All-Trans Retinoic Acid Release from Polyion-Complex Micelles of Methoxy Poly(ethylene glycol) Grafted Chitosan

Dong-Gon Kim, Young-Il Jeong, Jae-Woon Nah

Department of Polymer Science and Engineering, Sunchon National University, Jeonnam 540-742, Korea

Received 25 July 2006; accepted 26 January 2007
DOI 10.1002/app.26480
Published online 24 May 2007 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: We synthesized a methoxy poly(ethylene glycol) grafted chitosan (ChitoPEG) copolymer to prepare a retinoic acid (RA) encapsulated polymeric micelle. The RA-encapsulated polymeric micelle of the ChitoPEG copolymer had a particle size of 100–500 nm and a spherical shape when observed by transmission electron microscopy. In a ^{1}H-NMR study, the specific peaks of RA and chitosan as a drug-carrying inner core disappeared in deuterium oxide, and only the specific peak of methoxy poly (ethylene glycol) (MPEG) was observed, whereas specific peaks of MPEG, RA, and chitosan appeared in dimethyl sulfoxide. This indicated that the RA/ChitoPEG ion complexes were composed of a polymeric micelle with a core–shell structure and that free drug did not exist in the polymeric micelle formulations. Other evidence of drug incorporation into the polymeric micelle was witnessed in a differential scanning calorimetry analysis. The melting peaks of RA and chitosan were 182 and 220°C, respectively. The melting peak of the polymeric micelle was 200°C, whereas the melting peaks of the physical mixtures were those of both RA and the ChitoPEG copolymer. The lyophilized polymeric micelle was successfully reconstituted into phosphate-buffered saline without the aid of cryoprotectants. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 3246–3254, 2007

Key words: drug delivery systems; micelles; polyelectrolytes; polysaccharides; self-assembly

INTRODUCTION

Polymeric micelles have been extensively employed for drug delivery systems for intravenous injection during the last decade.[1–5] Polymeric micelles have peculiar properties that are based on a hydrophobic inner core and a hydrophilic outer shell; they indicate that the hydrophobic inner core acts as a drug-incorporating domain through hydrophobic interactions and/or ionic interactions, whereas the hydrophilic outer shell protects the drug-incorporating inner core from an attack by the reticuloendothelial system (RES) and the adsorption of proteins.[4,5] The advantages of polymeric micelle over conventional drug carriers are many: prolonging the blood circulation of drugs, passive tumor targeting, small particle size, solubilization of hydrophobic drugs, reduction of the adverse effects of anticancer drugs, preservation of bioactive agents in the micellar core for long duration, and so forth.[1–7] Recently, Kataoka's group[6,7] reported on polymeric micelles based on an ion-complex formation between poly(aspartic acid) and a water-soluble, metallic anticancer agent, cisplatin. They were composed of an ion-complex inner core of the cationic, metallic anticancer drug cisplatin and anionic poly(aspartic acid), whereas poly(ethylene glycol) formed the hydrated outer shell. The cisplatin-incorporated polymeric micelles showed prolonged blood circulation of the drug and enhanced anticancer effects. Alternatively, Thünemann and Beyermann[8] reported that poly(ethylene imine) (PEI) complexes with retinoic acid (RA) formed nanoparticles that allowed for the controlled release of RA. The synthesis of a nanoparticulate complex occurred between the anionic drug RA and cationic PEI with sizes ranging from 170 to 580 nm. Others have reported[8,9] complexes between poly(ethylene oxide)-*b*-poly(L-lysine) and RA and the formation of core–shell-type micelles.

In this study, we prepared an RA-incorporated polymeric micelle of methoxy poly(ethylene glycol) grafted chitosan (ChitoPEG) to develop an intravenously injectable drug delivery system. The RA-encapsulated polymeric micelle of ChitoPEG formed an ion-complex inner core composed of water-soluble cationic chitosan and the anionic drug RA, whereas methoxy poly(ethylene glycol) (MPEG) formed a hydrated outer shell. Chitosan is a natural polymer derived from chitin by deacetylation. Be-

Correspondence to: J.-W. Nah (jwnah@sunchon.ac.kr).
Contract grant sponsor: National Research Laboratory Program of the Korea Science and Engineering Foundation.

cause chitosan is regarded as biocompatible, biodegradable, and nontoxic, it is an interesting biomaterial for its ability to be used as a drug-carrying material and its ease of modification.[10] Furthermore, chitosan has been reported to enhance drug delivery across the nasal or mucosal layer without any damage incurred by the drug.[11,12] Because chitosan is soluble only in an acidic aqueous solution and its use is limited as a bioactive agent in such applications, water-soluble chitosan, which can be easily dissolved in a neutral aqueous solution, has recently been reported by several authors.[13–15] RA and its analogues, the retinoids, regulate cell behavior during development and play key roles in cell fate determination, cell division, and cell differentiation.[16] RA is especially effective in the treatment of epithelial and hematological malignancies such as breast cancer, head and neck cancers, ovarian adenocarcinoma, and acute promyelocytic leukemia.[17–22]

RA-encapsulated polymeric micelles of ChitoPEG were prepared through the ion complex of RA and chitosan, and their characteristics were studied with dynamic light scattering, ^{1}H-NMR, and transmission electron microscopy (TEM).

EXPERIMENTAL

Materials

Water-soluble chitosan (molecular weight = 10,000 Da, deacetylation degree = 97.0%) was a gift from Chittolife Co., Ltd. (Seoul, Korea). Methoxy poly(ethylene glycol)–*N*-hydroxysuccimide (MPEG–NHS; molecular weight = 2000 g/mol) was purchased from SunBio Co. (Ansan, Korea). All-trans RA, 4-dicyclohexylcarbodimide (DCC), and *N*-hydroxysuccimide (NHS) were purchased from Sigma Co., Ltd. (St. Louis, MO). High-pressure performance liquid chromatography (HPLC) grade ethyl alcohol (EtOH), dimethylformamide, dimethyl sulfoxide (DMSO), and dichloromethane (DCM) were purchased from Sigma. DMSO (*d*-form for the NMR study) and deuterium oxide (D_2O) were purchased from Sigma. The dialysis tube (molecular weight cutoff = 12,000 g/mol) was purchased from SpectraPor Co., Ltd. (Compton, CA). The dialysis tube was treated with hot water (100°C) for 30 min and then washed with tap water for 2 h before use.

Synthesis of the ChitoPEG copolymer

The synthesis procedure for ChitoPEG was as follows: 100 mg of chitosan was dissolved in 0.2 mL of deionized water and diluted with 9.8 mL of DMSO. To this solution, MPEG–NHS, dissolved in 2 mL of DMSO, was added, and the mixture reacted overnight under a nitrogen atmosphere. After that, the resulting solution was dialyzed against plenty of deionized water for 2 days, and this was followed by its lyophilization. The lyophilized solid was resuspended into plenty of DCM to remove unreacted MPEG–NHS three times and fractionated into deionized water, and this was followed by its lyophilization.

Gel permeation chromatography (GPC) measurements

The absolute molecular weight (molecular weight) and molecular weight distribution, represented as the polydispersity index, of the ChitoPEG copolymers were measured with a gel permeation chromatograph equipped with a multi-angle laser light scattering detector (18-angle detector, Wyatt, Santa Barbara, CA), as previously reported by Son et al.[23] The samples were dissolved in 0.5*M* ammonium acetate (pH 5.5; at more than 5 different concentrations ranging from 0 to 1.0 mg/mL), and the increments in the reflective index were measured with a Pot-LAB reflectometer (Wyatt). Then, the absolute molecular weight and molecular weight distribution of the ChitoPEG copolymers were obtained from the GPC chromatogram with light scattering data (Debye plot regressions). The mobile phase was a 0.5*M* ammonium acetate buffer (pH 5.5), and the flow rate was 0.5 mL/min. The injection volume was 0.2 mL (10 mg/mL).

HPLC measurements

To analyze RA encapsulated in the polymeric micelle by HPLC, 5 mg of the lyophilized polymeric micelle was redistributed into 0.3 mL of deionized water, and then 1.2 mL of EtOH was added slowly for 10 min with sonication (bar-type sonicator, Vibracell, Sonic & Materials, Inc.) at 4°C. This solution was treated with additional sonication for 10 min (25-s pulse with 5-s intervals, 20 times) at 4°C and magnetically stirred for 3 h. This solution (0.1 mL) was mixed with 0.9 mL of EtOH and then injected into the column.

The mobile phase (methanol/water = 95 : 5 v/v) was degassed by sonication under reduced pressure for 3 min before use. RA was separated by isocratic elution chromatography at a flow rate of 1.5 mL/min and at 50°C with a Phenomenex Sphereclone 5micro ODS(2) column (250 × 4.60 mm) (Torrance, CA). A 5-cm Phenomenex Sphereclone 5micro ODS(2) precolumn (30 × 4.60 mm) was placed between the injector and the column. UV absorbance detection was performed at 345 nm. For standard tests, RA was dissolved in EtOH, and 20 μL was injected into the column. The retention time of RA was about 3.8 min.

Preparation of the RA-encapsulated polymeric micelles of ChitoPEG

An RA-encapsulated polymeric micelle of ChitoPEG was prepared through an ion complex between RA

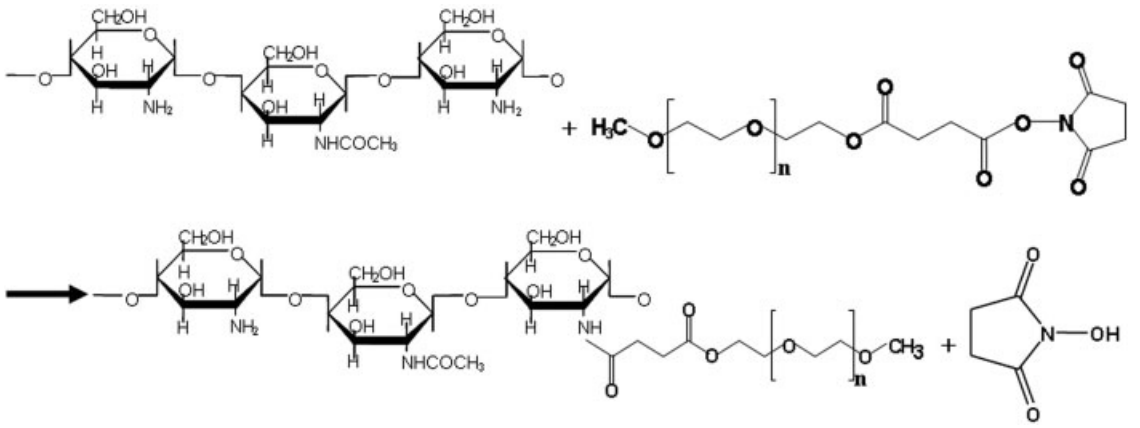

Figure 1 Synthesis scheme of the ChitoPEG copolymer.

and chitosan as follows: 20 mg of ChitoPEG was dissolved in 10 mL of deionized water (0.2% w/v). RA, dissolved in 0.1 mL of EtOH, was dropped into this solution with mild magnetic stirring at 25°C for 10 min. After that, EtOH was evaporated with a rotary evaporator for 30 min at 40°C under a vacuum. The resulting solution was analyzed or lyophilized for 2 days.

For the measurement of the drug contents and loading efficiency, 1 mL of the polymeric micelle solution was added to 9 mL of DMSO, and the contents of RA were estimated with UV spectrophotometry (345 nm, UV-1200, Shimadzu Co., Ltd., Kyoto, Japan) or the HPLC method as described previously. An equivalent amount of the ChitoPEG copolymer was used as a blank test.

All procedures were performed under dark conditions:

$$\text{Drug contents} = \frac{\text{Amount of RA in the polymeric micelles}}{\text{Weight of polymeric micelles}} \times 100$$

$$\text{Loading efficiency} = \frac{\text{Residual amount of RA in the polymeric micelles}}{\text{Feeding amount of RA}} \times 100$$

^{1}H-NMR spectrometry measurements

The ^{1}H-NMR spectra of the copolymer and polymeric micelle were measured in D_2O or DMSO (*d*-form) with a 400-MHz NMR spectrometer (Avance 400FT-NMR, Bruker, Germany).

Differential scanning calorimetry (DSC) measurements

A DSC study of the RA-encapsulated polymeric micelle of the ChitoPEG copolymer was carried out with a DSC 2910 differential scanning calorimeter (TA Instruments Co., Leatherhead, United Kingdom) to measure the melting and thermal decomposition temperatures from room temperature to 300°C under a nitrogen atmosphere (10°C/min).

Measurement of the particle size

The particle size and ζ potential of the polymeric micelles were measured with an ELS-8000 electrophoretic LS spectrophotometer (Nicomp 380 ZLS ζ-potential/particle sizer, Otsuka Electronics, Inc., Tokyo, Japan) equipped with a He–Ne laser beam at a wavelength of 632.8 nm at 25°C (scattering angle = 90°). A sample solution was used for the particle size measurement (concentration = 1 mg/mL).

TEM observations

For the TEM observations, a drop of the polyion-complex micelle suspension was placed on a carbon film coated on a copper grid. The observation was performed with a JEOL JEM-2000 FX II at 80 kV (Tokyo, Japan).

Reconstitution study of the RA-encapsulated polymeric micelles

For a reconstitution study, 5 mg of the lyophilized RA-encapsulated polymeric micelle was simply mixed with 5 mL of phosphate-buffered saline (PBS; pH 7.4, 0.1*M*), and this was followed by gentle magnetic stirring. The reconstituted polymeric micelle was used to measure the particle size distribution.

RESULTS

Characterization of the ChitoPEG copolymer

The ChitoPEG copolymer was composed of a nonionic, hydrophilic MPEG side chain and a cationic

TABLE I
Characterization of the ChitoPEG Copolymers

	MPEG feeding ratio (%)	M_n by ^{1}H-NMR	Molecular weight by GPC			DS of MPEG	
			M_n	M_w	Polydispersity	NMR[a]	GPC[b]
Chitosan	—		9,335	1,218	1.305 ± 0.918		
ChitoPEG-1	5	16,411	14,290	17,000	1.189 ± 0.294	6.1	4.3
ChitoPEG-2	10	22,907	19,630	25,040	1.276 ± 0.685	11.7	8.9
ChitoPEG-3	15	24,067	23,860	29,040	1.217 ± 0.684	14.7	12.5
ChitoPEG-4	20	31,839	32,280	44,560	1.380 ± 0.265	19.4	19.8

M_n = number-average molecular weight; M_w = weight-average molecular weight.
[a] DS of MPEG was evaluated as follows:

$$\mathrm{DS} = \frac{\text{Proton integration ratio of methyl group of MPEG}/3}{\text{Proton integration ratio of C1 position of chitosan} + (\text{Proton integration ratio of acetyl group of chitosan}/3)} \times 100$$

[b] DS of MPEG was evaluated by GPC as follows:

$$\mathrm{DS} = \frac{(M_n \text{ of ChitoPEG copolymer of GPC} - M_n \text{ of chitosan})/2000}{\text{Degree of polymerization of chitosan}} \times 100$$

chitosan main chain, as shown in Figure 1. The ChitoPEG copolymers were synthesized through the activation of carboxylic acid of MPEG with a DCC/NHS system and the introduction of MPEG into the chitosan main chains. Four different kinds of ChitoPEG copolymers were synthesized, each having a different number of MPEG side chains, as summarized in Table I. The higher molecular weight ChitoPEG copolymer was synthesized by an increase in the feed amount of activated MPEG. To remove the unreacted MPEG, the resulting copolymer was purified by precipitation into DCM because MPEG was freely soluble in DCM, whereas chitosan was insoluble. Figure 2 shows the ^{1}H-NMR spectra of chitosan, MPEG, and the ChitoPEG copolymer. As shown in Figure 2, chitosan had its specific peaks between 1.5 and 4.5 ppm, and the specific peak of MPEG was about 3.7 ppm. The ^{1}H-NMR spectra of ChitoPEG showed specific peaks of both chitosan and MPEG, indicating that MPEG was successively grafted to the chain of chitosan. Furthermore, the degree of substitution (DS) of MPEG with respect to chitosan was estimated from the proton integration ratio between the C1 position (4.5 ppm in a D_2O/DMSO solvent mixture), acetyl group (1.8 ppm) of chitosan, and methyl group of MPEG (3.4 ppm at $CDCl_3$ and

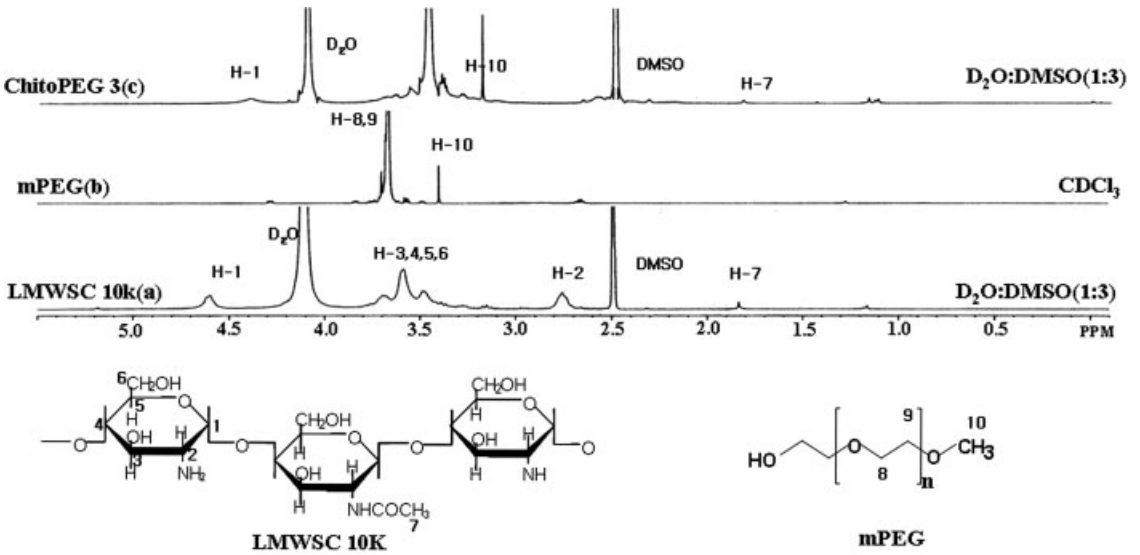

Figure 2 ^{1}H-NMR spectra of the ChitoPEG copolymer: (a) chitosan (LMWSC 10K) dissolved in a D_2O/DMSO (1/3) mixture, (b) the MPEG copolymer dissolved in $CDCl_3$, and (c) the ChitoPEG copolymer dissolved in a solvent mixture of D_2O and DMSO (1/3).

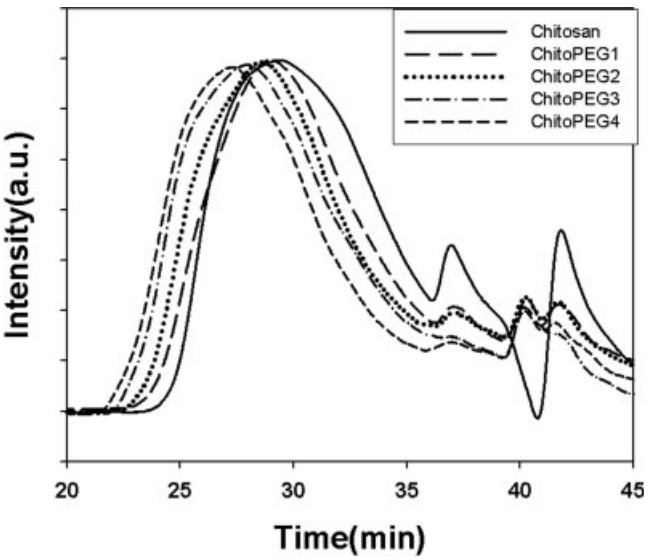

Figure 3 GPC chromatogram of the ChitoPEG copolymer.

3.3 ppm in a D_2O/DMSO solvent mixture). DS of MPEG was calculated with the proton integration ratio between the C1 position at 4.5 ppm, the acetyl group of chitosan at 1.8 ppm, and the methyl group of MPEG at 3.4 ppm. From these results, the DS of MPEG and the molecular weight of the ChitoPEG copolymer were calculated. We designed the feed ratio of MPEG to be 5, 10, 15, and 20% for the synthesis procedure, and the resulting DS values of MPEG were 6.1, 11.7, 14.7, and 19.4, respectively. Furthermore, a typical GPC profile is shown in Figure 3. As shown in Figure 3, the retention time of the ChitoPEG copolymer gradually decreased according to the increased DS of MPEG. The estimated molecular weights of the ChitoPEG copolymers are summarized in Table I. These results show that DS of MPEG corresponded to the feed ratio of MPEG.

Characterization of the RA-encapsulated polymeric micelles of the ChitoPEG copolymer

Figure 4(b) shows a TEM photograph of the RA-encapsulated polymeric micelle of ChitoPEG-2. As shown in Figure 4(b), spherical, nanosized particles were observed with TEM with a particle size of around 50–300 nm, and this indicated that it was possible to form spherical particles through ion-complex formation between chitosan and RA, as illustrated in Figure 4(a). As illustrated in Figure 4(a), nanosized particles formed a core–shell-type micellar structure, and this indicated that the ion complex between chitosan and RA formed an inner core, and the nonionic domain, MPEG, formed a hydrated outer shell. As summarized in Table II, the drug contents were 7.2–8.3 with the variation of the ChitoPEG copolymer. The higher the drug feed ratio was, the higher the drug contents were. The increased drug contents of the polymeric micelle resulted in an increased particle size and a decreased ζ potential. Furthermore, the higher the DS was of MPEG, the larger the particle size was and lower the ζ potential was.

Figure 5 shows the FTIR spectra of the RA-encapsulated polymeric micelle of the ChitoPEG copoly-

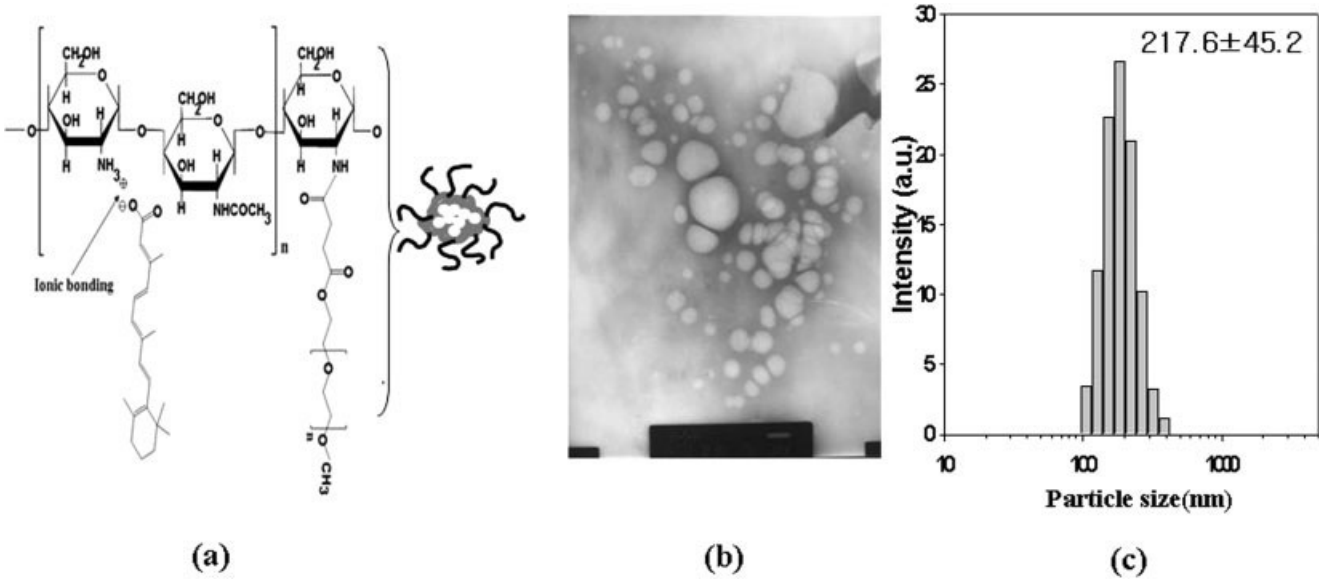

Figure 4 (a) Schematic illustration of core–shell-type nanoparticle formation between the ChitoPEG copolymer and RA, (b) TEM photograph of a core–shell-type nanoparticle of ChitoPEG-2/RA (ChitoPEG/RA = 20/2 w/w), and (c) particle size distribution of ChitoPEG-2/RA nanoparticles.

TABLE II
Characterization of ChitoPEG/RA Ion-Complex Polymeric Micelles

	Polymer/ drug ratio	Drug content (% w/w)	Loading efficiency (% w/w)	Particle size (nm)			ζ potential (mV)
				Intensity-average	Weight-average	Number-average	
ChitoPEG-1	20/2	7.2	79.3	232.9 ± 55.9	226.3 ± 43.1	213.8 ± 32.8	40.29
ChitoPEG-2	20/2	7.4	81.9	229.5 ± 51.4	191.8 ± 39.1	182.4 ± 41.4	27.53
ChitoPEG-3	20/1	4.0	83.7	321.5 ± 32.8	283.1 ± 42.1	228.5 ± 21.6	33.09
	20/2	7.8	85.3	352.8 ± 43.1	320.6 ± 57.3	285.4 ± 41.2	30.97
	20/3	12.6	96.4	405.8 ± 54.2	340.8 ± 61.7	259.7 ± 23.2	17.85
ChitoPEG-4	20/2	8.3	90.8	432.8 ± 32.9	404.6 ± 51.4	350.4 ± 21.1	29.42

mer. The carbonyl group of RA at 1690 cm^{-1} was relatively reduced in the polyion-complex micelle of RA/ChitoPEG, and this indicated that the ion complex was formed between the carboxyl group of RA and the amine group of chitosan.[8,9]

The core–shell micellar structure of the polyion-complex micelles was confirmed by a ^{1}H-NMR study. As shown in Figure 6, RA had its original characteristic peaks (in the region of 1.6 and 2.4 ppm). Because ChitoPEG was soluble in both D_2O and DMSO,[100] the characteristic peaks of MPEG (3.4–3.5 ppm) and chitosan (4.1 ppm) were observed in the mixture of D_2O and DMSO. In D_2O, the characteristic peaks of both chitosan and RA of the polymeric micelle disappeared, and only the peaks of MPEG were observed, whereas all the characteristic peaks were observed in DMSO or $CDCl_3$. These results confirmed that ion complexation between RA and chitosan formed a rigid inner core in an aqueous environment, whereas MPEG formed a hydrated outer shell.

Figure 7 shows a quantitative analysis of the RA-encapsulated polymeric micelle of ChitoPEG. As shown in Figure 7(a), the HPLC chromatogram of the RA polymeric micelle increased in proportion to the RA contents. These results indicate that RA in the polymeric micelle was not affected by the encapsulation process, and almost all of the encapsulated RA was decomplexed by its dissolution in a D_2O/DMSO mixture (1/9 v/v). Furthermore, RA in all the formulations of the polymeric micelles revealed a similar retention time in the HPLC chromatogram, and this indicated that RA was not changed by the complexation/decomplexation process. As shown in Figure 8, RA in the UV spectrophotometry measurements showed the maximum absorption peaks at 345 nm. The absorption peaks of the RA-encapsulated polymeric micelle became a broad spectrum with a maximum peak at 440 nm. However, when the polymeric micelle was dissolved in water/DMSO, RA from the polymeric micelle showed absorption spectra similar to that of RA itself. These results indicate that the peculiar properties of RA in the polymeric micelle were not affected by the ion-complexation process, and the decomplexed RA showed UV absorption spectra that were identical to that of RA itself.

Figure 9 shows the thermal characteristics of RA-encapsulated polymeric micelles of ChitoPEG-3 by DSC analysis. As shown in Figure 6, the intrinsic melting temperatures of RA and chitosan were 182 and 200°C, respectively. The polymeric micelle encapsulating RA showed the melting temperature of RA (200°C), whereas the melting temperature of physical mixtures showed both 182 (RA) and 220°C (ChitoPEG copolymer). These results indicate that RA was encapsulated into the core of the polymeric micelles by an ion complex with chitosan.

Figure 10 shows the reconstitution of the RA-encapsulated polymeric micelle of ChitoPEG-3. As shown in Figure 10, the lyophilized RA-entrapped polymeric micelle was 200–500 nm according to the series of formulations between RA and the ChitoPEG copolymer. After its reconstitution into a PBS aqueous solution, the particle size slightly decreased, and

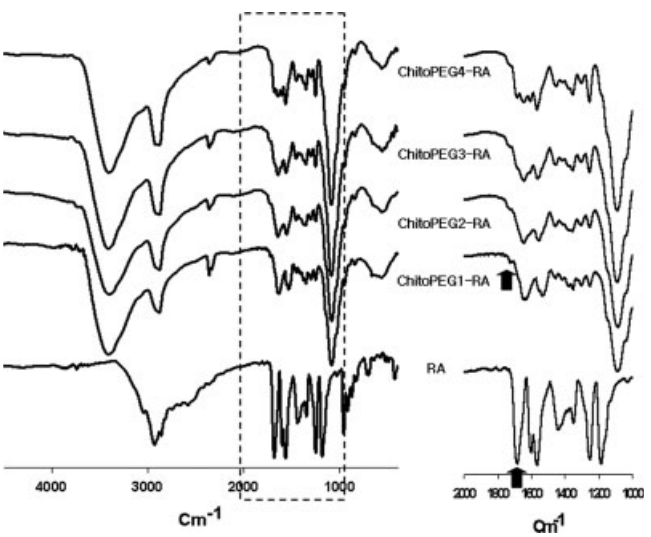

Figure 5 FTIR spectra of RA-encapsulated core–shell-type nanoparticles of the ChitoPEG copolymer versus the drug contents: (a) RA, (b) ChitoPEG-1/RA (weight ratio = 20/3), (c) ChitoPEG-2/RA (weight ratio = 20/3), (d) ChitoPEG-3/RA (weight ratio = 20/3), and (e) ChitoPEG-4/RA (weight ratio = 20/3).

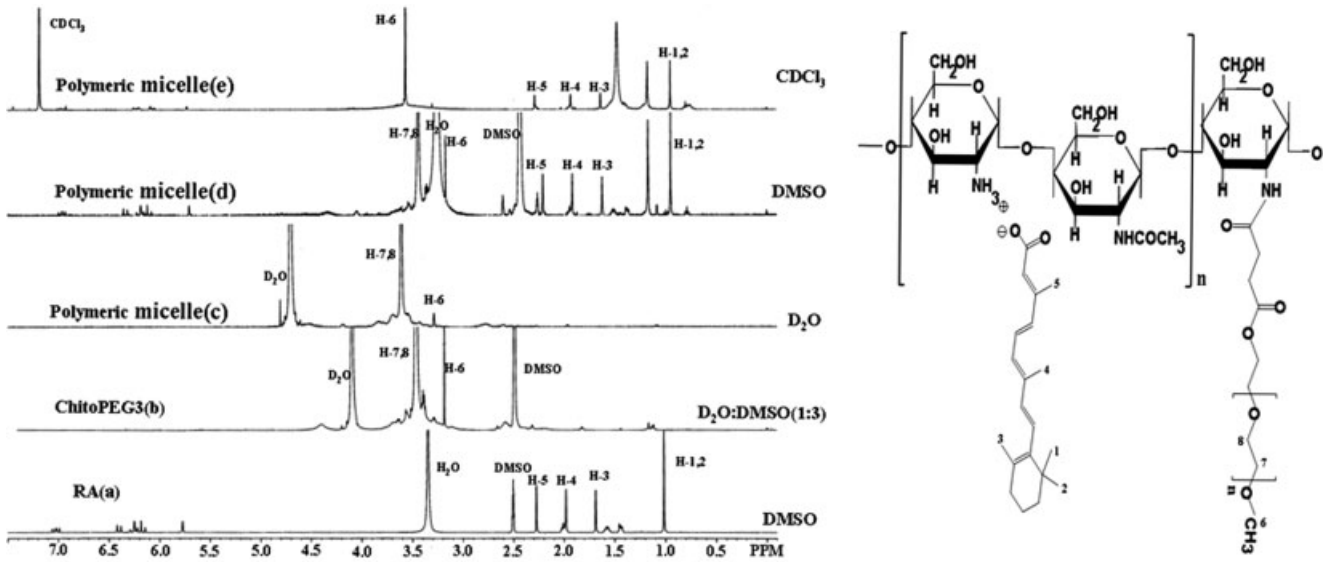

Figure 6 ^{1}H-NMR spectra of the core–shell-type nanoparticles of ChitoPEG-2/RA: (a) RA in DMSO, (b) the ChitoPEG copolymer in D_2O and DMSO, (c) the RA-encapsulated ChitoPEG core–shell-type nanoparticles in DMSO, (d) the RA-encapsulated ChitoPEG core–shell-type nanoparticles in D_2O, and (e) the RA-encapsulated ChitoPEG core–shell-type nanoparticles in $CDCl_3$.

there was no significant aggregation in the reconstitution process.

DISCUSSION

Polymeric micelles have been extensively investigated for their potential for resolving drug targeting issues.[1–7] A polymeric micelle is formed through the self-assembling process of amphiphilic polymers. Furthermore, the structural properties, that is, the hydrophobic inner core and the hydrophilic outer shell, are advantageous for the solubilization of hydrophobic drugs, avoidance of RES, reduction of side effects of anticancer drugs, and so forth. Several research groups have reported on polymeric micelle formation based on ion complexes between polymers and drugs.[6–9] Basically, the inner core of polyion-complex micelles is composed of an ion complex between a polymer and a drug, and the outer shell is composed of hydrophilic domains such as MPEG.

To make the RA-encapsulated polyion-complex micelles, we synthesized a graft copolymer composed of a chitosan backbone and MPEG side chains. It is expected that the cationic polymer, chitosan, may compose a polyion-complex core with the anionic drug, RA, and the MPEG domain may compose a hydrophilic outer shell. Because RA is a practically water-insoluble and photosensitive drug, nanoparticles[8] or polymeric micelles[9] are thought to be appropriate candidates for the effective intravenous injection of RA or another administration route.

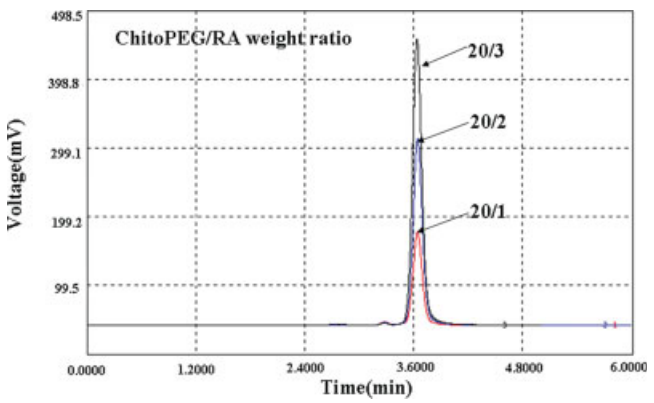

Figure 7 HPLC chromatogram of an RA-encapsulated polymeric micelle of the ChitoPEG copolymer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

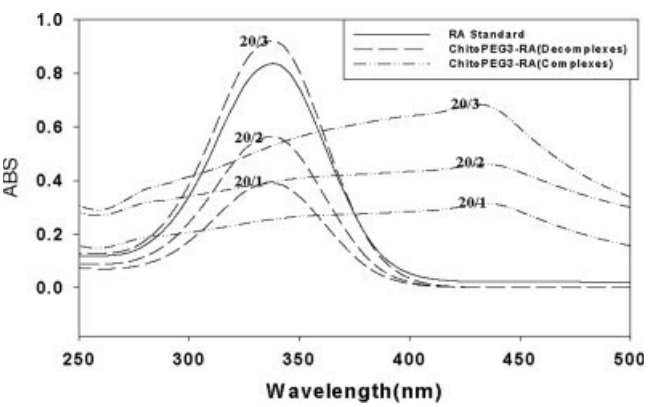

Figure 8 UV spectra of RA itself, the polymeric micelle, and RA extracted from the polymeric micelle in a water/DMSO mixture (1/9 v/v).

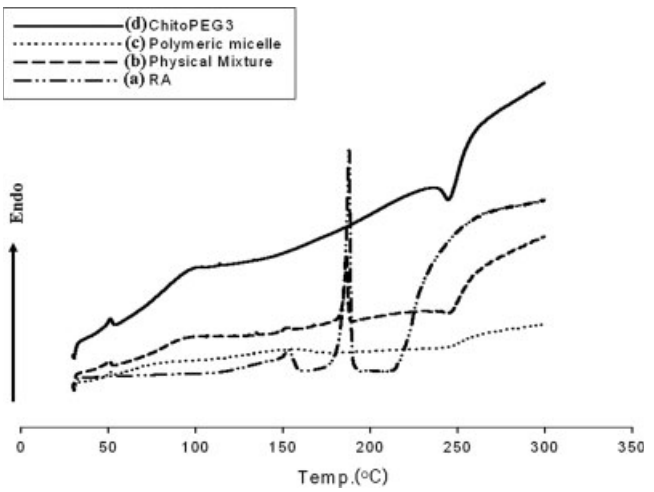

Figure 9 DSC of RA-encapsulated core–shell-type nanoparticles of ChitoPEG: (a) free RA, (b) the RA/ChitoPEG copolymer physical mixture (RA/ChitoPEG-3 = 3/20 w/w), (c) the RA-encapsulated polymeric micelle, and (d) ChitoPEG-3.

Because of the anionic characteristics of RA, a cationic polymer such as chitosan has been considered a good candidate for the preparation of nanoparticles or polymeric micelles.[8,9] Poly(L-lysine) or PEI as a polycationic vehicle for the formation of complexes with RA has been reported.[8,9] Researchers have reported the formation of RA-encapsulated core–shell-type nanoparticles based on a polyion complex. Their ideas of RA-encapsulated nanoparticles stimulated us to try a cationic polysaccharide, chitosan, and the ChitoPEG copolymer to make polymeric micelles. Because most drugs, proteins, peptides, and DNA drugs are sensitive to an acidic solution and can be easily inactivated in a highly acidic environment, we previously reported that water-soluble chitosan has great potential in drug delivery systems and in gene delivery systems.[23,24] The ChitoPEG copolymer enabled us to make the core–shell-type polymeric micelle when an ion complex was formed between RA and the chitosan main chain. These typical structural characteristics of polymeric micelles are shown in Figure 6. The specific peaks of chitosan and RA as a core of the polyion complex disappeared in water (D_2O), whereas the MPEG outer shells showed their specific peaks. However, in an organic solvent such as DMSO or $CDCl_3$, both the drug and MPEG showed their specific peaks, and this indicated that the chitosan main chain and RA formed the core of the polymeric micelle, and MPEG formed the outer shell of the polymeric micelle, as illustrated in Figure 4(a). Other evidence of ion-complex formation between chitosan and RA was observed in FTIR results (Fig. 5). The carbonyl group (at 1690 cm^{-1}) of RA disappeared when polymeric micelles were formed. Theses results were also reported by Thünemann and Beyermann[8,9] and Seo et al.[25] They also reported that the cationic group of the polymer and the anionic group of RA form an ion complex followed by self-assembly formation as nanoparticles or polymeric micelles. As shown in Figure 9, RA was incorporated into the core of the polyion complex; that is, the melting peaks of RA disappeared in the polymeric micelle, whereas the physical mixture of RA and the ChitoPEG copolymer showed the specific melting peaks of both RA and the ChitoPEG copolymer. These results indicate that RA in the polymeric micelle existed as ion complexes with chitosan and did not exist as a free drug.

Otherwise, RA was easily decomplexed in solvent mixtures of water and DMSO (1/9 v/v), and RA was not significantly changed during the process of ion-complex formation, as shown in Figures 7 and 8. Furthermore, almost all of the drug was decomplexed in the solvent mixtures of water and DMSO. Because RA is a photosensitive agent, the stability of RA is considered a primarily important factor for practical applications.

In the practical use of polymeric micelle formulations, their reconstitution in a lyophilized solid form is one of the most important factors in the whole process of polymeric micelle preparation and application. For the long-term storage of polymeric micelles, an aqueous solution of polymeric micelles is essentially required to lyophilize as a solid product, and the lyophilized solid polymeric micelle must be reconstituted into a physiological solution just as its original aqueous solution must be before its use.[26–29] Konan et al.[29] reported that sterilized nanoparticles (<200 nm) of a polyester appeared to

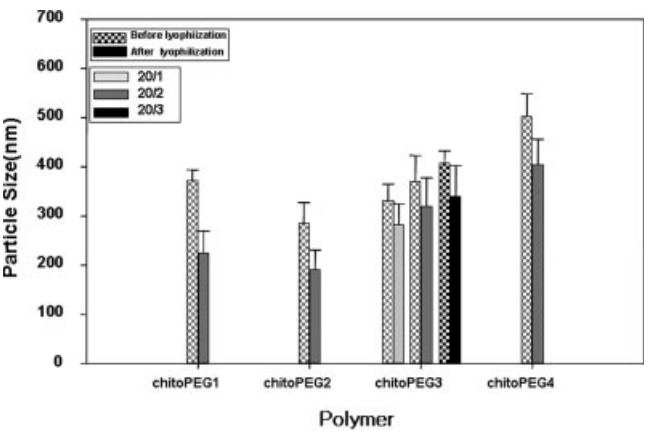

Figure 10 Reconstitution of the lyophilized RA-encapsulated polymeric micelle of ChitoPEG. The lyophilized RA-encapsulated polymeric micelle (5 mg) was simply reconstituted with 5 mL of PBS (pH 7.4, 0.1*M*), and this was followed by gentle magnetic stirring. The reconstituted polymeric micelle was used to measure the particle size.

be completely redispersed in the presence of tested lyoprotectants such as saccharides, whereas aggregation was observed without lyoprotectants. Jaeghere et al.[27] reported that poly(D,L-lactide)/poly(ethylene oxide) nanoparticles aggregated after freeze drying, and this problem could be circumvented by the use of trehalose as a cryoprotectant. Generally, lyophilized nanoparticles are significantly aggregated, and the particle size increases when they are reconstituted into an aqueous solution, although those nanoparticles are prepared in the presence of a surfactant or emulsifier.[26,29]

In our study, ChitoPEG was fully water-soluble, whereas other kinds of chitosan derivatives required an acidic solution for dissolution. Therefore, this suggests that the RA-encapsulated and lyophilized polymeric micelles of ChitoPEG are capable of redispersing into an aqueous solution without the aid of cryoprotectants. To investigate whether or not the lyophilized polymeric micelles could be reconstituted into aqueous solutions, aqueous solutions of RA-encapsulated polymeric micelles of the ChitoPEG copolymer were lyophilized and reconstituted into PBS (pH 7.4, 0.1*M*). The reconstituted polymeric micelles showed almost similar size distributions, as shown in Figure 10, in all formulations. Furthermore, the aggregation or precipitation of lyophilized polymeric micelles was not observed during the reconstitution process, and all the polymeric micelles were completely reconstituted in aqueous solutions. These results show that the RA-encapsulated polymeric micelle has potential as a drug delivery system for intravenous administration. These results led us to conclude that the RA-encapsulated polymeric micelle of the ChitoPEG copolymer could be stored in a solid form and easily reconstituted into an injection solution.

CONCLUSIONS

The ChitoPEG copolymer was synthesized to prepare the RA-encapsulated polymeric micelle. The RA-encapsulated polymeric micelle of the ChitoPEG copolymer had a particle size of 100–400 nm and appeared spherical in TEM observations. In our ^{1}H-NMR study, the specific peaks of RA and chitosan disappeared in D_2O, whereas the specific peak of MPEG was observed. When the polymeric micelle was dissolved in DMSO or $CDCl_3$, the specific peaks of MPEG, RA, and chitosan appeared, indicating that RA/ChitoPEG ion complexes composed of the polymeric micelle with a core–shell structure and free drug did not exist in the polymeric micelle formulations. Other evidence of drug encapsulation in the polymeric micelle was analyzed with an HPLC study and UV spectroscopy. RA was not affected by the ion-complexation process. In a DSC analysis, the melting peaks of RA and chitosan were 182 and 220°C, respectively. The melting peak of the polymeric micelle was 200°C, whereas those of the physical mixtures were those of both RA and the Chito-PEG copolymer. The lyophilized polymeric micelle was successfully reconstituted into PBS.

References

1. Nishiyama, N.; Kataoka, K. Adv Exp Med Biol 2003, 519, 155.
2. Kim, S. C.; Kim, D. W.; Shim, Y. H.; Bang, J. S.; Oh, H. S.; Wan Kim, S.; Seo, M. H. J Controlled Release 2001, 72, 191.
3. Jeong, Y. I.; Nah, J. W.; Lee, H. C.; Kim, S. H.; Cho, C. S. Int J Pharm 1999, 188, 49.
4. Yokoyama, M.; Okano, T.; Sakurai, Y.; Ekimoto, H.; Shibazaki, C.; Kataoka, K. Cancer Res 1991, 51, 3229.
5. Kim, T. Y.; Kim, D. W.; Chung, J. Y.; Shin, S. G.; Kim, S. C.; Heo, D. S.; Kim, N. K.; Bang, Y. J. Clin Cancer Res 2004, 10, 3708.
6. Nishiyama, N.; Okazaki, S.; Cabral, H.; Miyamoto, M.; Kato, Y.; Sugiyama, Y.; Nishio, K.; Matsumura, Y.; Kataoka, K. Cancer Res 2003, 63, 8977.
7. Nishiyama, N.; Kato, Y.; Sugiyama, Y.; Kataoka, K. Pharm Res 2001, 18, 1035.
8. Thünemann, A. F.; Beyermann, J. Macromolecules 2000, 33, 6878.
9. Thünemann, A. F.; Beyermann, J.; Kukula, H. Macromolecules 2000, 33, 5906.
10. Hirano, S. Polym Int 1999, 48, 732.
11. Fernandez-Urrusuno, R.; Calvo, P.; Remunan-Lopez, C.; Vila-Jato, J. L.; Alonso, M. J. Pharm Res 1999, 16, 1576.
12. Kotez, A. F.; De Leeuw, B. J.; Lueben, H. L.; DeBoer, A. G.; Verhoef, J. C.; Junginger, H. E. Int J Pharm 1997, 159, 243.
13. Jang, M. K.; Jeong, Y. I.; Cho, C. S.; Yang, S. H.; Kang, Y. E.; Nah, J. W. Bull Korean Chem Soc 2002, 23, 914.
14. Kato, Y.; Onishi, H.; Machida, Y. Biomaterials 2004, 25, 907.
15. Bhattarai, N.; Ramay, H. R.; Gunn, J.; Matsen, F. A.; Zhang, M. J Controlled Release 2005, 103, 609.
16. Retinoids in Normal Development and Teratogenesis; Morriss-Kay, G., Ed.; Oxford Science: Oxford, 1992.
17. Noll, E.; Miller, R. H. Development 1994, 120, 649.
18. Rotan, R. Semin Cancer Biol 1991, 2, 197.
19. Kalmekerian, G. P.; Jasti, R. K.; Celano, P.; Nelkin, B. D.; Marby, M. Cell Growth Differ 1994, 5, 55.
20. Giannini, F.; Maestro, R.; Vukosa, T.; Vljevic, T.; Pomponi, F.; Boiocchi, M. Int J Cancer 1997, 70, 194.
21. Krupitza, G.; Hulla, W.; Harant, H.; Dittrich, E.; Kallay, E.; Huber, H. Int J Cancer 1995, 61, 649.
22. Huang, E. J.; Ye, Y. C.; Chen, S. R.; Chai, J. R.; Lu, J. X.; Zhoa, L.; Gu, L. J.; Wang, Z. Y. Blood 1988, 72, 567.
23. Son, S.; Chae, S. Y.; Choi, C.; Kim, M. Y.; Ngugen, V. G.; Jang, M. K.; Nah, J. W.; Kweon, J. K. Macromol Res 2004, 12, 573.
24. Chae, S. Y.; Son, S.; Lee, M.; Jang, M. K.; Nah, J. W. J Controlled Release 2005, 109, 330.
25. Seo, S. J.; Kim, S. H.; Sasagawa, T.; Choi, Y. J.; Akaike, T.; Cho, C. S. Eur J Pharm Biopharm 2004, 58, 681.
26. Chacon, M.; Molpeceres, J.; Berges, L.; Guzman, M.; Aberturas, M. R. Eur J Pharm Sci 1999, 8, 99.
27. Jaeghere, F. D.; Allemann, E.; Leroux, J. C.; Stevels, W.; Feijen, J.; Doelker, E.; Gurny, R. Pharm Res 1999, 16, 859.
28. Zimmermann, E.; Muller, R. H.; Mader, K. Int J Pharm 2000, 196, 211.
29. Konan, Y. N.; Gurny, R.; Allemann, E. Int J Pharm 2002, 233, 239.