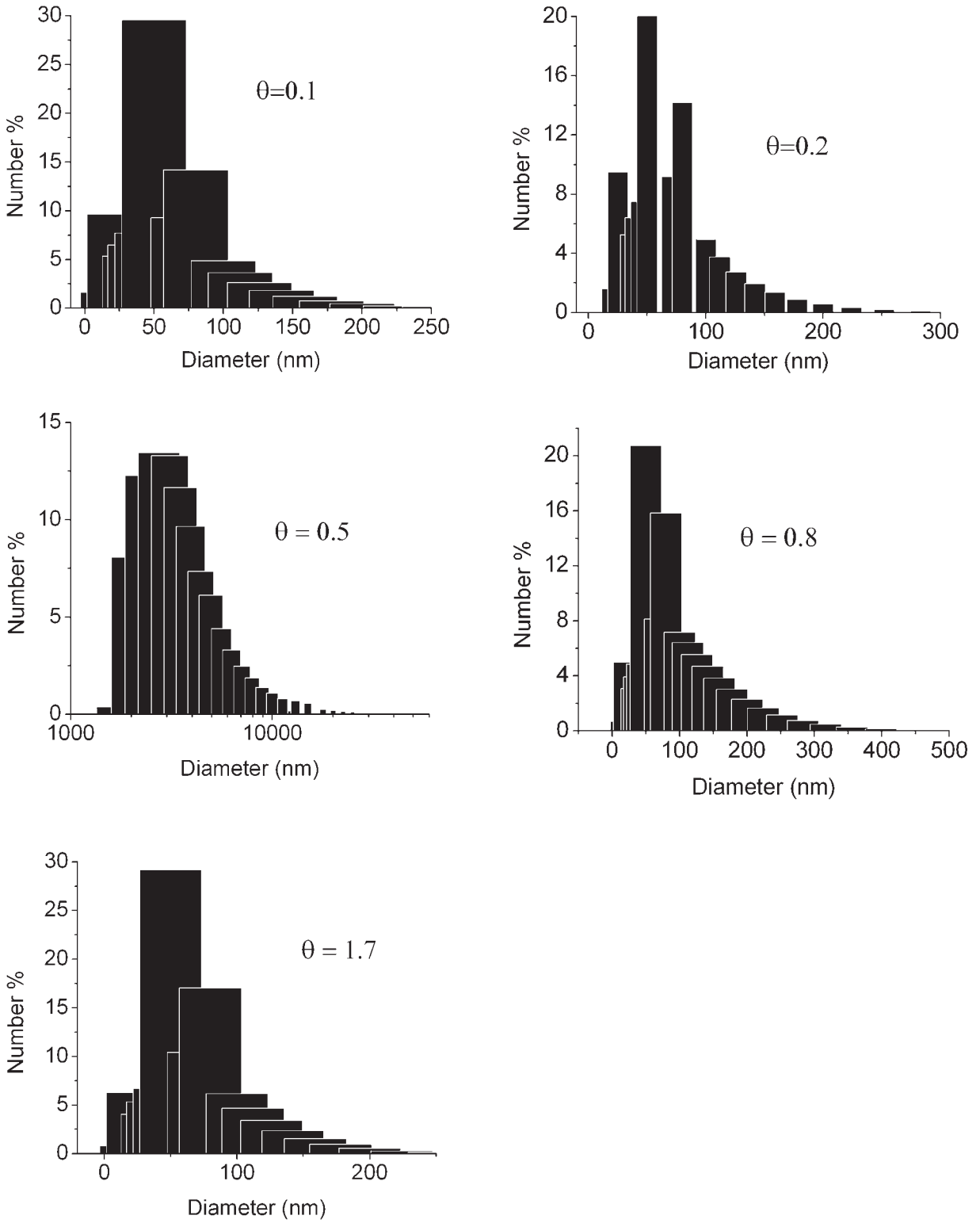
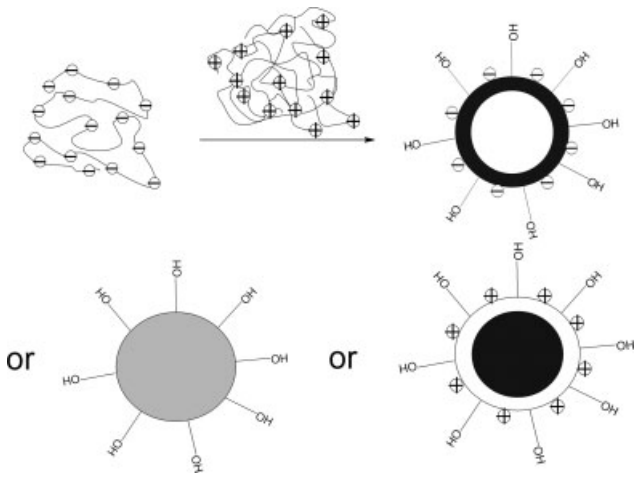


Figure 7 Particle diameter number distributions of complex at varied θ values.

PCT, NH stretching vibration occurs at 3384.5 cm^{-1}. Symmetric and asymmetric stretching vibration of CH_2 occurs separately at 2945.2 cm^{-1} and 2836.5 cm^{-1}. Peak at 1616.8 cm^{-1} is attributed to NH_2 bending vibration. Peaks at 1459.2 $cm^{-1}$and 1358.7 cm^{-1} are attributed to CH_2 bending vibration. Peak at 1309.8 cm^{-1} is ascribed to O—H bending vibration. Peak at 1116.1 cm^{-1} is attributed to C—N stretching vibration. Peak at 1053.5 cm^{-1} is attributed to C—O stretching vibration. When PAA and PCT formed complex, FTIR spectrum also produced some changes. In the spectrum of complex, peak of carboxylic acid



Scheme 2 Formation and structures of complex nanoparticles of PCT with PAA.

groups is weakened due to ammonium formation, peak at 3432.3 cm^{-1} becomes narrower. NH_2 and NH bending vibration peaks dissapear and peak characteristic of $=NH_2^+$ at 1552.4 cm^{-1} appears.

Size and morphology of complex nanoparticle

The mass ratio of PCT to PAA is defined as θ. When θ is increased, complex particle mean diameter showed a bell-shaped change (Fig. 5), which accorded with common complex of cationic polymer with DNA. When either component was much excess, that is to say at lower or higher θ, charged particle formed. Electrostatic repulsion prevented from aggregation among particle, so small complex particles were obtained. However at middle θ values, neutral or approximate neutral particles were obtained. These particles were easy to aggregate resulting in large mean particle diameter and broad diameter distribution (Figs. 6 and 7, Scheme 2). By comparison of Figure 6 with Figure 7, it was found that although at various θ values, there were some large particles forming during complexation, their amounts were very low even neglectable. When PCT complexed PAA at 1.7 of θ, complex particles were approximately global (Fig. 8).

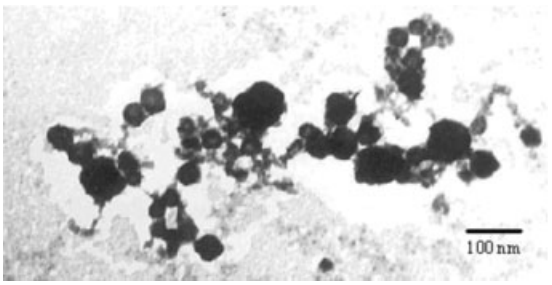


Figure 8 TEM micrograph of complex nanoparticles at 1.7 of θ.

Large particles scatter light more strongly than small ones, so when complexation was performed at middle θ values, complex solutions seemed very turbid. While when complexation was carried out at higher or lower θ values, complex solutions seemed very clear. Complex solutions at various θ values were examined in terms of absorbance (Fig. 9). When complex formed at θ of 0.5, solution had higher apparent absorbance at more than 300 nm wavelengths. The order of apparent absorbance accorded very well with that of mean diameter size, which further testifies the relation between complex particle size and θ value and indicates that UV-Vis is easy and credible way of characterizing indirectly particle size. At less than 300 nm wavelengths, complex solution at θ of 0.5 has lower apparent absorbance possibly because its particle number is lower than other solutions in which a large number of small particles contribute significantly to scattering of shortwave light.

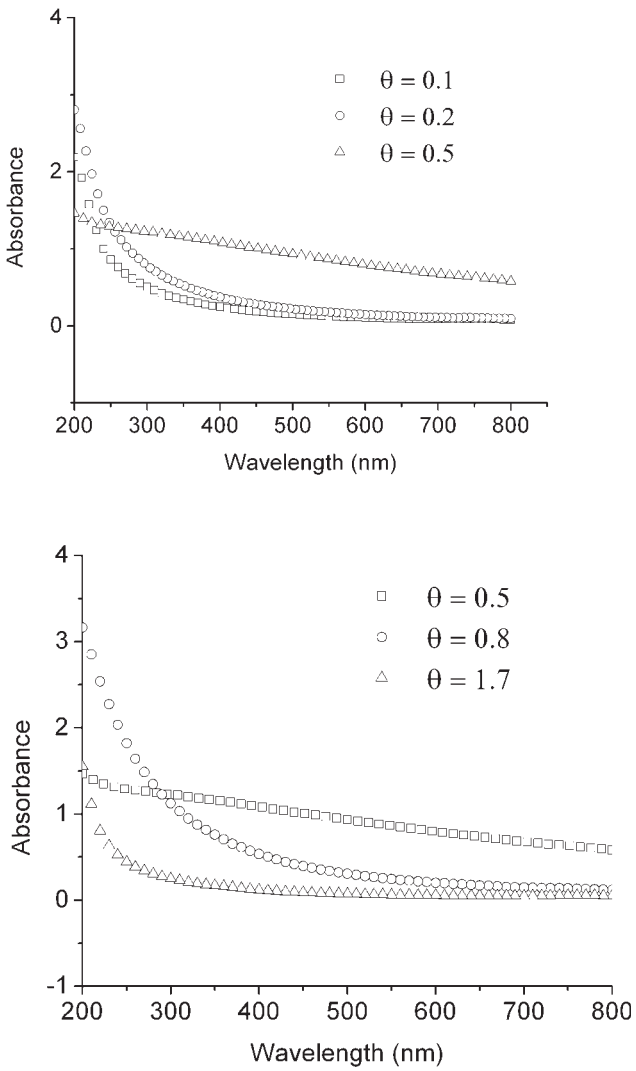


Figure 9 Apparent absorbance of complex solutions at various θ values.

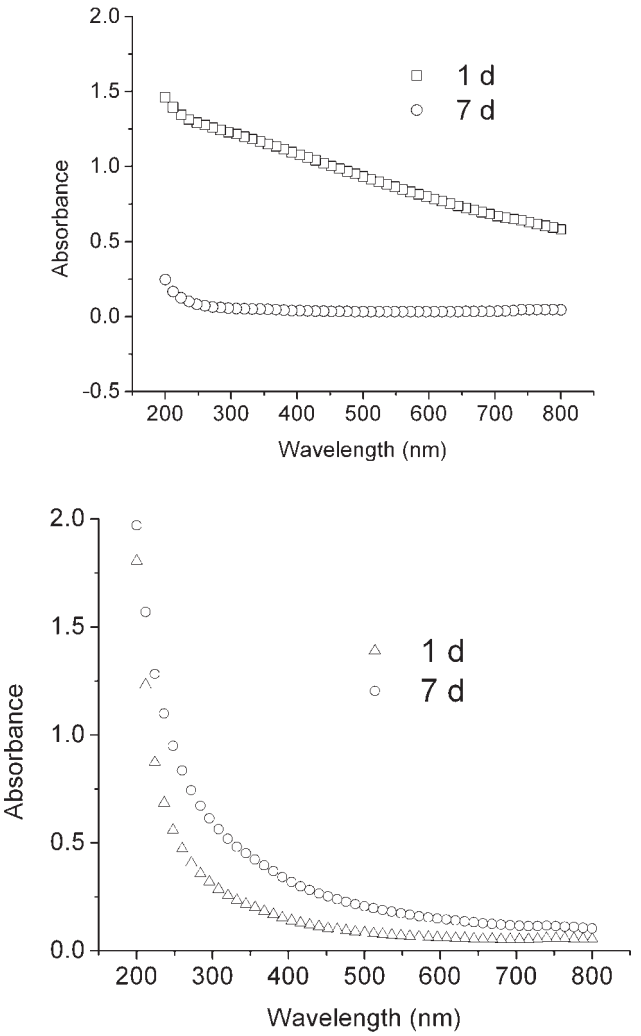


Figure 10 Apparent absorbance changes of complex solutions at θ of 0.5 (above) and 1.7 (below).

Stability of complex solution

In PCT there are a lot of quaternized amino groups and hydrophilic hydroxy groups. When PCT complexes with polyanion, a nucleus-shell construct with the stabilization of surficial hydroxy groups will form (Scheme 2). We supposed that short hydrophilic groups and quaternized amines possibly enhance cationic polymer's hydrophilicity but do not result in serious charge shielding. Adsorbance was measured at different times (Fig. 10). Apparent absorbance of complex solution forming at θ of 0.5 shows important change during 7 days. However, solution forming at θ of 1.7 has small change. This indicates that charged small particle solution has stronger stability than nearly uncharged one. Since all solutions forming at different θ values have discernable absorbance changes, it is considered that short hydroxy groups contributes weakly to solution stability. This is possibly because the hydrophobicity of unreacting chloromethyl groups disturbs hydrophilic hydroxy groups working.

CONCLUSIONS

A potential polycation gene vector PCT was synthesized by using commercially facile CEP and TEPA as monomers through two mechanisms circle-opening and quaternization. The charge density of PCT is 17.7 mmol N/g, which is larger than that of polylysine (PLL) but lower than that of PEI. So PCT is considered as a discretely charged polycation with high charge density. There are a lot of hydroxy and quaternized amino groups on PCT contributing to complex hydrophilicity. Acid base titration showed that PCT had good proton sponge effect, which possibly resulted from secondary amino groups of PCT. PCT complexed with PAA, a weak polyacid, into approximately global nanoparticles at proper mass ratio. With increasing mass ratio, mean diameter and its distribution of complex nanoparticle first increased then decreased because of electrostatic interaction. At higher or lower mass ratios, smaller complex nanoparticles were obtained and they had stronger stability than at middle mass ratios.

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References

1. Koike, H.; Tomita, N.; Azuma, H.; Taniyama, Y.; Yamasaki, K.; Kunugiza, Y.; Tachibana, K.; Ogihara, T.; Morishita, R. J Gene Med 2005, 7, 108.
2. Eliyahu, H.; Sian, S.; Azzam, T.; Domb, A. J.; Barenholz, Y. Biomaterials 2006, 27, 1646.
3. Lavertu, M.; Méthot, S.; Tran-Khanh, N.; Buschmann, M. D. Biomaterials 2006, 27, 4815.
4. Onishi, Y.; Eshita, Y.; Murashita, A.; Mizuno, M.; Yoshida, J. J Appl Polym Sci 2005, 98, 9.
5. Wen, J.; Mao, H. Q.; Li, W. P.; Lin, K. Y.; Leong, K. W. J Pharm Sci 2004, 93, 2142.
6. Kikuchi, H.; Suzuki, N.; Ebihara, K.; Morita, H.; Ishii, Y.; Kikuchi, A.; Sugaya, S.; Serikawa, T.; Tanaka, K. J Control Release 1999, 62, 269.
7. Herweijer, H.; Wolff, J. A. Gene Ther 2003, 10, 453.
8. Cheng, N.; Liu, W. G.; Cao, Z. Q.; Jia, W. H.; Liang, D. C.; Guo, G.; Zhang, J. Y. Biomaterials 2006, 27, 4984.
9. Affleck, D. G.; Yu, L.; Bull, D. A.; Bailey, S. H.; Kim, S. W. Gene Ther 2001, 8, 349.
10. Boussif, O.; Delair, T.; Brua, C.; Veron, L.; Pavirani, A.; Kolbe, H. V. J. Bioconjugate Chem 1999, 10, 877.
11. Bielinska, A. U.; Chen, C. L.; Johnson, J.; Johnson, J.; Baker, J. R. Bioconjugate Chem 1999, 10, 843.
12. Boussif, O.; Lezoualch, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Berh, J. P. Proc Natl Acad Sci USA 1995, 92, 7297.
13. van de Wetering, P.; Moret, E. E.; Schuurmans-Nieuwenbroek, N. M. E.; van Steenbergen, M. J.; Hennink, W. E. Bioconjugate Chem 1999, 10, 589.

14. von Harpe, A.; Petersen, H.; Li, Y. X.; Kissel, T. J Control Release 2000, 69, 309.
15. Werth, S.; Urban-Klein, B.; Dai, L.; Hobel, S.; Grzelinski, M.; Bakowsky, U.; Czubayko, F.; Aigner, A. J Control Release 2006, 112, 257.
16. Park, M. R.; Han, K. O.; Han, I. K.; Cho, M. H.; Nah, J. W.; Choi, Y. J.; Cho, C. S. J Control Release 2005, 105, 367.
17. Erbacher, P.; Bettinger, T.; Belguise-Valladier, P.; Zou, S. M.; Coll, J. L.; Behr, J. P.; Remy, J. S. J Gene Med 1999, 1, 210.
18. Barbani, N.; Lazzeri, L.; Cristallini, C.; Cascone, M. G.; Polacco, G.; Pizzirani, G. J Appl Polym Sci 1999, 72, 971.
19. Chun, M.-K.; Cho, C.-S.; Choi, H.-K. J Appl Polym Sci 2004, 94, 2390.
20. Garay, M. T.; Llamas, M. C.; Iglesias, E. Polymer 1997, 38, 5091.
21. Lau, C.; Mi, Y. L. Polymer 2002, 43, 823.
22. Mathieu, F.; Ugazio, S.; Carnelle, G.; Ducinf, Y.; Legrand, J. J Appl Polym Sci 2006, 101, 708.
23. Jin, S. P.; Liu, M. Z.; Chen, S. L.; Chen, Y. Eur Polym Mater 2005, 41, 2406.
24. Kircheis, R.; Wightman, L.; Wagner, E. Adv Drug Deliv Rev 2001, 53, 341.