Preparation of Monomethyl Poly (ethylene glycol)-*g*-Chitosan Copolymers with Various Degrees of Substitution: Their Ability to Encapsulate and Condense Plasmid DNA

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ABSTRACT: Chitosan (CS) has great potential as a nonvirus gene delivery vector, but its application is limited because of poor water solubility. Monomethyl poly(ethylene glycol) (mPEG)-*graft*-CS copolymers were synthesized by the reaction of mPEG–aldehyde (oxidized mPEG) with amino groups on CS chains; they showed enhanced solubility in water. Copolymers with various mPEG degrees of substitution (DS) and CS molecular weights were obtained, and their capabilities of DNA encapsulation were compared through gel retardation assay and particle size and ζ potential measurements. The effects of different ratios of primary amines on CS to the phosphate groups on DNA (N/P ratios), DS, and molecular weights on particle size and encapsulation efficiency were investigated. The results show that high N/P ratios and proper DS were necessary for the formation of well-distributed complex particles. Among all of these samples, mPEG (3.55)–CS (50 kDa)/DNA complexes [where the parentheses following mPEG indicate DS (%), and the parentheses following CS indicate the molecular weight of CS] raised the ζ potential from negative to positive most quickly, yielded the smallest particle size, and were retarded in agarose gel at the lowest N/P ratio; this indicated the best efficiency of DNA encapsulation. On the contrary, mPEG (0.80)–CS (50 kDa)/DNA complexes raised the ζ potential to positive most slowly, fluctuated around the value 0 from N/P ratios of 15 : 1 to 30 : 1, and were retarded in agarose gel at the highest N/P ratio; this indicated the lowest efficiency of encapsulating plasmids. Copolymers with desirable efficiencies of DNA encapsulation could be promising gene carriers. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 108: 2958–2967, 2008

Key words: chitosan; PEG; nonviral gene delivery system; DNA condensation

INTRODUCTION

Cationic polymers have recently aroused more and more attention for their application as nonviral gene delivery vectors. The development of a safe and efficient delivery system is a critical issue in gene therapy. Although viruses are generally thought to be more efficient at transfecting mammalian cells, the immunoresponse risk and low DNA carrying capacity are major drawbacks.[1,2]

About 30 years ago, it was discovered that multivalent cations cause DNA to collapse from spreading chains into toroid-shaped complexes.[3] The complexes condense DNA into nanoscale particles and protect DNA from nuclease.[4] Among all of these cationic polymer vectors, such as polyethyleneimine and polyamidoamine dendrimers, chitosan (CS) has made a name for its biocompatible, biodegradable, and nontoxic features.

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CS is a polysaccharide produced by the alkaline *N*-deacetylation of chitin, one of the most abundant natural polymers, and it contains 51–100% of 2-amine-2-deoxy-(1–4)-β-D-glucopyranose residues (D-glucosamine units). It is widely applied in medicine and biomaterial fields, but its application is severely hindered by its poor water solubility at physiological pH. Water-soluble chitosans could be obtained by degradation, with molecular weights of 5 kDa and below. However, these low-molecular-weight chitosans can only form weak complexes with DNA, and results in physical instability and low transfection efficiency under physiological conditions.[5] With a greater length of the polycation chain, a significant degree of compaction could be attained.[6] Stable polyplexes capable of transfection were formed only when the CS chain had a high density of positive charges, that is, a greater value of the chain length, or a higher degree of deacetylation.

To overcome its poor solubility, the introduction of hydrophilic chains onto CS has been considered. Poly(ethylene glycol) (PEG) is a polymer widely used as a pharmacological product with preferred hydrophilicity and biocompatibility. The modifica-

tion with a hydrophilic polymer would be expected to produce hydrophilic chitin or CS while keeping the fundamental skeleton intact.[7] Also, the surface stabilization of nanoparticles modified by PEG has proved to be one of the most successful approaches for keeping the particles in long-term circulation.[8]

Previous studies have investigated the influence of CS structure parameters, that is, the degree of acetylation and the degree of polymerization, on the formation of DNA–CS polyelectrolyte complexes. Studies on PEG-*g*-CS copolymers include those on their physicochemical properties,[9–11] blood compatibility,[12,13] function as thermoreversible hydrogels,[14,15] and drug-delivery vehicles[16–20] but have rarely focused on their behaviors as nonviral gene vectors.[21,22] To the best of our knowledge, research on the influence of different degrees of substitution (DS) of PEG-*g*-CS and different ratios of the number of primary amines on CS to the number of phosphate groups on DNA (N/P ratios) on the properties of PEG-*g*-CS/DNA complexes is unavailable in the literature. In this study, a series of monomethyl poly (ethylene glycol) (mPEG)-*g*-CS copolymers were prepared with different DS or molecular weights, and with DNA compacted in different N/P ratios. The effects of those factors of CS on nanoparticle formation and encapsulation efficiency, were investigated through gel electrophoresis and ζ potential and nanoparticle size measurements.

EXPERIMENTAL

Materials

CS (molecular weights = 5 and 20 kDa, degree of de acetylation = 90%) was a gift from Golden-Shell Co., Ltd. (Zhejiang, China). mPEG (molecular weight = 5 kDa) was purchased from Fluka. Trichlorisocyanuric acid (97%) and 2,2,6,6-tetramethylpiperidin-1-yloxy (free radical, 98%) was purchased from Aldrich (St. Louis, MO), and $NaCNBH_3$ was purchased from Sigma (St. Louis, MO). Other reagents were from Guangzhou Chemical Reagent Factory (Guangzhou, China). Encoding green fluorescent protein (4.7 kb; a gift from West China Center of Medical Science) was propagated in *Escherichia coli* and purified by a HQ&Q Plasmid Mini kit (U-Gene Biotechnology Co., Ltd., Anhwei, China). The purity of the plasmids consisting of supercoiled, open circular, and closed circular forms was determined by electrophoresis on a 0.9% agarose gel, and the concentration of DNA was determined by the measurement of UV absorbance at 260 and 280 nm.

Synthesis of mPEG-*g*-CS

To improve the solubility of CS at physiological pH and neutral conditions, we modified CS with mPEG. First, ω-OH of mPEG was oxidated to aldehyde according to ref. 23, in which an efficient approach of turning primary alcohols to the corresponding aldehydes was reported. Second, mPEG-aldehyde was grafted to CS by a widely accepted method.[7,14,15]

Previously dried mPEG (5 g, 1 mmol) was dissolved in 10 mL of CH_2Cl_2. 2,2,6,6-Tetramethylpiperidin-1-yloxy (20 mg, 0.13 mmol) was added, followed by the addition of 122 mg of trichlorisocyanuric acid (0.52 mmol). The reaction mixture was stirred at room temperature for 8 h and was kept overnight to ensure a complete reaction. The mixture was then precipitated by the addition of diethyl ether (250 mL), filtered, rinsed thoroughly with diethyl ether, and dried *in vacuo*. The dried white powder was then dissolved in several milliliters of distilled water and mixed with a CS solution that was made previously by the dissolution of CS into a mixture of a 2% AC_2O aqueous solution and methyl alcohol with a volume ratio of 2 : 1.

The mixture was continuously stirred for 48 h, and then stirring was paused to adjust the pH value to 6.0 by a 0.5 mol/L NaOH solution; subsequently, a proper amount of $NaCNBH_3$ was added. Then, the reaction mixture was kept stirring for the last 24 h and dialyzed against distilled water by a dialysis membrane (molecular weight cutoff = 12 kDa) for 48 h; then it was freeze dried. The yellowish mass was rinsed with acetone to remove unreactive mPEG[24] and then dried *in vacuo*. The synthesis schedule is shown in Figure 1.

Characterization of mPEG-*g*-CS

^{1}H-NMR spectra was carried out with 10 mg of polymers in a 5-mm sample tube on an NMR spectrometer (Varian, Palo Alto, CA, Mercury-Plus 300) at 300 MHz and with a 25°C probe temperature.

IR spectra of the polymers were measured by a 683-type spectrometer (PerkinElmer, Waltham, MA).

X-ray diffraction (XRD) measurements were performed with a powder diffractometer (D/MAX IIIA, Rigaku, Japan).

A differential scanning calorimeter was used to measure the thermology of the polymers. The differential scanning calorimetry (DSC) thermograms covered the temperature range 25–190°C at a scanning rate of 20 K/min on a DSC204 (Netzsch, Germany).

Preparation of the mPEG-*g*-CS/DNA complexes

The mPEG-*g*-CS copolymers were diluted in deionized water to the chosen concentrations. After vortexing, the appropriate amount of DNA solution was added to the mPEG-*g*-CS solutions to adjust the final concentrations to 40 μg/mL. The mixed solution was revortexed and incubated for at least 30 min. We calculated the

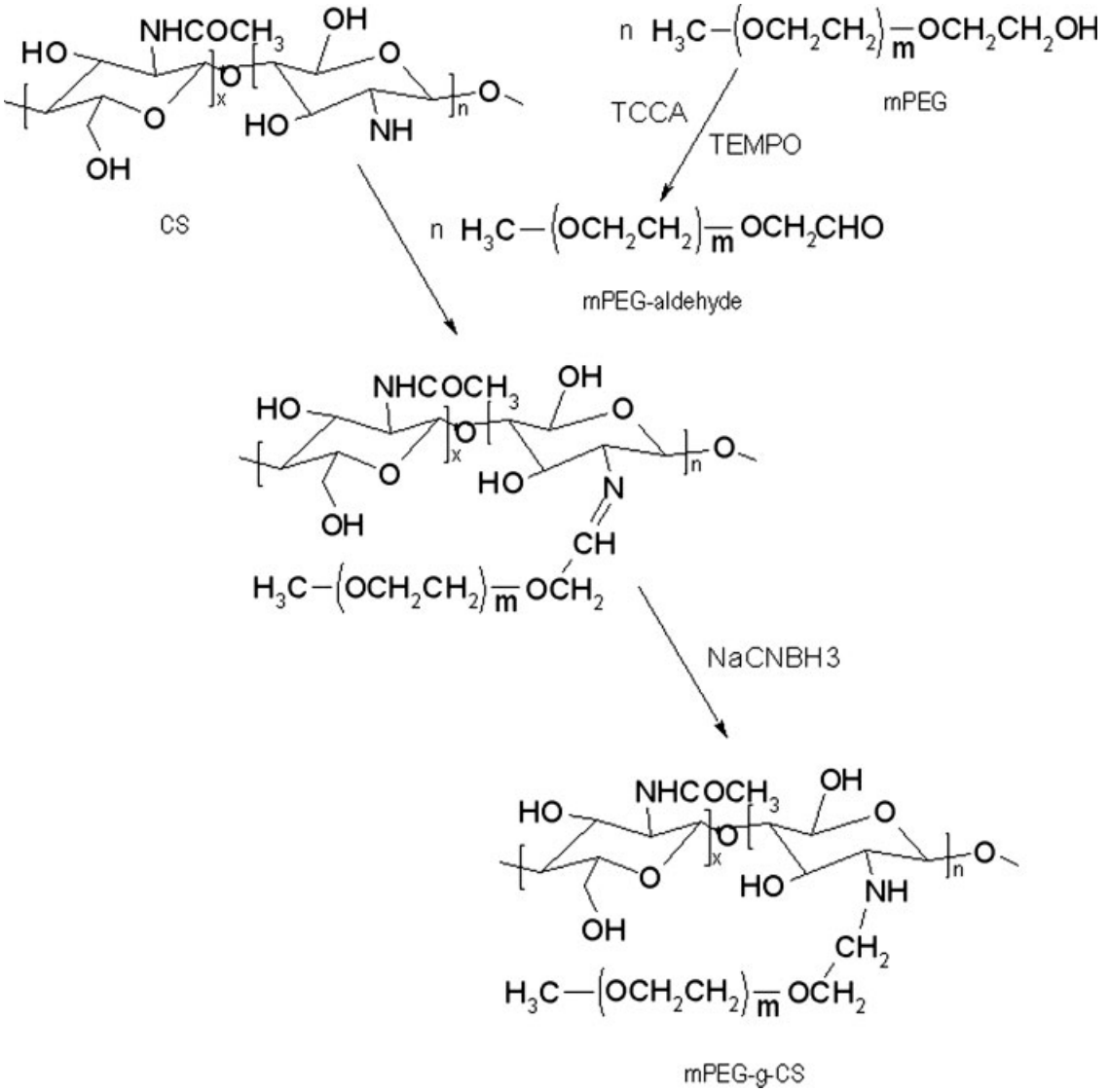

Figure 1 Schematic representation of mPEG-*g*-CS synthesis.

required amount of mPEG-*g*-CS, according to the DNA concentration and N/P ratio needed, by taking into account the fact that 1 μg of DNA contained 3 nmol of phosphate and that 1 μg of CS (degree of deacetylation = 90%) held 6.2 nmol of amine nitrogen.

DNA retardation assay

Agarose gel electrophoresis was performed to detect the migration of DNA in agarose gel with ethidium bromide (EtBr). The DNA binding ability of polymers was evaluated. The movement of negatively charged DNA to the positive electrode in an electric field can be retarded by positively charged polymers. mPEG-*g*-CS/DNA complexes (9 μL) mixed with 1 μL of loading buffer were loaded onto an EtBr-containing 0.9% agarose gel. Gel electrophoresis was performed at room temperature in TBE (45 mM Tri-HC1, 44 mM boric acid, and 10 mM EDTA) buffer at 80 V for 60 min. DNA bands were visualized by a UV (254 nm) illuminator.

Nanoparticle size and ζ potential measurements

The particle size reflects the degree of DNA condensation, and the ζ potential corresponding to the surface charge indicates the degree of coverage of positive charges over negative charges within the nanoparticles. The size of the mPEG-*g*-CS/DNA complexes was determined by dynamic light scattering at 25°C, with a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK). Each sample was scanned 30 times. The ζ potential of the complexes was measured by laser Doppler anemometry on the same instrument with an aqueous flow cell at 25°C, and each sample was scanned 30 times. All of the testing complex solutions were in the same concentrations, and N/P ratios as used in the DNA retardation measurement were used.

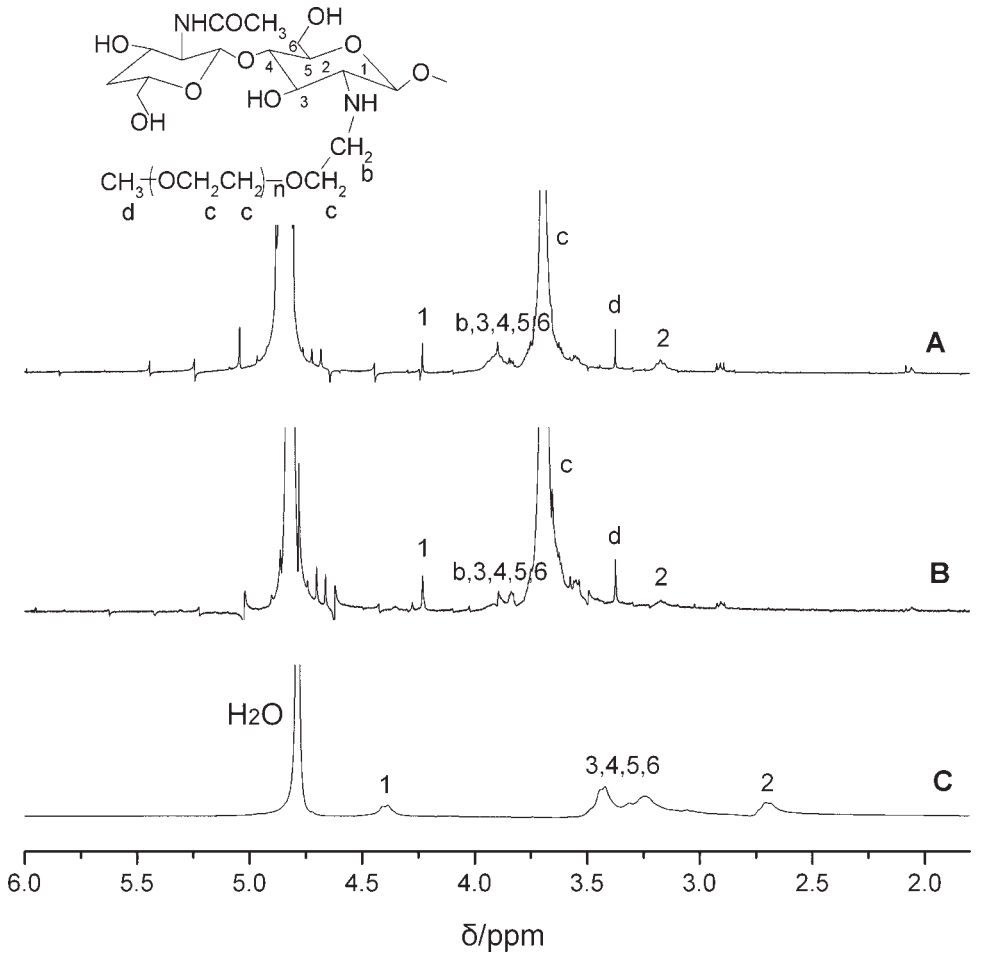

Figure 2 ^{1}H-NMR spectra of (A) mPEG (10.60)–CS (200 kDa), (B) mPEG (3.55)–CS (50 kDa), and (C) CS (50 kDa) with D_2O/CF_3COOD as the solvent.

RESULTS AND DISCUSSION

Synthesis and characterization of mPEG-*g*-CS

The mPEG was used for PEGmodification instead of PEG to avoid the crosslinking of copolymers. By varying the amount ratio of mPEG-aldehyde to CS in the reaction solution, we synthesized polymers with different DS. Increasing the amount ratio of mPEG-aldehyde to CS resulted in higher DS, but it was not linear dependent. The DS of mPEG to CS was calculated from the following formula (where M indicates the molecular weight of the polymers within the batch of the reaction and 5000 and 161 are the molecular weights of mPEG and CS saccharide residue, respectively):

$$\mathrm{DS}(\%) = \frac{(M_{\text{mPEG-}g\text{-CS}} - M_{\text{CS}})/5000}{M_{\text{CS}}/161} \times 100\%$$

When changing the molecular weight of CS from 50 to 200 kDa, only one usable sample was obtained, and the others resulted in gels for the strong intermolecular hydrogen bands. All of the resulting copolymers show enhanced water solubility compared to CS.

The ^{1}H-NMR spectrum of mPEG–aldehyde showed δ's of 3.37 (—OCH_3), 3.69 (—OCH_2—), and 9.78 ppm (—CHO; data not shown). The oxidation ratio (OR) was obtained by the calculation of the signal ratio of —CHO to —OCH_3. The experiments were repeated three times, and the weighted average value of OR was 26.8%. The white powder obtained in the first step was the mixture of mPEG–aldehyde and the unreactive mPEG. The latter could be removed by dialysis and acetone rinsing.

The ^{1}H-NMR data of mPEG-*g*-CS (Fig. 2) showed δ's of 4.25 (H-1), 3.5–3.8 (H-3, H-4, H-5, H-6, —O—CH_2—), 3.37 (CH_3O—), and 3.2 (H-2). The assignments of CS shifted to δ's of 4.4 (H-1), 3.2–3.5 (H-3, H-4, H-5, H-6), and 2.7 (H-2) for more CF_3COOD in solvent. The IR spectra of CS and mPEG-*g*-CS are shown in Figure 3. The characteristic peaks associated with mPEG at 952 and 846 cm^{-1} increased in mPEG-*g*-CS compared with CS. The

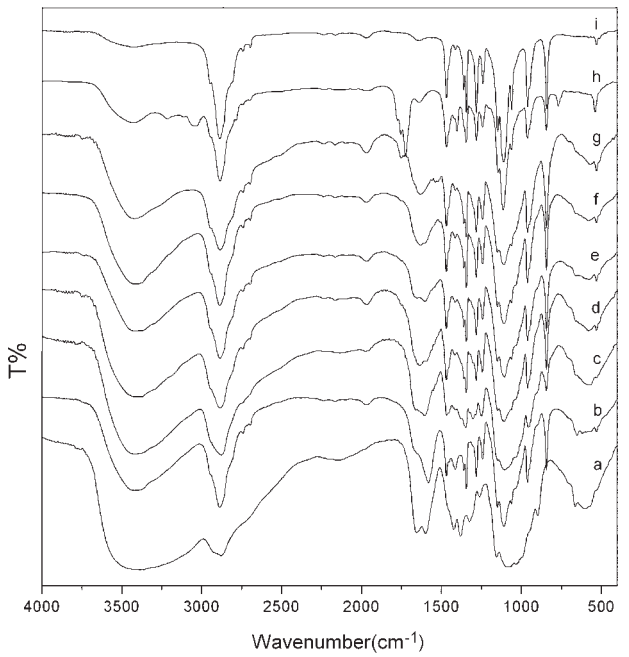

Figure 3 IR spectra of (a) CS, (b) mPEG (0.80)–CS (50 kDa), (c) mPEG (1.81)–CS (50 kDa), (d) mPEG (2.41)–CS (50 kDa), (e) mPEG (3.55)–CS (50 kDa), (f) mPEG (4.30)–CS (50 kDa), (g) mPEG (10.60)–CS (200 kDa), (h) mPEG–CHO, and (i) mPEG. T% is the percentage transmission.

intensities of the peaks corresponding to the hydroxyl groups, amino groups, and amide groups of CS at 3300–3500 cm^{-1} were significantly reduced as a result of mPEG grafting. The peaks of amide I at 1656 cm^{-1} and amide II at 1598 cm^{-1} slightly shifted, and their intensity significantly decreased, whereas the peak of —CHO in mPEG–CHO [Fig. 3(b)] at 1700 cm^{-1} disappeared in the spectrum of mPEG–CS, which showed that part of the $—NH_2$ groups of CS had reacted with mPEG–CHO. The batch weight, yield, and DS values of mPEG to CS are shown in Table I.

As is generally known, CS is a semicrystalline polymer, whereas PEG is highly crystalline with a well-defined crystal structure. We measured the XRD patterns of freeze-dried copolymers and compared them with pure mPEG and CS (Fig. 4). The pure CS showed strong reflections at $2\theta = 10$ and 20°.These two peaks disappeared with the incorporation of mPEG moieties, and strong reflections around $2\theta = 19$ and 23°, coming from the ordered arrangement of PEG, appeared and increased with mPEG content.

DSC measurements were performed on a Netzsch DSC 204 (Burlington, VT). The DSC curve of CS [Fig. 5(a)] was obtained from the second heating run at a rate of 20 K/min after the first run of heating up to 190°C and cooling to 25°C at the same rate of 20 K/min under a nitrogen atmosphere. Other samples underwent only one heating process. The large peak near 83°C could be seen in the curve of CS in the first heating [Fig. 5(b)], which corresponded to the water of crystallization. CS did not show any melting behavior, whereas mPEG–CS showed a melting peak in the range 40–55°C, corresponding to the melting of the PEG chains. As expected, the PEG melting peak increased and the peak of crystal water decreased as the proportion of mPEG became larger and larger.

Formation and characterization of the mPEG-*g*-CS/DNA complexes

The formation of the complexes was confirmed through the gel retardation assay and nanoparticle size measurement. EtBr intercalated between the base pairs of the DNA double helix, yielding a highly fluorescent DNA–EtBr complex[25] which could be detected and displayed the location of DNA in the gel. Negative DNA migrated to the positive electrode in an electric field, and three bands of free plasmids denoted three different plasmid conformations: supercoil, closed circle, and open circle, respectively. The neutral or positively charged complexes were detained and fluoresced inside the loading wells (Fig. 6).

TABLE I
Yields and DS Values of mPEG-*g*-CS

Sample[a]	CS (g)	mPEG–aldehyde (g)[b]	Output (g)		Yield (%)[d]	DS (%)[e]	
			Practical	Theoretical[c]		Practical	Theoretical
mPEG (10.60)–CS (200 kDa)	0.21	1.34	0.90	1.55	67.2	10.60	20.6
mPEG (4.30)–CS (50 kDa)	0.96	1.29	2.24	2.25	99.6	4.30	4.33
mPEG (3.55)–CS (50 kDa)	0.50	0.64	1.05	1.14	92.1	3.55	4.13
mPEG (2.41)–CS (50 kDa)	1.01	0.63	1.77	1.64	107.9	2.41	2.03
mPEG (1.81)–CS (50 kDa)	1.00	0.64	1.56	1.64	95.1	1.81	2.06
mPEG (0.80)–CS (50 kDa)	1.50	0.64	1.87	2.14	87.4	0.80	1.38

[a] The parentheses following mPEG indicate DS (%), and the parentheses following CS indicate the molecular weight of CS.
[b] The weight of mPEG–aldehyde calculated by the OR from the products of oxidated mPEG.
[c] Sum of the weight of CS and the weight of mPEG–aldehyde in one reaction.
[d] Ratio of the practical output to the theoretical output.
[e] Molar ratio of mPEG to amino groups of CS.

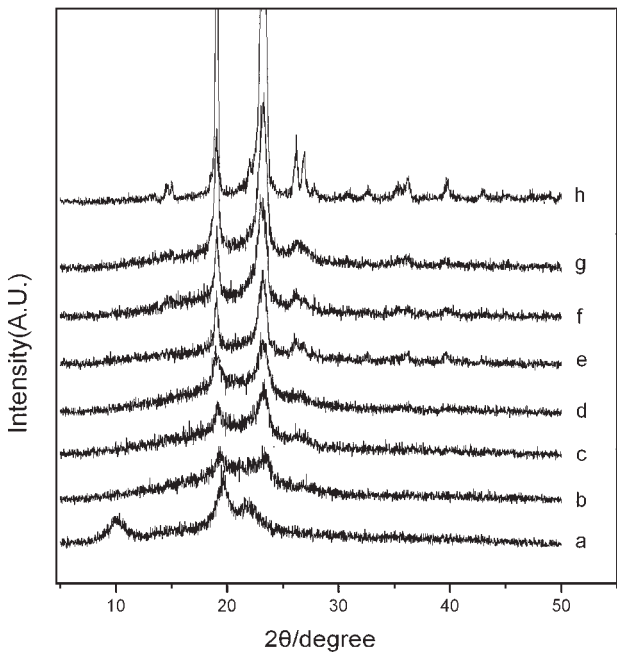

Figure 4 XRD patterns of (a) CS (50 kDa), (b) mPEG (0.80)–CS (50 kDa), (c) mPEG (1.81)–CS (50 kDa), (d) mPEG (2.41)–CS (50 kDa), (e) mPEG (3.55)–CS (50 kDa), (f) mPEG (4.30)–CS (50 kDa), (g) mPEG (10.60)–CS (200 kDa), and (h) mPEG.

Displacement of EtBr by the mPEG-*g*-CS copolymers

Upon polycation binding to DNA, EtBr is expelled from DNA–EtBr complexes, which results in a decrease in fluorescence.[26,27] Comparing with polyethylenimine (PEI) which showed a strong binding affinity with DNA and displaced EtBr at low N/P ratios,[28] CS was not so efficient as PEI. The fluorescence in the loading wells did not disappear even at N/P ratio of 80 : 1 (data not shown), indicating that CS could not displace EtBr totally.

Effect of the N/P ratio on the surface charges of the mPEG-*g*-CS/DNA complexes

In solution, the negatively charged DNA were neutralized gradually by the addition of positively charged copolymers. With increasing amounts of the mPEG-*g*-CS copolymers, the surface charges of the mPEG-*g*-CS/DNA complexes increased from negative to positive. When the surface charges were neutralized totally and became positive, the mPEG-*g*-CS/DNA complexes stopped moving to the positive electrode; this is so-called DNA retardation. Generally, the N/P ratio is used to indicate the molar ratio of amino groups on CS chains and phosphate group on DNA chains and is also used to represent the proportion of polymers and DNA within complexes. However, the N/P ratio is not equal to plus or minus the charge ratio under these experimental conditions for the incomplete protonation of CS amino groups at a neutral pH. When the N/P ratios increased, a portion of the positive mPEG-*g*-CS copolymers increased, and negatively charged plasmids were neutralized. All of the complex samples showed the same characteristics, in which the migration bands weakened gradually with increasing N/P ratio and disappeared at higher N/P ratios; this corresponded to the behaviors in which copolymers gradually retard DNA from partly to totally. We define LRR as the lowest N/P ratio totally retarding DNA. According to Figure 6, LRR indicates the N/P ratio where the bands disappeared very first.

Effect of the N/P ratio on the particle formation of complexes

Some interesting models have been established for DNA compaction by polycations.[6,29] These theoretical studies are important for helping us understand the mechanism and processes of DNA compaction. Upon low positive charge concentrations, the compaction of much larger DNA molecules by sparing or shorter positive chains is not complete, and the

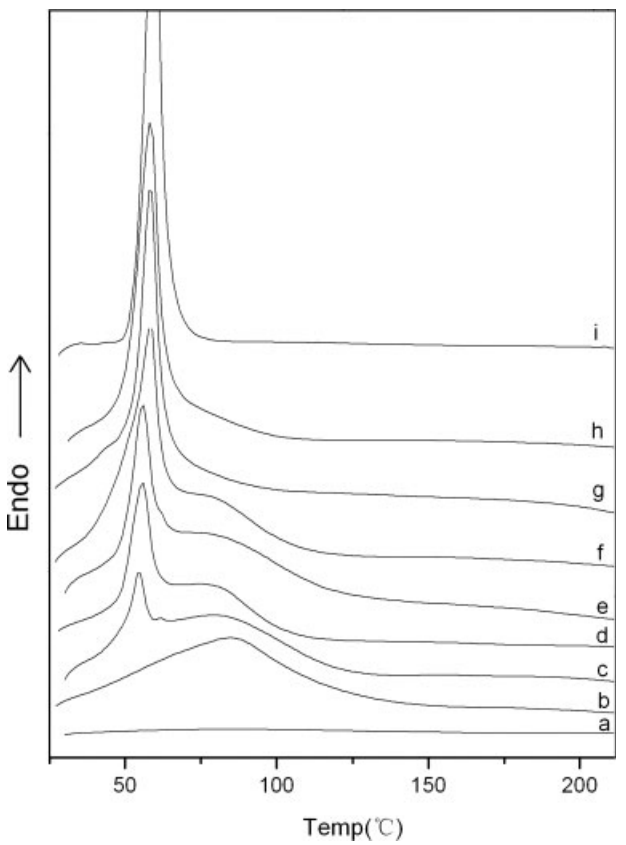

Figure 5 DSC curves of (a) CS (50 kDa) obtained from the second heating run and (b) CS (50 kDa), (c) mPEG (0.80)–CS (50 kDa), (d) mPEG (1.81)–CS (50 kDa), (e) mPEG (2.41)–CS (50 kDa), (f) mPEG (3.55)–CS (50 kDa), (g) mPEG (4.30)–CS (50 kDa), (h) mPEG (10.60)–CS (200 kDa), and (i) mPEG obtained from only one heating run at a rate of 20 K/min.

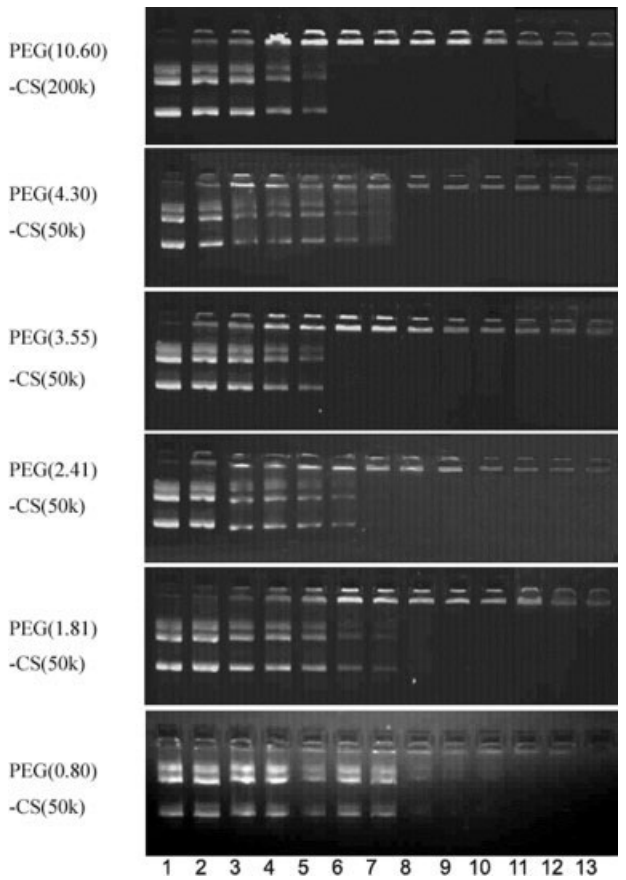

Figure 6 Agarose gel retardation of the mPEG-*g*-CS/DNA complexes. Lanes 1–13 indicate N/P ratios of 0 (naked plasmid DNA), 0.5 : 1, 1 : 1, 2 : 1, 3 : 1, 6 : 1, 8 : 1, 10 : 1, 15 : 1, 20 : 1, 30 : 1, 40 : 1, and 50 : 1, respectively. With increasing N/P ratio, the three bands of plasmid DNA gradually attenuated and finally disappeared. All of the copolymers with different molecular weights and DS's of mPEG could efficiently retard DNA, although different N/P ratios were needed for complete retardation.

complexes are formed in less well-defined shapes. When the positive charge concentration is increased to comparable with the negative charge concentration, the complexes are well shaped in a consistent particle size.[30]

For most of our samples, when the N/P ratio was as low as 1 : 1 (Fig. 7), the particle size was less well distributed, and two main peaks of the size value were obtained. The greater size values were in the range 300–600 nm, and the smaller ones were mainly in the range 100–250 nm when mPEG (1.81)–CS (50 kDa) [where the parentheses following mPEG indicate DS (%), and the parentheses following CS indicate the molecular weight of CS] even presented a size of less than 100 nm. This phenomenon was not exceptional because Erbacher et al.[31] showed two main peaks partly in the size distribution of CS/DNA complexes. The reason is that when the positive charge concentration is low, the DNA loops are condensed incompletely (this results in large particle sizes) or are condensed in parts of their domain (this results in small particle sizes). As the N/P ratio increased, most samples maintained a consistent particle size at LRR or above in a range 100–250 nm, except mPEG (4.30)–CS (50 kDa), whose particle size split to two values at LRR = 10 : 1 and even as high as 40 : 1 and 50 : 1. The explanation is that the complexes were less compact because of the steric effects caused by excess PEG chains. However, the coexisting particle size values of mPEG (4.30)–CS (50 kDa) at higher N/P ratios were smaller than those at lower N/P ratios, which indicated that the high positive charge concentrations had predominated effect on the particle size.

Influence of different DS values on the particle sizes of complexes

Because the complexes mainly showed good shapes and homogeneous size distributions when the positive charges predominated,[30] we calculated the weight-average values of nanoparticle size for every sample at LRR or above. The size value of mPEG (0.80)–CS (50 kDa) at a N/P ratio of 15:1 below LRR was counted because its ζ potential at this N/P ratio was very close to zero. Two values at one N/P ratio were averaged for mPEG (4.30)–CS (50 kDa). The results are shown in Figure 8. The sizes of these particles were not significantly different and were in the range 170–205 nm. Jiang et al.[21] reported a kind of CS-*g*-PEG/DNA complex formed at a size of about 200 nm. Lavertu et al.[32] reported that CS/DNA complexes of CS (40, 80, and 150 kDa) with different degrees of deacetylation (98, 92, 80, and 72%) were found to be in the range 200–400 nm. Strand et al.[33] used CS oligomers and obtained DNA–CS complexes in the size range 50–250 nm. All of these reports mentioned previously and our research all used the Zetasizer by Malvern for nanoparticle size measurement and revealed the fact that the molecular weight or degree of modification of CS made no significant difference to the ability of DNA condensation.

Influence of different DS values on the encapsulation efficiency

At one N/P ratio, different samples show different retardation characteristics. The samples with the same CS chain length but different DS's had different LRRs (Fig. 9). We presumed that the lower the LRR was, the higher the encapsulation efficiency of the copolymer was. mPEG (3.55)–CS (50 kDa) had the lowest LRR, although it had a medium DS. The substitution of mPEG onto CS chains had both advantages and disadvantages. The advantages of substitution were the introduction of hydrophilic chains and the destruction of the intermolecular and intramolecular hydrogen bonds, both of which

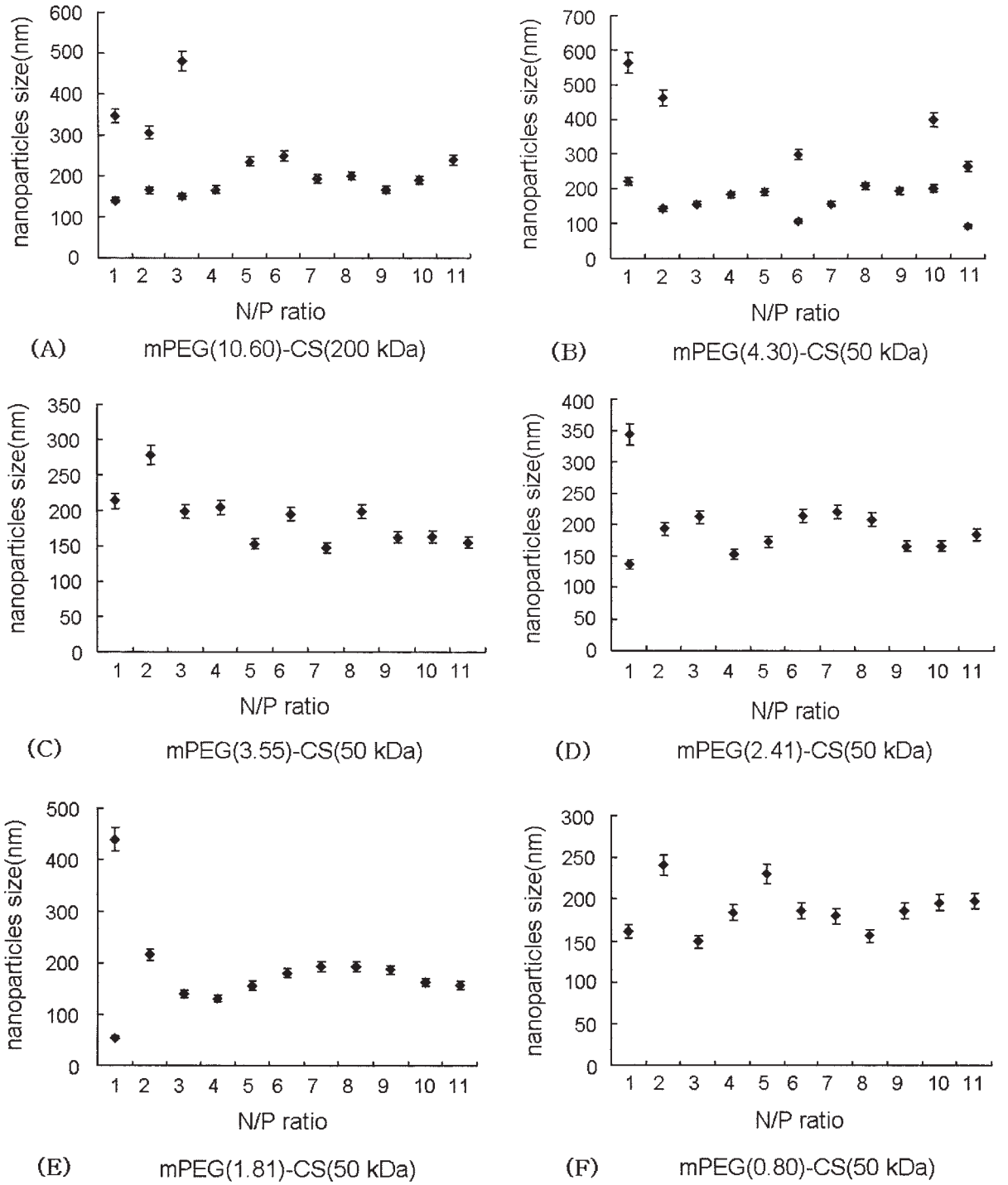

Figure 7 Different nanoparticle sizes of mPEG-*g*-CS/DNA complexes at different N/P ratios ($n = 3$). This assay was performed with a Zetasizer Nano ZS90. N/P ratios 1–11 indicate 1 : 1, 2 : 1, 3 : 1, 6 : 1, 8 : 1, 10 : 1, 15 : 1, 20 : 1, 30 : 1, 40 : 1, and 50 : 1, respectively.

enhanced the water solubility of CS. The disadvantages were the shielding of amino groups on the CS main chain and the formation of steric hindrance for DNA combination. Higher DS value, meaning larger mPEG moieties in the mPEG-*g*-CS copolymers, caused greater steric hindrance of mPEG on the CS chains; the lower DS value, meaning smaller mPEG moieties, did not efficiently enhance the water solubility of CS. In both of these situations, the copolymers could not expose enough amino groups, which was the reason why too high or too low a DS reduced the encapsulating efficiency.

On the other hand, the ζ potential curve could be used to explain the encapsulation efficiency of differ-

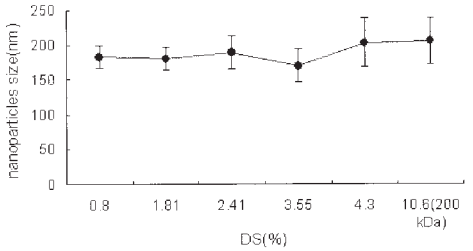

Figure 8 Average nanoparticle size values of mPEG-*g*-CS samples at LRR or above. The error bars show variance corresponding to the degree of size consistency. Smaller variances indicated more consistency in particle size.

ent samples (Fig. 10). All of the sample curves shared a similar trend: with increasing N/P ratio, ζ potential increased from a negative value (ca. −50 to −30 mV) to a positive value (ca. +10 to +30 mV). At every N/P ratio, different samples usually had different ζ potential values, and mPEG (3.55)–CS (50 kDa), with a medium DS, usually had the highest value while mPEG (0.80)–CS (50 kDa), with the smallest DS value, has the lowest. As shown in Figure 10, the curve for mPEG (3.55)–CS (50 kDa) raised most quickly and to the highest level, whereas the curve for mPEG (0.80)–CS (50 kDa) raised most slowly and fluctuated around the value 0 at a much higher N/P ratio of 15 : 1 to 30 : 1. This phenomenon indicated the highest encapsulation efficiency for mPEG (3.55)–CS (50 kDa) and the lowest efficiency for mPEG (0.80)–CS (50 kDa). The highest DS samples, mPEG (4.30)–CS (50 kDa) and mPEG (10.60)–CS (200 kDa), only had medium encapsulation efficiencies. Hence, it was not difficult to conclude that a DS of about 3.5% was the optimal value for the exposure of enough amino groups of CS (50 kDa) for DNA encapsulation.

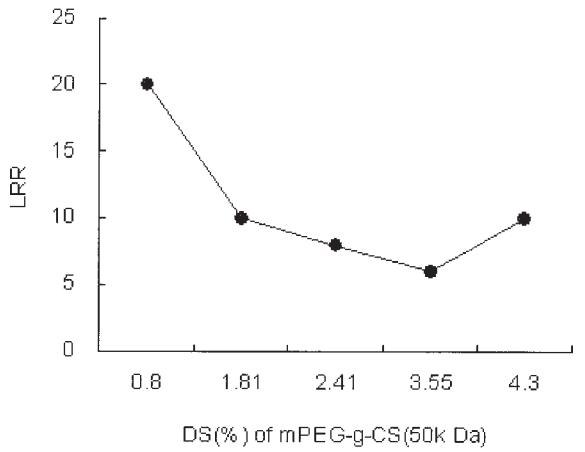

Figure 9 LRR as a function of DS of mPEG-*g*-CS (50 kDa). The values of LRR were determined by the agarose gel retardation assay, as shown in Figure 4.

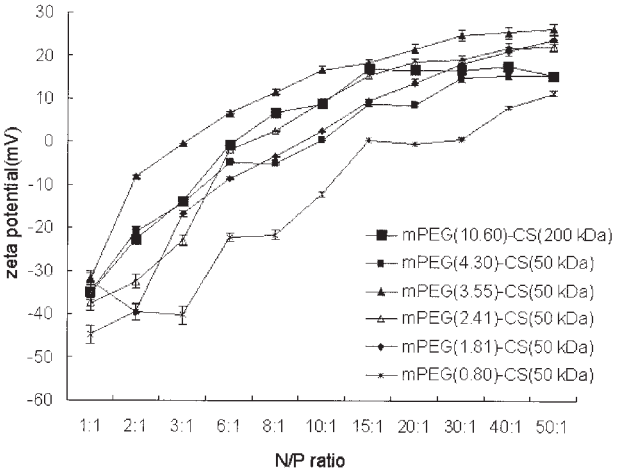

Figure 10 ζ potential of different mPEG-*g*-CS copolymers at different N/P ratios ($n = 3$). This assay was performed with a Zetasizer Nano ZS90.

Effect of the molecular weight on the encapsulation efficiency

Although mPEG (10.60)–CS (200 kDa) had a much higher DS and larger CS chains than the other samples, we could still compare mPEG (10.60)–CS (200 kDa) with mPEG (4.30)–CS (50 kDa), which had the highest DS among the CS (50 kDa) samples. Although the DS of mPEG (10.60)–CS (200 kDa) was more than double that of mPEG (4.30)–CS (50 kDa), their average size values and consistency of complexes were comparable (Fig. 8), and the ζ potential curves were also comparable (Fig. 10). mPEG (10.60)–CS (200 kDa) has a lower LRR and a better distributed particle size than that of mPEG (4.30)–CS (50 kDa) (Figs. 7 and 8). The explanation was that the greater length of the CS chains with a higher density of positive charges had a much greater encapsulation capability,[4,6] even though the substitution was much higher than that of the shorter chains.

CONCLUSIONS

MPEG-*g*-CS copolymers were successfully synthesized with various mPEG DS in the range 0.8–10.60%. MPEG-*g*-CS/DNA complexes were easily prepared in solution, and the formation procedure was affected by some parameters, such as N/P ratio, DS, and molecular weight. The plasmids were totally retarded in agarose gel at a certain N/P ratio, which we defined as LRR, for the lowest N/P ratio totally retarding DNA. The DNA encapsulating and condensing efficiencies were revealed through the LRR, ζ potential curves, and size of nanoparticles. Increasing the N/P ratio, meaning an increase in the polymer-to-plasmid ratio, resulted in positive complexes with smaller and more consistent particle sizes at

LRR or above. The lower the LRR was, the higher the DNA encapsulating efficiency of the copolymers was. The optimal DS for DNA compaction was neither too high nor to low because mPEG (3.55)–CS (50 kDa) with a medium DS value (3.55%) showed the highest encapsulating efficiency. MPEG (10.60)–CS (200 kDa) showed a comparable efficiency with mPEG (4.30)–CS (50 kDa), which demonstrated that a high density of positive charges was important for DNA compaction and that different molecular weight did not much affect DNA condensation or complex size. The copolymers maintaining good capability of encapsulating and condensing DNA could thus be potentially used as nonviral gene carriers.

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References

1. Dincer, S.; Turk, M.; Piskin, E. Gene Ther 2005, 12, S139.
2. Akhtar, S. Gene Ther 2006, 13, 739.
3. Gosule, L. C.; Schellman, J. A. Nature 1976, 259, 333.
4. Koping-Hoggard, M.; Tubulekas, I.; Guan, H.; Edwards, K.; Nilsson, M.; Varum, K. M.; Artusson, P. Gene Ther 2001, 8, 1108.
5. Koping-Hoggard, M.; Mel'nikova, Y. S.; Varum, K. M.; Lindman, B.; Artursson, P. J Gene Med 2003, 5, 130.
6. Dias, R. S.; Pais, A. A. C. C.; Miguel, M. G.; Lindman, B. J Chem Phys 2003, 119, 8150.
7. Sugimoto, M.; Morimoto, M.; Sashiwa, H.; Saimoto, H.; Shigemasa, Y. Carbohydr Polym 1998, 36, 49.
8. Moghimi, S. M.; Szebeni, J. Prog Lipid Res 2003, 42, 49.
9. Kulkarni, A. R.; Hukkeri, V. I.; Sung, H. W.; Liang, H. F. Macromol Biosci 2005, 5, 925.
10. Kolhe, P.; Kannan, R. M. Biomacromolecules 2003, 4, 173.
11. Liu, L.; Li, F.; Fang, Y.; Guo, S. Macromol Biosci 2006, 6, 855.
12. Sharon, S.; Mai-Ngam, K. Colloids Surf B 2005, 42, 147.
13. Mansoor, M. A. Carbohydr Polym 1997, 32, 193.
14. Bhattarai, N.; Matsen, F. A.; Zhang, M. Macromol Biosci 2005, 5, 107.
15. Bhattarai, N.; Ramay, H. R.; Gunn, J.; Matsen, F. A.; Zhang, M. J Controlled Release 2005, 103, 609.
16. Prego, C.; Torres, D.; Fernandez-Megia, E.; Novoa-Carballal, R.; Quinoa, E.; Alonso, M. J. J Controlled Release 2006, 111, 299.
17. Mao, S.; Germershaus, O.; Fischer, D.; Linn, T.; Schnepf, R.; Kissel, T. Pharm Res 2005, 22, 2058.
18. Aktas, Y.; Yemisci, M.; Andrieux, K.; Gursoy, R. N.; Alonso, M. J.; Fernandez-Megia, E.; Novoa-Carballal, R.; Quinoa, E.; Riguera, R.; Sargon, M. F.; Celik, H. H.; Demir, A. S.; Hincal, A. A.; Dalkara, T.; Capan, Y.; Couvreur, P. Bioconjugate Chem 2005, 16, 1503.
19. Mao, S.; Shuai, X.; Unger, F.; Wittmar, M.; Xie, X.; Kissel, T. Biomaterials 2005, 26, 6343.
20. Huh, K. M.; Cho, Y. M.; Chung, H.; Kwon, I. C.; Jeong, S. Y.; Oova, T.; Lee, W. K.; Sasaki, S.; Yui, N. Macromol Biosci 2004, 4, 92.
21. Jiang, X.; Dai, H.; Leong, K. W.; Goh, S. H.; Mao, H. Q.; Yang, Y. Y. J Gene Med 2006, 8, 477.
22. Yun, Y. H.; Jiang, H.; Chan, R.; Chen, W. J Biomater Sci Polym Ed 2005, 16, 1359.
23. Luca, L. D.; Glacemeili, G.; Porcheddu, A. Org Lett 2001, 3, 3041.
24. Muslim, T.; Morimoto, M.; Saimoto, H. Carbohydr Polym 2001, 46, 323.
25. Lepecq, J. B.; Paoletti, C. J Mol Biol 1967, 27, 87.
26. Izumrudov, V. A.; Zhiryakova, M. V.; Kudaibergenov, S. E. Biopolymers 1999, 52, 94.
27. Parker, A. L.; Oupicky, D.; Dash, P. R.; Seymour, L. W. Anal Biochem 2002, 302, 75.
28. Strand, S. P.; Danielsen, S.; Christensen, B. E.; Varum, K. M. Biomacromolecules 2005, 6, 3357.
29. Dias, R. S.; Pais, A. A. C. C.; Miguel, M. G. Colloids Surf A 2004, 250, 115.
30. Danielsen, S.; Varum, K. M.; Stokke, B. T. Biomacromolecules 2003, 5, 928.
31. Erbacher, P.; Zou, S.; Bettinger, T.; Steffan, A. M.; Remy, J. S. Pharm Res 1998, 15, 1332.
32. Lavertu, M.; Methot, S.; Tran-Khanh, N.; Buschmann, M. D. Biomaterials 2006, 27, 4815.
33. Strand, S. P.; Danielsen, S.; Christensen, B. E.; Varum, K. M. Biomacromolecules 2005, 6, 3357.