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International Journal of Pharmaceutics 254 (2003) 109–121
international journal of pharmaceutics
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Characterization of hydrophobized pullulan with various hydrophobicities

Sun-Woong Jung [a], Young-Il Jeong [b], Sung-Ho Kim [a,*]

[a] *College of Pharmacy, Chosun University, #375 Seosuk-dong, Dong-gu, Gwangju 501-759, South Korea*
[b] *Research Institute of Medical Sciences, Chonnam National University, Gwnggju 501-746, South Korea*

Received 18 March 2002; received in revised form 2 December 2002; accepted 18 December 2002

Abstract

In this study, we prepared self-assembling nanospheres of hydrophobized pullulan. Pullulan acetate (PA), as hydrophobized pullulan, was synthesized by the acetylation of pullulan. PA derivatives were synthesized by changing the degree of acetylation. PA was characterized by Fourier transform infrared (FT-IR), X-ray diffractometry (XRD), and differential scanning calorimetry (DSC). The particle size distribution of the PA was determined using photon correlation spectroscopy (PCS) and the number-average particle size was found to depend upon the degree of acetylation of PA. Morphology by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) showed the PA nanospheres were spherical in shape. The fluorescence probe technique was used to study the self-association behavior of hydrophobized pullulans in water using pyrene as a hydrophobic probe. The critical association concentration (CAC) values were determined from the fluorescence excitation spectra, CAC values were dependent upon the degree of acetylation. Drug release studies using clonazepam (CNZ) as a hydrophobic model drug showed that the increased drug contents and increased degree of acetylation resulted in a slower release rate of drug from the nanospheres.

Keywords: Pullulan acetate; Acetylation; Nanospheres

1. Introduction

Since nanoparticles are solid colloidal particles ranging in size from 10 to 1000 nm (Kreuter, 1991), they are suitable device for parenteral injection. For parenteral use it is desirable to limit the size of the particles in order to minimize possible irritant reactions at the injection site. Nanoparticles or colloidal drug delivery systems offer a number of advantages over conventional dosage forms. Targeting the desired site of action would not only increase the therapeutic efficiency of a drug but would also allow a reduction in the amount of drug administered, thus minimizing undesirable side effects. The use of a colloidal carrier is attractive because a wide variety of systems are available and particles with different physicochemical properties and loading characteristics can be constructed. The various carriers used for drug targeting include liposomes (Gregoriadis, 1981, 1983; Alving, 1982; Weinstein and Leserman, 1984), emulsions (Davis, 1984; Stevenson and Sefton, 1987), polymeric microspheres (Davis and Illum, 1988; Bodmeier and Chen, 1989; Maulding, 1987; Benoit et al., 1986; Tabata and Ikada, 1988; Sah et al.,

* Corresponding author. Tel.: +82-62-230-6379; fax: +82-62-222-5414.
E-mail address: shkim@chosun.ac.kr (S.-H. Kim).

0378-5173/03/$ – see front matter
doi:10.1016/S0378-5173(03)00006-1

1994; Crotts and Park, 1997; Yoshioka et al., 1981), nanoparticles (Yoshioka et al., 1981; Davis and Illum, 1985; Kreuter, 1991; Allemann et al., 1993; Dunn et al., 1997), and natural carriers, such as cells (erythrocytes) and lipoproteins. Problems associated with conventional drug carriers include; poor disease site selectivity, polymer toxicity, and the free diffusion of drugs throughout the body. Novel types of drug delivery systems are required to provide solution to these problems.

Supramolecular self-assembly has been investigated intensively during recent decades, and the nanosize concept has received considerable attention due to its extensive applications in such areas as, colloid science, electronics, environmental technology, biotechnology, and biomedical engineering (Whitesides et al., 1991; Lehn, 1993; Freedman, 1991). The use of the self-aggregating properties of amphiphilic macromolecules in selective solvents has become an attractive means for producing lipid bilayer membranes (Walker et al., 1997), polymeric micelles (Kown et al., 1993), and Langmuir–Blodgett films (Cho et al., 1995a,b). In the case of drug delivery systems, the self-aggregating characteristics of amphiphilic polymers in aqueous solutions have received considerable attention as a means of developing effective targetable drug carriers, such as polymeric micelles (Kataoka et al., 1993) and hydrophobized polysaccharides (Akiyoshi et al., 1993). Generally, self-assembled nanoparticles are composed of an inner hydrophobic core and an outer shell composed of more hydrophilic elements. The amphiphilic nature of self-assembled nanoparticles is believed to offer real advantages in terms of their use as drug carriers because the inner hydrophobic core offers a drug incorporation site and the outer hydrophilic shell can be used to prevent attack by biologic entities, such as macrophages and plasma proteins. Due to their thermodynamic stability and small size, self-assembled nanoparticles are considered effective vehicles for the prolonged blood circulation of drugs and for targeting solid tumors (Yokoyama et al., 1994). Yokoyama et al. (1990, 1991) reported that adriamycin-conjugated poly(ethylene glycol)–poly(aspartic acid) block copolymeric micelles were effective tools for the treatment of solid tumors, and added that they have the potential of prolonged blood circulation times. Akiyoshi et al. (1993, 1997) reported that hydrophobized polysaccharide has amphiphilic characteristics in water, and that it can form stable nanoparticulate self-assemblies in aqueous media. They also reported upon the thermoresponsiveness of hydrogel nanoparticles (Akiyoshi et al., 1997). Nishikawa et al. (1994) observed a stable macromolecular complexation with proteins on heating a self-assembly of hydrophobized polysaccharide. However, in spite of their potential importance, the self-assembly of hydrophobically modified polysaccharide has not been adequately investigated in terms of its potential use as a colloidal carriers for sustained drug release.

In this study, we describe the preparation of self-assembling nanospheres of hydrophobized pullulans in water, and report upon its potential use as a drug carrier using clonazepam (CNZ) as a hydrophobic model drug. CNZ is an anticonvulsant benzodiazepine that is used for the treatment of panic disorder. It is hydrophobic with a water solubility (phosphate-buffered saline (PBS), pH 7.4, 0.1 M at 37 °C) of less than 14.66 μg/ml, and it has a high affinity for proteins in vivo (O'Hare et al., 1989; Wu and Wu, 1988). Accordingly, the half-life of CNZ could be extended if protein adsorption and uptake by non-target organs were avoided. The self-assembling behaviors, morphological shapes, and particle sizes of hydrophobized pullulans were characterized by fluorescence spectroscopy, transmission electron microscopy (TEM), and photon correlation spectroscopy (PCS). In addition, CNZ release rates from the nanospheres were determined in vitro. Particle size, critical micelle concentration, drug-loading capacity, and their physicochemical properties with respect to various levels of acetylation of pullulan acetate (PA) and solvents were investigated in vitro.

2. Materials and methods

2.1. Materials

Pullulan (MW = 200,000) was purchased from the Hayashibara Company, Japan, CNZ from Roche, Switzerland, and dimethylformide (DMF), dimethyl sulfoxide (DMSO), acetone, and 1,4-dioxane were of reagent grade.

2.2. Synthesis of hydrophobized pullulans

PA, as hydrophobized pullulan, was synthesized by the Motozato's method (Motozato et al., 1986) as follows: 2 g of pullulan, suspended in 20 ml of formamide, was dissolved by vigorous stirring at 54 °C. To this solution, pyridine (6 ml), and various amounts of acetic anhydride (15, 7.5, and 5 ml of acetic anhydride were added to change the acetylation degree, and designated as PA1, PA2, PA3, respectively.) were then added, and the mixture stirred at 54 °C for 48 h. A dark-brown precipitate was thus obtained and purified by reprecipitation with 1000 ml of distilled water and 500 ml of methanol. The solid material was vacuum-dried for 3 days, and a white powder was obtained.

2.3. Preparation of CNZ-loaded nanospheres

Nanospheres of PA were prepared by the dialysis method (Cho et al., 1995a,b, 1997; Jeong et al., 1998; Kim et al., 1997). PA (40 mg) was dissolved in 10 ml of DMSO. To form the nanospheres, the solutions formed were dialyzed using a molecular weight cut-off (MWCO) 12,000 g/mol dialysis tube (Sigma Chemical Co., St. Louis, USA) against distilled water. The distilled water was exchanged every 1 h for 3 h and 3 h for additional 21 h, the dialyzed solution was then analyzed or freeze-dried.

Drug-loaded nanospheres were prepared as follows: PA and CNZ were dissolved in DMSO, and dialyzed as described earlier. The dialyzed solutions obtained were freeze-dried.

To measure the drug-loading contents, freeze-dried samples of CNZ-loaded nanospheres were suspended in methanol, vigorously stirred for 2 h, and sonicated for 15 min. The resulting solutions were centrifuged at 3000 rpm for 30 min, and the drug concentrations were measured on the supernatants using a UV spectrophotometer (Shimadzu UV-1201, Shimadzu Co. Ltd., Tokyo, Japan) at 309 nm.

2.4. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy (Nicolet, Magna IR 550) was used to confirm the synthesis of the hydrophobized pullulans. The degree of acetylation was also calculated.

2.5. X-ray diffractometry (XRD)

X-ray powder diffractograms were obtained using a Rigaku D/Max-1200 (Rigaku) with Ni-filtered Cu Kα radiation (35 kV, 15 mA) to determine the crystallinity of the polymer.

2.6. Differential scanning calorimetry (DSC)

The melting points of pullulan and the PA were measured by DSC at a temperature range of −30 to 250 °C under nitrogen and at a rate of °C/min.

2.7. Photon correlation spectroscopy (PCS)

Particle sizes were measured with a Zetasizer 3000 (Malvern Instruments, UK) with a He–Ne laser beam at a wavelength of 633 nm at 25 °C (scattering angle of 90°). The nanospheres presented in the sample suspension were at 1 g/l and measurements were made without filtering.

2.8. Scanning electron microscope and transmission electron microscope measurements

The morphology of the nanoparticles was observed using an SEM (Jeol, JSM 5400, Japan). A drop of the nanoparticle suspension was placed on a graphite surface, and freeze-dried. The sample was then coated with gold/palladium by Ion Sputter (Jeol, JFC-1100). Coating was performed at 20 mA for 4 min, and observation was made at 25 kV.

A drop of nanosphere suspension containing 0.01% of phosphotungstic acid was placed on a TEM copper grid coated with carbon film and dried at 25 °C. Observations were performed at 80 kV with a JEM-2000 FX II (Jeol, Japan).

2.9. Fluorescence spectroscopy

Fluorescence spectroscopy (Shimadzu RF-5301 PC spectrofluorophotometer, Shimadzu Co. Ltd., Japan) was performed to prove the self-assembly potential of PA. PA suspensions were prepared without an incorporated drug as follows: 40 mg of PA was dissolved in 10 ml of DMSO and dialyzed against distilled water for up to 2 days using the method described earlier.

The resultant suspension was adjusted to various nanosphere concentrations.

The critical association concentration (CAC) of the PA was estimated using pyrene as a hydrophobic probe (Kalyanasundaram and Thomas, 1977; Wilhelm et al., 1991). To prepare sample solutions, a known amount of pyrene in acetone was added to each of a series of 20 ml vials, and the acetone was evaporated. The final concentration of pyrene was 6.0×10^{-7} M. To each vial was added 10 ml of various concentrations of nanosphere suspensions, and then the mixtures were heated for 3 h at 65 °C. Equilibration of the pyrene and the PA nanospheres was achieved by allowing the solutions to stand overnight at room temperature. The fluorescence excitation spectra were measured at an emission wavelength of 390 nm. The excitation and emission bandwidths were both 1.5 nm.

2.10. In vitro drug release studies

The release experiment was carried out in vitro as follows: CNZ-loaded PA nanospheres (5 mg) and 1 ml of PBS (0.1 M, pH 7.4) were placed in dialysis tubes (MWCO = 12,000 g/mol). The tubes were then introduced into a 100 ml bottle containing 50 ml of PBS, and the medium was stirred at 100 rpm and 37 °C. At specific time intervals, the medium was removed and replaced with fresh PBS to prevent saturation. The concentration of the CNZ released into the PBS was determined using a UV spectrophotometer at 309 nm (Jeong et al., 1998).

3. Results and discussion

Pullulan consists of α-1,4 and α-1,6 glycosidic linkages and is a water-soluble, neutral, linear polysaccharide. In the present study, pullulan was modified by replacing the hydroxyl groups of the glucose unit with acetate groups to produce a hydrophobically modified pullulan, PA.

Fig. 1a–d show the FT-IR spectra of pullulan, PA1, PA2, and PA3. The spectra demonstrated the introduction of the acetate group, as indicated by C=O stretching at 1752 cm^{-1}, CH_3 deformation at 1372 cm^{-1}, and O–C=O bonds at 602 cm^{-1}. We evaluated the degree of acetylation of PA; the results are summarized in Table 1.

Fig. 2 shows that the XRD was used to investigate the physicochemical characteristics of pullulan and PA derivatives. A specific broad peak associated with unmodified pullulan was observed (Fig. 2a), and these specific crystalline peaks of pullulan decreased as the pullulan was converted into PA1 (Fig. 2b), PA2 (Fig. 2c), and PA3 (Fig. 2d). This result was supported by the results of the thermal analysis as shown in Fig. 3. The melting point of pullulan was observed at 107.14 °C, but the melting points of the various PAs were at lower temperatures, i.e. 101.08 °C (PA1), 95.30 °C (PA2), and 91.77 °C (PA3). It is thought that the specific crystallinity of pullulan is decreased at increased levels of acetylation.

PA is readily soluble in DMSO, DMF, tetrahydrofuran, dichloromethane, chloroform, acetone, and 1,4-dioxane. To make nanospheres using the dialysis method, only water-miscible solvents were considered because the solvents had to be exchanged in a water media by dialysis. Moreover, the solvent selected to dissolve the polymer can influence the particle size, drug loading, and the physicochemical properties of the PA nanospheres, due to the complex partitions possibilities in this multi-component, multi-phase system involving solvent, polymer, water, and drug. Therefore, solvents are of primary importance in the formation of nanospheres by the dialysis method. In this study, four water-miscible solvents, namely, DMSO, DMF, acetone, and 1,4-dioxane were used. Table 2 shows the particle sizes of empty nanospheres

Table 1
The estimation of acetylation degree, ΔT_m, ΔH, CAC, and particle size of pullulan and pullulan acetate derivatives

	Acetylation degree (%)	ΔT_m (°C)	ΔH (J/g)	CAC $\times 10^{-4}$ (g/l)	Particle size (nm)
Pullulan	–	53.69	289.6	–	–
PA1	87.39	58.82	32.72	3.0	127.6 ± 71.00
PA2	82.95	52.65	31.25	4.0	294.4 ± 52.30
PA3	72.94	51.08	10.89	5.0	415.8 ± 167.4

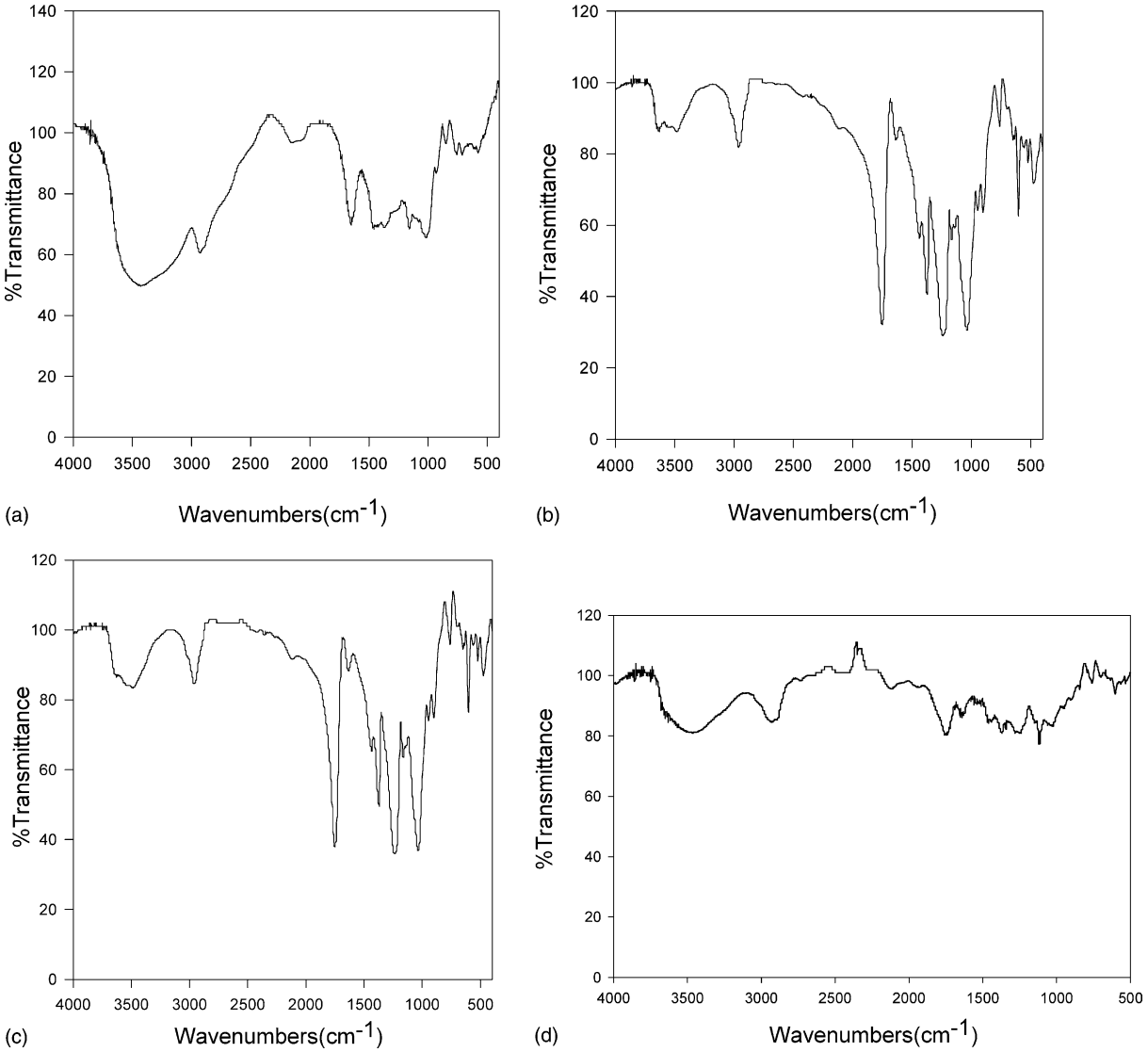

Fig. 1. FT-IR spectra of pullulan (a), PA1 (b), PA2 (c), and PA3 (d).

Table 2
Particle size and drug-loading contents of pullulan acetate nanospheres against various initial solvents used

Solvent	Particle size (nm)	CAC $\times 10^{-4}$ (g/l)	Loading contents (wt.%)	Loading efficiency (wt.%)
DMSO	139.7 ± 4.9	9.0	18.0	54.6
DMF	84.2 ± 75.9	2.8	19.4	58.8
Acetone	71.1 ± 4.3	4.0	23.2	70.3
1,4-Dioxane	380.1 ± 23.9	8.9	23.2	70.3

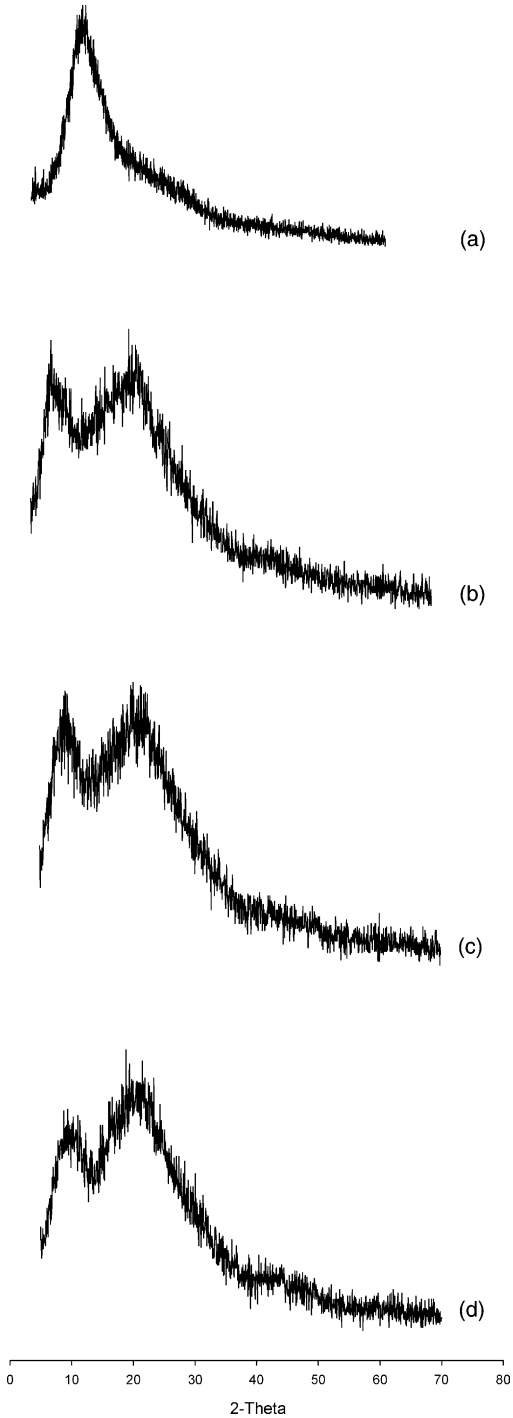

Fig. 2. X-ray diffraction patterns of pullulan (a), PA1 (b), PA2 (c), and PA3 (d).

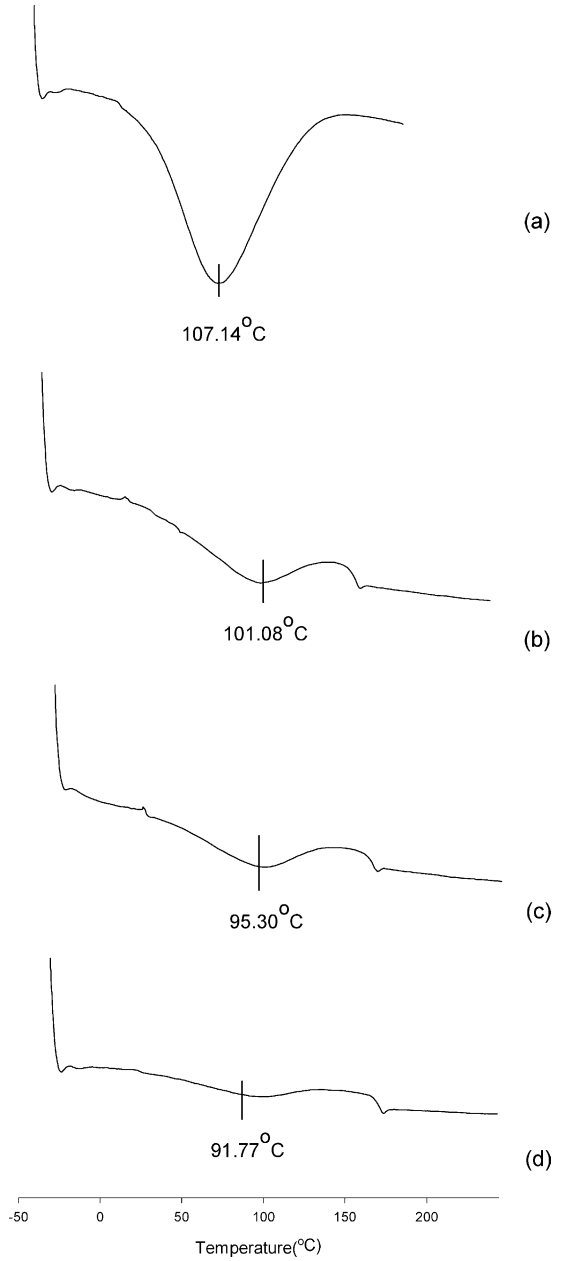

Fig. 3. DSC thermograms of pullulan (a), PA1 (b), PA2 (c), and PA3 (d).

and drug-loaded nanospheres for the various solvents. DMF and acetone resulted in relatively smaller particle sizes than DMSO and 1,4-dioxane. In particular, 1,4-dioxane resulted in the highest particle size.

However, the drug loadings were not in accordance with the particle size. DMSO and DMF resulted in lower drug loadings than acetone and 1,4-dioxane. Although the differences were not remarkable, acetone was associated with higher drug loading than DMSO or DMF in spite of its smaller particle size. 1,4-Dioxane was similar to acetone in terms of drug loading despite having a particle size some five times greater. These results indicate that the solvents used in the preparation significantly affect the physico-

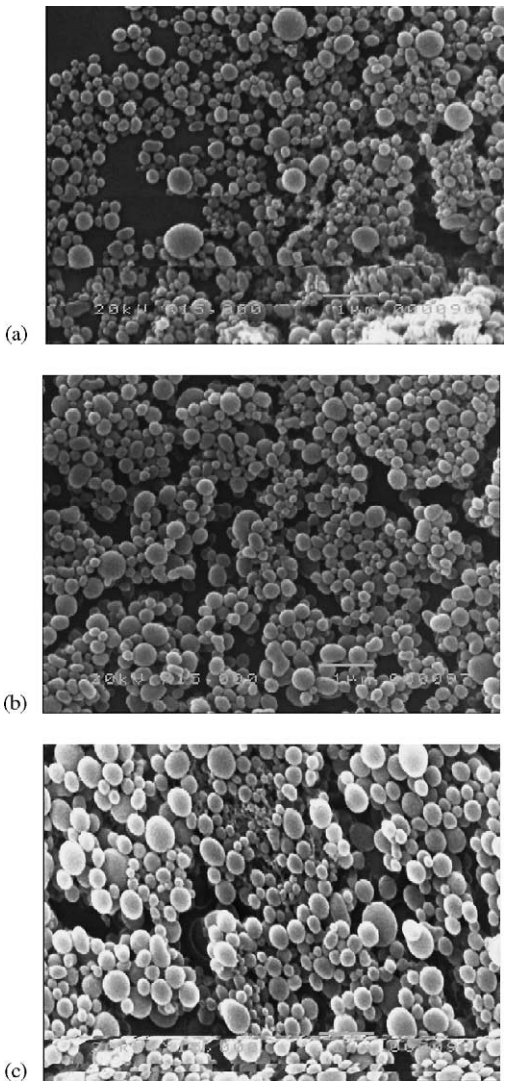

Fig. 4. Scanning electron microscopy (SEM) photographs of PA1 (a), PA2 (b), and PA3 (c).

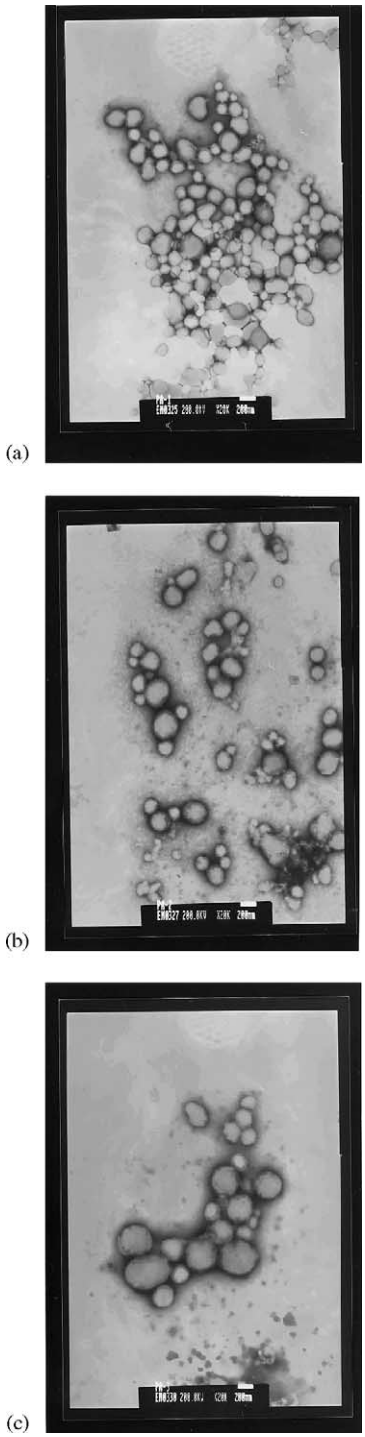

Fig. 5. Transmission electron microscopy (TEM) photographs of PA1 (a), PA2 (b), and PA3 (c).

chemical properties, and that particle size and drug loading can be changed by solvent choice.

Fig. 4 shows the SEM photographs of various PAs. The shapes of the nanoparticles were spherical and their sizes ranged from 100 to 450 nm in diameter. TEM observations of PA derivatives nanoparticles are also shown in Fig. 5. They are spherical in appearances and they range from 100 to 450 nm, which is similar to that found by SEM. As shown by Table 1, the acetylation degree of PA was increased as the particle size decreased.

Generally, block and graft copolymers, and other amphiphilic materials show self-assembling potential in aqueous media (Wilhelm et al., 1991; Jeong et al., 1998). To characterize the self-assembling behavior of PA derivatives in aqueous media, the fluorescence

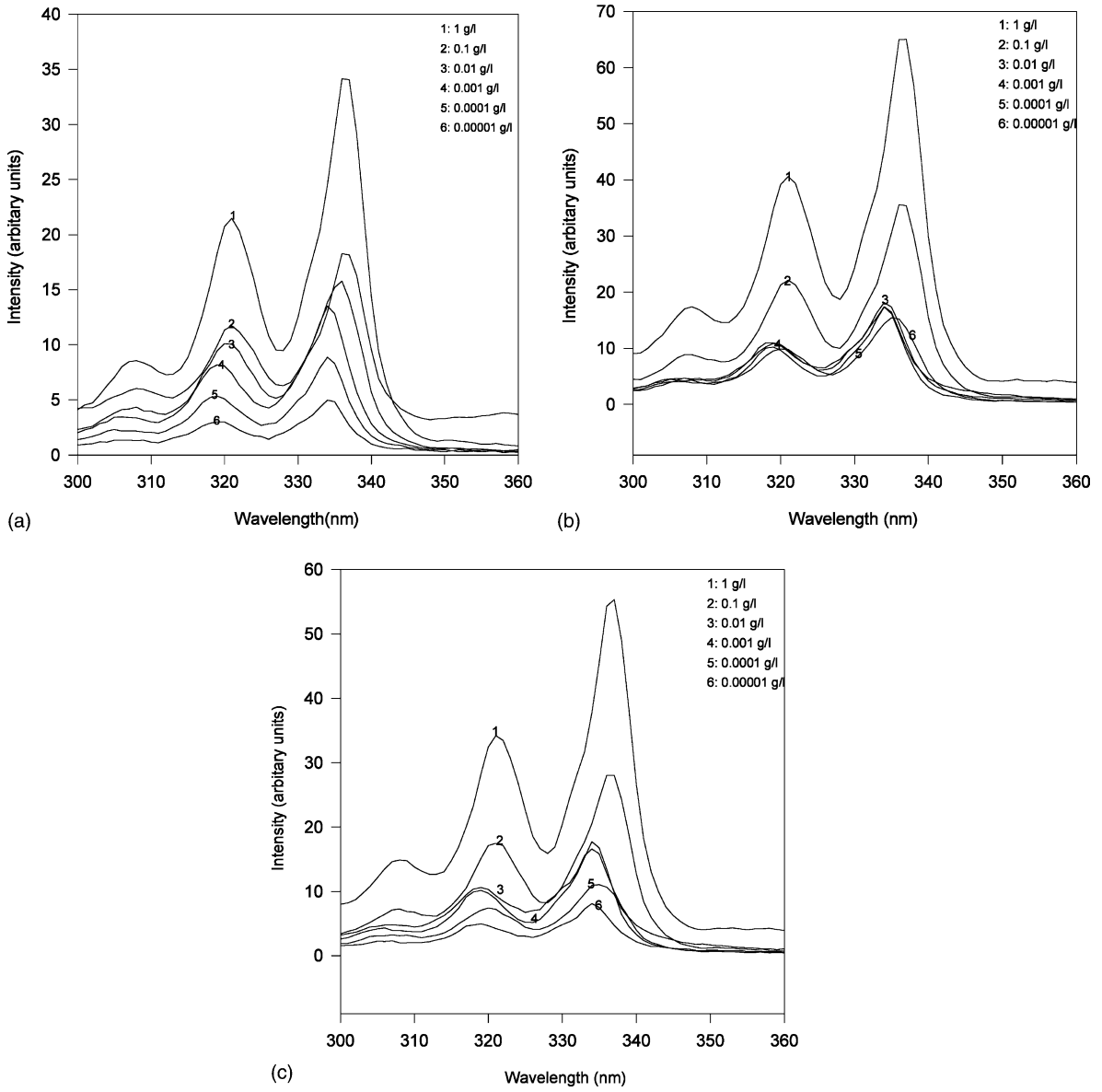

Fig. 6. Fluorescence excitation spectra of pyrene (6.0×10^{-7} M) vs. the concentration of PA in distilled water ($\lambda_{em} = 390$ nm).

probe technique was introduced using pyrene as a hydrophobic probe. Fig. 6 shows the fluorescence excitation spectra of pyrene at various concentrations of PA. The fluorescence intensity of pyrene was found to increase with increasing concentrations of PA, which indicates self-assembly of the hydrophobized pullulan in water. In addition, in the excitation spectra a red shift was observed with increasing PA concentration. It is thought that pyrene is preferentially solubilized into the nanospheres, composed of a core-shell structure when it was introduced into aqueous phase using a good solvent (Kown et al., 1993). The in-

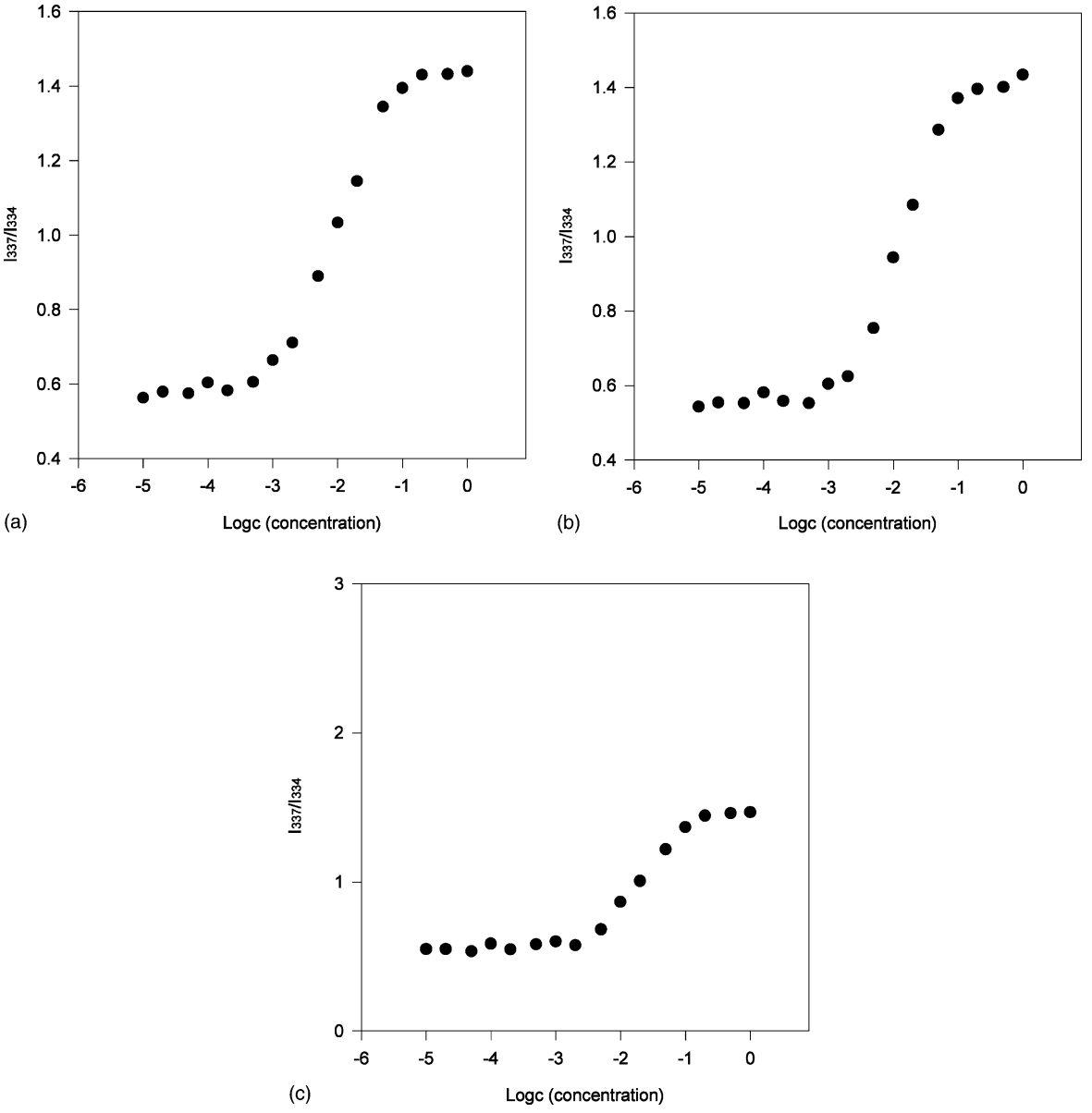

Fig. 7. Plots of the intensity ratios I_{337}/I_{334} from the pyrene excitation spectra vs. log c of the various PAs in distilled water. [Pyrene] $= 6.0 \times 10^{-7}$ M.

tensity ratio of I_{337}/I_{334} versus log c of PA in the pyrene excitation spectra is shown in Fig. 7. A flat region at extremely low concentration and a sigmoid change in the crossover region were observed. This result indicates that a signal change in the crossover region could be evaluated to the CAC value of PA. As shown in Table 2, the CAC values were significantly dependent upon the solvent used. The CAC of the nanospheres was lowest for DMF. In the case of DMSO and 1,4-dioxane, which had relatively larger particle size, the CAC values were higher than those of DMF and acetone, which had smaller particle sizes.

To study the drug release behavior, the CNZ-loaded PA nanospheres were simply resuspended in PBS (pH 7.4, 0.1 M) and the drug release determined in vitro. Fig. 8 shows the in vitro drug release from PA nanosperes versus the solvent used in the preparation. Fig. 9 shows the in vitro drug release from PA nanospheres versus the degree of acetylation. At constant particle size with different drug loadings, the higher drug loadings were found to result in decreased drug release rates. In addition, for different particle sizes with the same drug loadings, higher particle size resulted in significantly lower drug release rates. These phenomena have been previously reported (Wilhelm et al., 1991; Gref et al., 1994; Jeong et al., 1998). Gref et al. (1994) reported that crystallization of hydrophobic drugs occurred inside the nanoparticles, especially at higher drug loadings, in which case phase separation tended to occur that led to crystallization. This was used to explain why hydrophobic drugs loaded into nanospheres were released more slowly at higher drug loadings. This behavior distinguishes between the hydrophobic and hydrophilic water-soluble drugs. In addition, we observed that CNZ release was reduced from nanospheres with higher drug loadings. It is believed likely that at low drug loadings, CNZ exists as a molecular dispersion inside the nanospheres (Jeong et al., 1998), and that in this form the drug is more likely to be dissolved rapidly. These characteristics of drug release behavior are supported by the results of calorimetric analysis

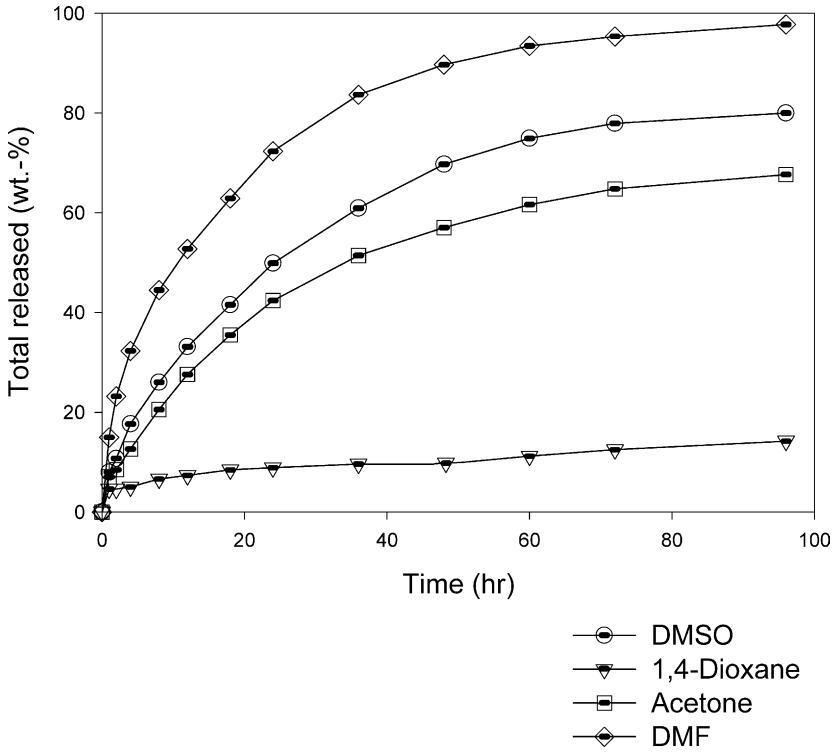

Fig. 8. CNZ release from PA1 nanospheres for different initial solvents.

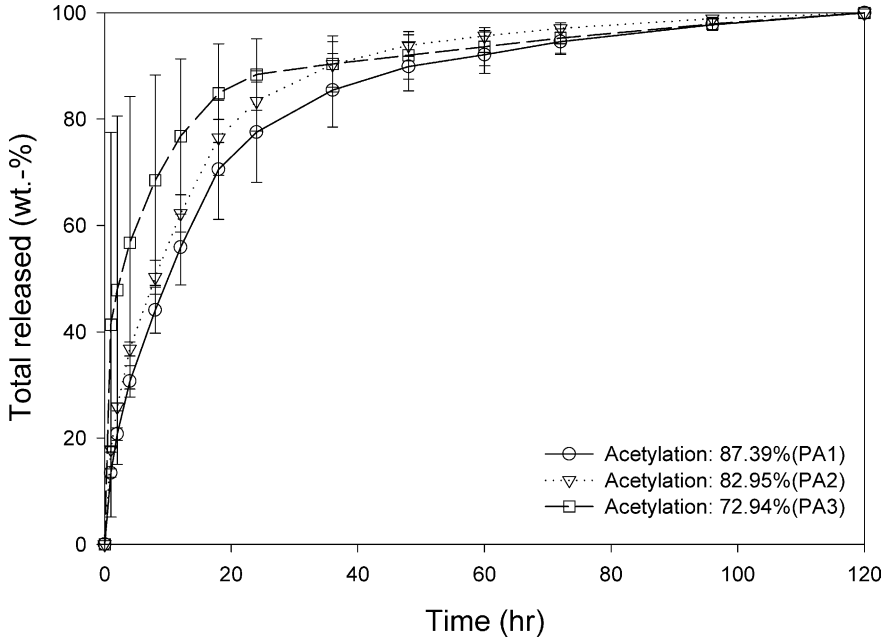

Fig. 9. CNZ release from PA nanospheres for different hydrophobicities.

(Gref et al., 1994). Also, because of the different diffusion rates of drug molecules through the particles to the outer aqueous phase, the kinetics of drug release is affected not only by drug-loading contents but also by the size of the nanoparticles.

In this study, we prepared self-assembling nanospheres of hydrophobized pullulan. PA, as hydrophobized pullulan, was synthesized by the acetylation of pullulan, and PA derivatives were synthesized by changing the degree of acetylation. PA was characterized by FT-IR, XRD, and DSC. From the results of XRD, specific broad peaks associated with unmodified pullulan were observed, and these specific crystalline peaks were found to decrease as pullulan was converted into PA. This result is supported by the thermal analysis. It is believed that the specific crystallinity of pullulan was decreased by the increased degree of acetylation of PA. The particle size distribution of the PA was measured by PCS and their number-average particle size was found to change according to the degree of acetylation of PA. Morphology studies by TEM and SEM showed that the PA nanospheres had a spherical shape, and their size range from 100 to 450 nm. CAC values were evaluated from the fluorescence excitation spectra, and also changed according to the degree of acetylation. Drug release studies using CNZ, as a hydrophobic model drug, showed that the increased drug contents and degree of acetylation reduced the release rate of the drug from the nanospheres. Controlled drug release could be achieved by optimizing the chemical nature of the polymers, the initial solvents used and by using nanospheres with different hydrophobicities, drug loadings, and particle sizes.

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