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Self-Assembled Polymeric Nanoparticles of Poly(ethylene glycol) Grafted Pullulan Acetate as a Novel Drug Carrier

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Self-assembling nanospheres of hydrophobized pullulan have been developed. Pullulan acetate (PA), as hydrophobized pullulan, was synthesized by acetylation. Carboxymethylated poly(ethylene-glycol) (CMPEG) was introduced into pullulan acetate (PA) through a coupling reaction using N,N'-dicyclohexyl carbodiimide (DCC). A synthesized PA-PEG-PA (abbreviated as PEP) conjugate was confirmed by Fourier transform-infrared (FT-IR) spectroscopy. Since PEP conjugates have amphiphilic characteristics in aqueous solution, polymeric nanoparticles of PEP conjugates were prepared using a simple dialysis method in water. From the analysis of fluorescence excitation spectra primarily, the critical association concentration (CAC) of this conjugate was found to be 0.0063 g/L. Observations by scanning electron microscopy (SEM) showed the spherical morphologies of the PEP nanoparticles. The particle size distribution of the PEP conjugates was determined using photon correlation spectroscopy (PCS) and the intensity-average particle size was 193.3 ± 13.53 nm with a unimodal distribution. Clonazepam (CNZ), as a model drug, was easy to entrap into polymeric nanoparticles of the PEP conjugates. The drug release behavior was mainly diffusion controlled from the core portion.

Key words: Pullulan, Poly(ethylene glycol), Polysaccharide, Self-assembly, Stealth properties

INTRODUCTION

Controlled drug delivery technology represents one of the frontiers of science contributing to human health care, which involves a multidisciplinary scientific approach. These delivery systems offer numerous advantages compared to conventional dosage forms, they include, improved efficacy, reduced toxicity, improved patient compliance and convenience (Dumitriu and Dumitriu, 1994; Holinger, 1995; Langer, 1976; Langer, 1989; Lewis, 1990; Robinson and Lee, 1987). Such systems often use macromolecules as carriers for the drugs. By doing so, treatments that would not otherwise be possible are now in conventional use. This field of pharmaceutical technology has grown and diversified rapidly in recent years. Of the different dosage forms reported, nanoparticles and microparticles have attained much importance, due to a tendency to accumulate in inflamed areas of the body. The desirable properties of nano and microparticles give them a unique position in drug delivery technology (Majeti and Ravi Kumar, 2000).

Since nanoparticles are solid colloidal particles ranging in size from 10 to 1000 nm (Kreuter, 1991), they are suitable 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 not only increases the therapeutic efficiency of a drug but also allows a reduction in the quantity of drug administered, thus minimizing undesirable side effects. The use of a colloidal carrier is attractive, because a wide range of different physicochemical properties and loading characteristics can be constructed. The various carriers used for drug targeting include liposomes (Estey *et al.*, 1990), emulsions (Davis *et al.*, 1984; Stevenson *et al.*, 1987), polymeric microspheres (Choi *et al.*, 2001), nanoaprticles (Gref *et al.*, 1994; Jeong

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et al., 1998) and natural carriers such as cells. 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 solutions to these problems.

The self-assembling characteristics of amphiphilic macromolecules in aqueous solutions, have received considerable attention as a means of developing nanoparticulate drug carriers, such as, polymeric micelles (Kataoka *et al.*, 1993; Lehn, 1993) and hydrophobized polysaccharide (Akiyoshi *et al.*, 1993). Self-assembled nanoparticles are composed of a hydrophobic inner-core and a hydrophilic outer-shell. Thus, self-assembled nanoparticles have potential as a drug carrier due to their amphiphilic characteristics. These properties of self-assembled nanoparticles may offer favorable biodistribution of drugs and targeting of drugs to solid tumors (Yokoyama *et al.*, 1994). Yokoyama *et al.* reported that adriamycin conjugated, block copolymeric micelles, were effective tools for the treatment of solid tumors and have the potential for prolonged blood circulation (Yokoyama *et al.*, 1990; Yokoyama *et al.*, 1991). Akiyoshi *et al.* (1997) reported that hydrophobized polysaccharide has amphiphilicity in water and also, that it forms nano-sized self-assemblies (Akiyoshi *et al.*, 1993). I It was also reported that nanoparticles of hydrophobized polysaccharide had a thermo-responsiveness (Akiyoshi *et al.*, 1997) and maintained stable macromolecular complexation with proteins during temperature increases (Nishikawa *et al.*, 1994). Jung *et al.*, 2003, reported that pullulan acetate (PA), as amphiphilic macromolecules, has potential as a drug carrier and that the physicochemical properties of PA were changed by hydrophobicies However, alterations to PA are still necessary to achieve better physicochemical properties, such as, stealth properties and active targeting potentials (Dunn *et al.*, 1997).

In this study, amphiphilic polymers composed of hydrophobized pullulan and poly(ethylene glycol) (PEG) have been synthesized, to give stealth properties as novel drug carriers. PEG is a popularly used water-soluble polymer for stealth properties, due to the following characteristics, good water-solubility, non-toxic, non-immunogenic and biocompatible. PEG represents the protective behavior of the blood protein and reticuloendothelial system (RES) uptake by its hydrophilic nature (Lee *et al.*, 1989). Pullulan is a water-soluble, viscous polysaccharide, consisting of three α-1,4-linked glucose molecules that are repeatedly polymerized by α-1,6-linkages on the terminal glucose. It has been used extensively as an additive in the food industry. Among the drug delivry systems, polysaccharides are one of the most promising carriers for delivery of both drugs and enzymes (Poznansky and Cleland, 1980; Molteni, 1979; Schacht *et al.*, 1985; Kaneo *et al.*, 1989a; Kaneo *et al.*, 1989b). The principal advantages of pullulan, a nonionic polysaccharide, as a macromolecular drug carrier are, high water solubility, multiple hydroxyl groups that can readily be modified chemically, no toxicity, lack of immunogenicity, and usefulness as a plasma expander (Yuen, 1974; Jeanes, 1977).

Introduction of PEG into a hydrophobized polysaccharide, pullulan acetate (PA), will induce the increased stealth properties of nanoparticles and valuable physicochemical properties. The self-assembling behaviors, morphological shapes and particle sizes of polymeric nanospheres, were characterized by fluorescence spectroscopy, scanning electron microscopy (SEM) and photon correlation spectroscopy (PCS). In addition, CNZ release rates from the nanoparticles were determined *in vitro*.

MATERIALS AND METHODS

Materials

Pullulan, with a number-average molecular weight of 200,000, was purchased from the Hayashibara Company, Japan, Clonazepam (CNZ) from Roche, Switzerland, poly (ethylene glycol) (PEG) with a number-average molecular weight of 2,000, was purchased from Sigma Co. USA. The coupling agents *N,N'*-dicyclohexyl carbodiimide (DCC) and 4-(dimethylamino)-pyridine (DMAP), were obtained from the Aldrich Chemical Company (Milwaukee, USA). N-hydroxysuccinimide (NHS) was purchased from the Sigma Chemical Co. (St. Louis, USA). The dialysis membranes with a molecular weight cut-off (MWCO) of 12,000 g/mol were purchased from Spectra/Pro™ Membranes. Dimethyl sulfoxide (DMSO), was of reagent grade and used without further purification. All of the reagents used were extra reagent grade without need of further purification.

Synthesis of pullulan acetate

PA, as hydrophobized pullulan, was synthesized by the Motozatos method (Motozato *et al.*, 1986) as follows: 4 g of pullulan, suspended in 40 mL of formamide, was dissolved by vigorous stirring at 54°C. To this solution, pyridine (12 mL), and 10 mL of acetic anhydride were added, and the mixture stirred at 54°C for 48 h. A dark-brown precipitate was 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. The degree of acetylation was evaluated as 72.9% from the results of Fourier transform infrared (FT-IR) spectroscopy as described previously (Jung *et al.*, 1993).

Synthesis of pullulan acetate-PEG conjugate

The carboxymethylated PEG (CMPEG) was prepared

as follows (Royer and Anantharmaiah, 1979) : PEG (4 g) and potassium *tert*-butoxide (10 g) were dissolved in *tert*-butyl alcohol (150 mL) at 40°C. Ethyl bromoacetate (5 mL) was dropped in slowly for 10 min and stirred magnetically for 2 h. After that, the solvent was evaporated with a rotary evaporator and the residue was dissolved in 100 mL NaOH solution (1 N). After 2 h, the resulting solution was adjusted to pH 2 with HCl solution (6 N). CMPEG was extracted with chloroform several times and then, following filtration, dried with anhydrous $MgSO_4$. After evaporation of the chloroform, CMPEG was dried in a vacuum oven for 2 days. The final product of CMPEG was 1.57 g (yield: 78.5%, w/w). The carboxylation of the end group in the PEG was confirmed by FTIR at an absorption of 1730 cm^{-1} (C=O of COOH, data not shown). ^{1}H-NMR spectra of CMPEG in $CDCl_3$ identified absorption peaks at 4.2 ppm (OCH_2COOH, data not shown) and the carboxymethylation degree was evaluated at approximately 78.2%.

The PA-CMPEG (PEP) conjugate was prepared by conjugating the carboxylic acid of CMPEG and the residual hydroxyl group of PA, using DCC/DMAP as a coupling agent. PA (2 g) and CMPEG (0.5 g for PEP-1, 0.75 g for PEP-2) were dissolved in DMSO with 1.3 equivalent of DCC/DMAP. The resulting solution was stirred for 12 h at room temperature. In the reaction mixture, dicyclohexylurea (DCU) was formed, which was then filtered to remove the DCU. The resulting solution was placed in a dialysis membrane (MWCO 12,000, Sigma Co.) and dialyzed against distilled water for 3 days. The dialyzed solution was freeze-dried. The freeze-dried PEP conjugate was purified with chloroform five times to remove unconjugated CMPEG from the final product. Subsequently, the purified sample was filtered and dried in a vacuum oven for 3 days at room temperature. The dried sample was carefully weighted and the yield was 2.21 g for PEP-1 (yield : 88.4 % (w/w)), 2.29 g for PEP-2 (yield : 83.3 % (w/w)) and the conjugation yield of CMPEG into the PA backbone was evaluated as approximately 42 % (w/w) of PEP-1 ((0.21/0.5)*100) and 38.7 % (w/w) of PEP-2 ((0.29/0.75)*100).

Preparation of PEP nanoparticles

Polymeric nanoparticles of PEP were prepared by the dialysis method (Jeong *et al.*, 1998). 40 mg of PEP conjugate was dissolved in 10 mL of DMAc. To form polymeric nanoparticles, the solution was dialyzed against deionized water using a molecular weight cut-off (MWCO) 12,000 g/mol dialysis tube. The distilled water was exchanged hourly for the first 3 h and 3hourly for an additional 21 h, the dialyzed solution was then used to observe morphology, or freeze-dried.

Fourier transform-infrared (FT-IR) spectroscopy

Fourier transform-infrared (FT-IR) spectroscopy measurement (FT-IR Magna IR 550, Nicolet) was used to confirm the synthesis of a PEP conjugate.

Differential scanning calorimetry (DSC) measurement

The melting temperatures (Tm) of PEP polymeric nanoparticles were measured with a Universal V2.4F (TA instruments) differential scanning calorimeter. The measurements were carried out at temperatures ranging from -80 to 250°C, under nitrogen, at a scanning rate of 10°C/min.

Wide angle X-ray diffractometer (WAXD) measurement

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

Photon correlation spectroscopy (PCS)

Particle size distributions were measured by PCS using a Zetasizer 3000 (Malvern Instruments, UK) with a He-Ne laser beam, at a wavelength of 633 nm (scattering angel of 90°) at 25°C. Polymeric nanoparticle suspension (concentration: 1 g/L) was used for particle size measurement without filtering.

Scanning electron microscope (SEM) measurements

The morphology of the polymeric nanoparticles was observed using a SEM (Jeol, JSM 5400, Japan). A drop of the polymeric 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, Japan). The coating was performed at 20 mA for 4 min, and observation was made at 25 Kv.

Fluorescence spectroscopy

To investigate the core-shell structure formation and the critical association concentration (CAC), the PEP conjugate suspension was prepared as follows: 40 mg of the PEP conjugate was dissolved in 10 mL of DMAc and dialyzed using a MWCO 12,000 g/mol dialysis membrane, against distilled water, for 2 days. The resultant solution in the dialysis membrane was then adjusted to the concentrations of the PEP conjugates.

The CAC of the PEP conjugate was estimated to demonstrate the potential for nanoparticle formation, by a spectrofluorophotometer (Shimadzu RF-5301 PC, Tokyo, Japan) using pyrene as a hydrophobic probe (Kalyanasundaram and Thomas, 1977; Wilhelm *et al.*, 1991). The samples were prepared by adding a known

amount of pyrene in acetone to a vial and then evaporating the acetone. The pyrene concentration was adjusted to give a final concentration of 6.0×10^{-7} M in 10 mL of various concentrations of the PEP conjugate solution. The resulting solution was heated for 3 h at 65°C to equilibrate the pyrene and the polymeric nanoaprticles, and then left to cool overnight at room temperature. The emission wavelength was 390 nm for the excitation spectra. The excitation and emission bandwidths were 1.5 and 1.5 nm, respectively.

Drug loading and *in vitro* release studies

Clonazepam (CNZ)-loaded polymeric nanoaprticles were prepared as follows: 20 mg of the PEP conjugates were dissolved in 3 mL of DMAc, and 10 mg of CNZ in 1 mL DMSO was added to this solution. To form the polymeric nanoparticles and remove the free drug, the solution was dialyzed against 1 L×7 of distilled water for 24 h using a MWCO 12,000 g/mol dialysis membrane. The medium was replaced hourly for the first 3 h and 2 h for an additional 9 h, then the volume of the final aqueous solution of nanoparticles was adjusted to 10 mL for the drug release experiment (i.e. 20 mg of polymer/10 mL of water). The nanoparticle aqueous solution was used for further analysis or lyophilized for measurement of drug content.

To measure the drug contents, lyophilized samples of CNZ-loaded polymeric nanoparticles were suspended in methanol, vigorously stirred for 2 h, and sonicated for 15 min. The resulting solutions were centrifuged at 3,000 rpm for 30 min, and the drug concentration was measured in the supernatants using an UV-spectrophotometer (Shimadzu UV-1201, Shimadzu Co. Ltd., Tokyo, Japan) at 309 nm. For a control test, empty nanoparticles of PEP conjugates were used.

$$\text{Drug contents} = \frac{\text{Amount of drug in the nanoparticles}}{\text{Weight of nanoparticles}} \times 100$$

$$\text{Loading efficiency} = \frac{\text{Residual amount of drug in the nanoparticles}}{\text{Feeding amount of drug}} \times 100$$

The release experiment was carried out *in vitro* as follows: 2 mL of a dialyzed solution of nanoparticles (4 mg of polymer/2 mL water as described above) were placed into a dialysis membrane (MWCO 12,000 g/mol), then the dialysis membrane was put into a 200 mL bottle with 98 mL of PBS. The medium was stirred at 100 rpm at 37°C. At predetermined time intervals, the entire medium was removed and replaced with the same amount of fresh PBS. The amount of CNZ released from the polymeric nanoparticles was measured with a UV spectrophotometer at 309 nm (Jeong *et al.*, 1998).

RESULTS AND DISCUSSION

Pullulan was modified by replacing hydroxyl groups of the glucose unit with acetate groups to produce hydrophobically modified pullulan, pullulan acetate (PA). As reported previously (Jung *et al.*, 2003), PA itself also has self-association properties in an aqueous system and formed nanoparticles. CMPEG introduction into PA resulted in PEP graft copolymer, which has desirable properties, such as, avoidance of uptake by macrophages, absorption of proteins and favorable biodistribution.

Fig. 1 shows the FT-IR spectra of Pullulan (a), PA (b), and the PEP conjugate (c). The spectra demonstrated the introduction of the acetyl group, as indicated by C=O stretching at 1752 cm^{-1}, CH_3 deformation at 1732 cm^{-1}, and O-C=O bonds at 602 cm^{-1}. Four characteristic peaks on this spectra, i.e. C=O stretching at 1747 cm^{-1}, amide deformation at 3330 cm^{-1} and amide bending at 1570 cm^{-1},

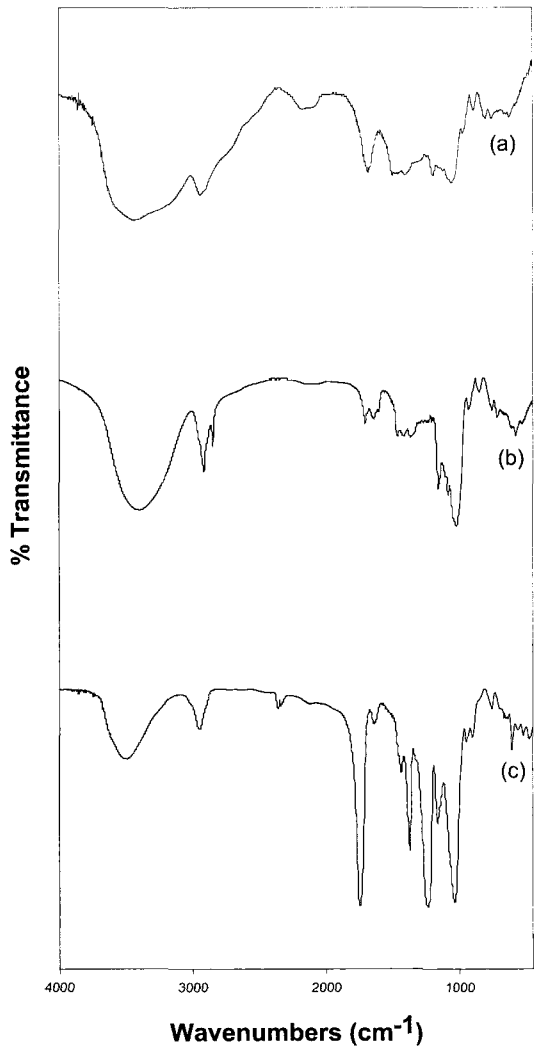

Fig. 1. FT-IR spectra of pullulan (a), pullulan acetate (b), and the PEP conjugate.

may be used to confirm that PEG conjugated to PA.

Generally, block and graft copolymers, as well as other amphiphilic materials, show self-assembling potential in aqueous media (Wilhelm *et al.*, 1991). To evaluate the CAC of the polymeric nanoparticles of the PEP conjugate, fluorescence spectroscopy was performed and pyrene was used as a hydrophobic probe. Fig. 2 shows the fluorescence excitation spectra of pyrene at the self-assembly of PA and PEP conjugates. The fluorescence intensity of pyrene was found to increase with increasing concentrations of PA and PEP conjugates, which indicates self-assembly of the hydrophobized pullulan and PEP conjugates in water. In addition, in the excitation spectra, a red shift was observed with increasing PA and PEP conjugation. It is thought that pyrene is preferentially solubilized into the nanospheres, composed of a core-shell structure, when it is introduced into the aqueous phase using a good solvent (Kwon *et al.*, 1993). The intensity ratio of I_{337}/I_{334} versus log c of PA and PEP in the pyrene excitation spectra is shown in Fig. 3. 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 as the CAC value of PA and PEP conjugates. As shown in Table I, the CAC value of PEP was higher than PA itself, indicating that the association concentration was increased by introduction of PEG and PEP nanoparticles more hydrophilic than PA.

Table I shows the drug-loading contents and loading efficiency of PA and PEP. When PEG was introduced, drug contents and loading efficiency were decreased. The reason for these results might be that PEP, compared to PA itself, became more hydrophilic by the introduction of PEG. This resulted in a relatively weak hydrophobic interaction between drug and polymer, resulting in decreased drug contents. Furthermore, drugs can easily leak during the dialysis procedure. Fig. 4 shows the SEM photographs of PA and PEP nanoparticles. As shown in Fig. 4(a) and

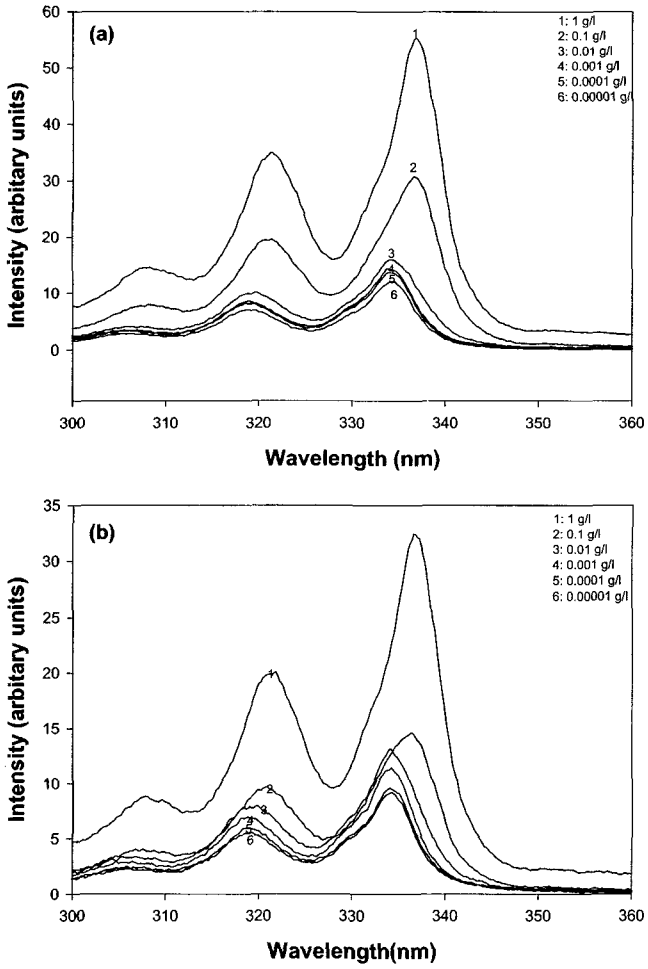

Fig. 2. Fluorescence excitation spectra of pyrene (6.0×10^{-7} M) versus the concentration of PA (a) and the PEP-1 conjugate (b) in distilled water (λ_{em} = 390 nm).

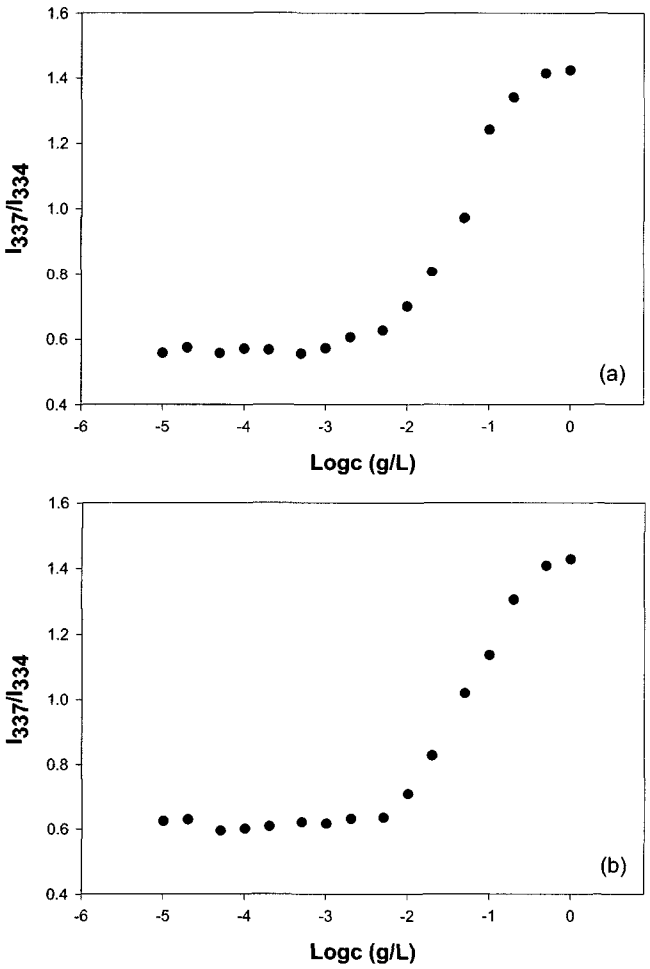

Fig. 3. Plots of intensity ratios I_{337}/I_{334} from the pyrene excitation spectra versus log c of the PA (a) and PEP-1 conjugate (b) in distilled water [pyrene]= 6.0×10^{-7} M.

Table I. Drug contents and loading efficiency of PA and PA-PEG

	CAC (g/L)	Particle size (nm)	Drug contents (%, w/w)		Loading efficiency (%, w/w)
			Theoretical	Experimental	
PA	0.0040	203.760.0	33.3	12.2	27.8
PEP-1	0.0063	193.313.5	33.3	9.1	20.0
PEP-2	0.0081	179.232.4	33.3	7.8	16.9

(b), both the nanoparticles of PA and PEP has spherical shapes and their particle sizes ranged from 200-1000 nm.

Fig. 5 shows the powder XRD scan of CNZ-loaded PEP noparticles. It can be observed that the XRD patterns showed broad peaks in the PEP-1 conjugates (Fig. 5(a)) and sharp peaks in CNZ drug crystals (Fig. 5(b)). In addition, specific drug crystal peaks were observed in the physical mixture of CNZ/PEP-1 empty nanoparticles (Fig. 5(c)) whereas specific peaks of drug crystals had disappeared in the CNZ-entrapped PEP-1 nanoparticles (Fig. 5(d)). It was thought that a crystalline drug showed sharp, specific, crystal peaks when it existed as drug crystals but, in this case, the drug existed as a molecular dispersion within the nanoparticles after the drug was incorporated into them (Gref *et al.*, 1994). These results

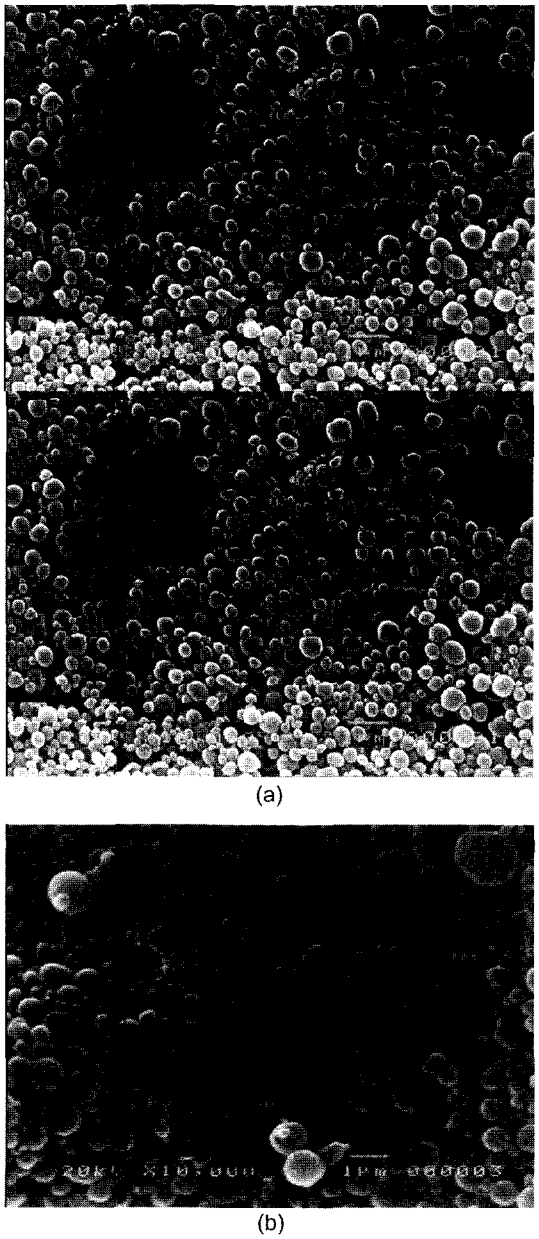

Fig. 4. Scanning electron microscopy photograph (SEM) of PA (a) and the PEP-1 conjugate (b).

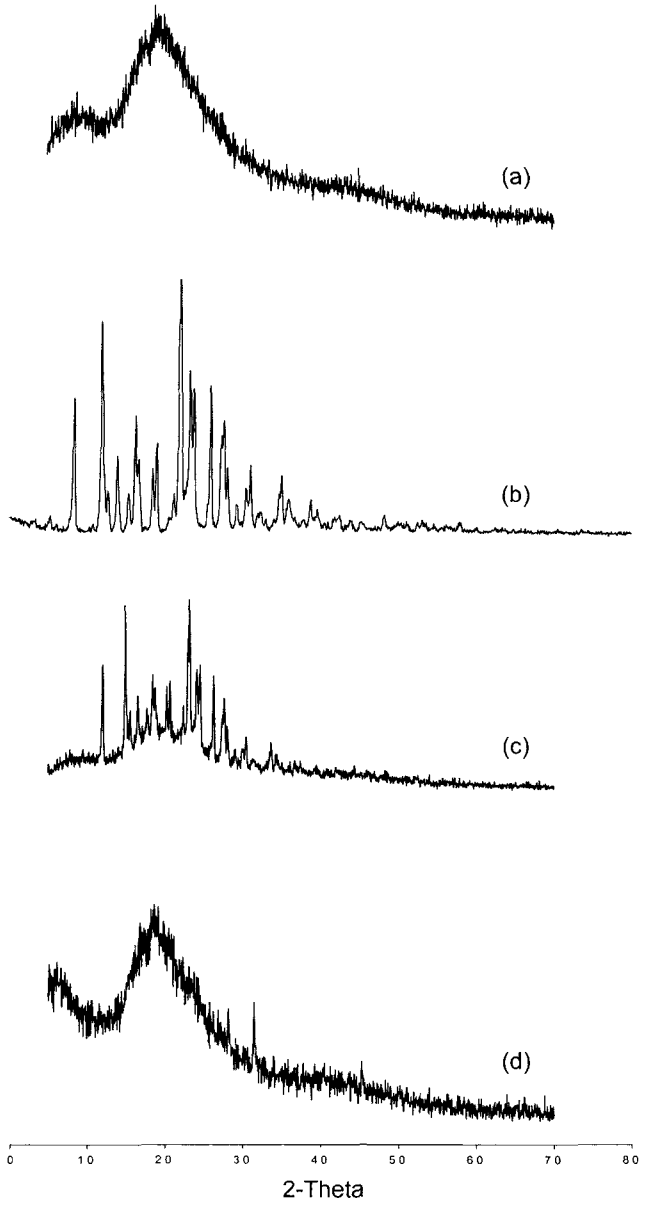

Fig. 5. X-ray diffraction patterns of PEP-1 empty nanoparticles (a), CNZ (b), CNZ/PEP-1 nanoparticle physical mixture (c), and CNZ-entrapped PEP-1 nanoparticles (d).

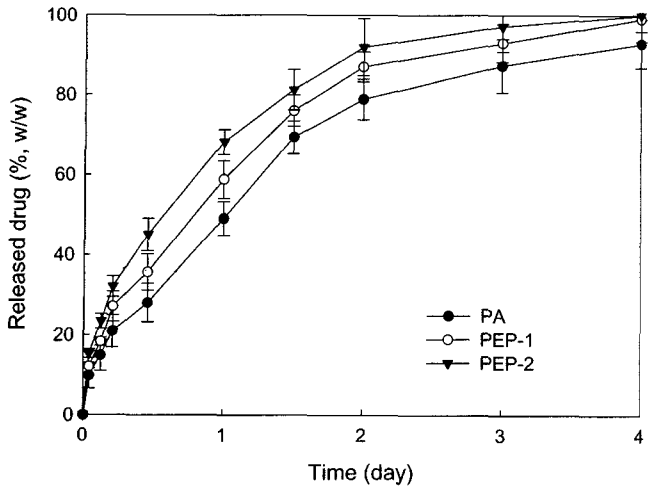

Fig. 6. CNZ release from PA and PEP nanoparticles.

showed that the CNZ was successfully entrapped into the nanoparticles as a molecular dispersion.

To study the drug release behavior, the CNZ-loaded nanoparticles of PA and PEP conjugates were simply redistributed in PBS (pH 7.4, 0.1 M) without surfactant. Fig. 6 shows the release kinetics of CNZ from nanoparticles of PA and PEP conjugates. It was observed that CNZ had sustained released from the nanoparticles for 4 days and the release rate was increased by introduction of PEG. These results indicated, that introduction of PEG into PA induces a more hydrophilic microenvironment into nanoparticles, resulting in the drug being diffused out more easily through hydrophilic aqueous channels by diffusion mechanisms. In addition, hydrophobic interaction between the drug and the polymer chain may become weaker by the introduction of PEG. Thus, the increased solubility of the drug in the nanoparticles induces easier drug release. Gref *et al.* (1994) reported that crystallization of hydrophobic drugs occurred inside the nanoparticles, especially at higher drug loadings, in these cases, phase separation tended to occur which led to crystallization. This reasoning was used to explain why hydrophobic drugs, loaded into nanoparticles, were released more slowly at higher drug loadings.

In conclusion, self-assembling nanospheres were prepared using PEG-grafted and hydrophobically modified polysaccharide, PEP. The self-assembling characteristics of PEP were confirmed using ^{1}H-NMR spectroscopy and fluorescent spectroscopy. Observations of SEM showed the spherical morphologies of the PEP nanoparticles and particle size distribution by PCS was 193.3 ± 13.53 nm with a unimodal distribution. Clonazepam (CNZ), as a model drug, was easy to entrap into polymeric nanoparticles of PEP conjugates and the drug release rate was increased by introduction of PEG. This study found that PA and the PEP conjugate are attractive systems for drug delivery because the materials have good biocompatibility with the possibility of manipulating the release characteristics of hydrophobic drugs.

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