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RESEARCH PAPER

Self-Assembling Nanospheres of Hydrophobized Pullulans in Water

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ABSTRACT

In this report, we have prepared self-assembling nanospheres of hydrophobized pullulans. Pullulan acetate as a hydrophobized pullulan was synthesized by acetylation of pullulan and characterized by Fourier transform infrared (FTIR) measurement. From the results of photon correlation spectroscopy (PCS), hydrophobized pullulans could be self-assembled in water as nanospherical aggregates, and their number-average particle size was 74.3 ± 38.2 nm with a unimodal distribution. Also, morphological studies observed by transmission electron microscopy (TEM) showed that self-assembly of hydrophobized pullulans results in nice spherical shapes with a size range of about 50–100 nm, which was in accordance with PCS measurements. Their size and morphology have acceptable properties for intravenous injectable drug-targeting carriers. The fluorescence probe technique was used for self-association of hydrophobized pullulans in water using pyrene as a hydrophobic probe.

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From the fluorescence measurement, the fluorescence intensity of pyrene increased with increasing concentration of hydrophobized pullulans, which indicates self-assembly formation of hydrophobized pullulans in water. Also, in the fluorescence excitation spectrum, a red shift was observed with increasing concentration of hydrophobized pullulans. These results also revealed that hydrophobized pullulans could be self-assembled in water, and from the plot of I_{337}/I_{334} *versus log* c *of hydrophobized pullulans, the critical association concentration was 0.0022 g/l, which was considerably lower than that of low molecular weight surfactants or poloxamer. A drug loading study was performed using clonazepam (CNZ) as a hydrophobic model drug. We observed that the higher the feeding amount of drug was, the more the drug loading contents were, the lower the drug loading efficiency was, and the larger the particle size was. CNZ was released from nanospheres via pseudo-zero-order kinetics, and the increased drug loading contents led to slower release of the drug.*

INTRODUCTION

Supramolecular self-assembly has been investigated intensively in recent decades, and the nanosize structure has seen considerable attention due to the extensive applications of the self-assemblies in such areas as colloid science, electronics, environmental technology, biotechnology, and biomedical engineering (1–6). The use of the self-aggregation properties of amphiphilic macromolecules in selective solvents has become an attractive strategy for such self-assembly as lipid bilayer membranes (7), polymeric micelles (8), and Langmuir-Blodgett films (9). In drug delivery systems especially, the self-aggregation characteristics of amphiphilic polymers in aqueous solutions have had explosive attention for development of effective targetable drug carriers such as polymeric micelles (10), hydrophobized polysaccharide (11), and aquasomes (12).

Amphiphilic block copolymers with hydrophobic and hydrophilic segments are generally self-assembled in the form of micelles in aqueous media. In these micelles, the hydrophobic blocks constitute the core, and the hydrophilic blocks form the corona. Due to their thermodynamic stability and small size structure (below 100 nm), polymeric micelles are considered effective drug delivery carriers for prolonged blood circulation and tumor targeting (13). Yokoyama et al. reported that adriamycin-conjugated poly(ethylene glycol)–poly(aspartic acid) block copolymeric micelles were effective tools for treatment of solid tumors and have the potential for prolonged blood circulation time (14,15). Akiyoshi et al. reported that hydrophobized polysaccharide has characteristics that are amphiphilic in water, and it can form stable self-assembly in aqueous media as nanoparticles (11). Also, they reported the thermoresponsiveness of hydrogel nanoparticles (16), and there was stable macromolecular complexation with proteins on heating of a self-assembly of hydrophobized polysaccharide (17). But, in spite of its importance, the self-assembly of hydrophobically modified polysaccharide has been little investigated for use as injectable colloidal drug carriers.

In drug delivery systems, nanoparticles or colloidal carriers have been widely accepted for intravenous (i.v.) injection of drugs and for drug targeting issues (18–20). The possibility of drug targeting to specific organs or tissues would provide great benefit in the therapy of several disease states (21,22). The use of nanoparticles has attracted considerable interest for achieving these objectives. It is widely accepted that the fate of nanoparticles after intravenous injection is influenced greatly by their interaction with the biological environment and their physicochemical properties. The effect of nanoparticle size has been shown to be of primary importance (18,23). For example, administered particles several micrometers in diameter become filtered by the lung capillaries (24,25). Also, smaller, submicron particles are normally rapidly cleared by the reticuloendothelial system (RES), such as the Kupffer cells of the liver, a major barrier to effective targeting to other sites (26–28).

In this study, we described the preparation of self-assembling nanospheres of hydrophobized pullulans in water and studied the potential of drug carriers using clonazepam (CNZ) as a hydrophobic model drug. CNZ is an anticonvulsant benzodiazepine that is efficacious for the treatment of panic disorder and is considerably hydrophobic, with water solubility (phosphate buffered saline [PBS], pH 7.4, 0.1 M at 37°C) of less than 14.66 μg/ml; especially, it has a high interaction with proteins

in vivo (29,30). Therefore, there is a need to extend the half-life of CNZ to avoid protein adsorption and rapid clearance by unwanted organs or tissues when the CNZ is injected into the body and to solve the drug solubility. Also, shapes and sizes of the self-assembling nanospheres of hydrophobized pullulans were characterized using fluorescence spectroscopy, photon correlation spectroscopy (PCS), and transmission electron microscopy (TEM). Also, CNZ release from the nanospheres was performed in vitro.

MATERIALS AND METHODS

Materials

Pullulan (MW 200,000) was purchased from Hayashibara Company, Japan. The clonazepam (CNZ) was purchased from Roche, Switzerland. Reagent grade dimethyl sulfoxide (DMSO) was used without further purification.

Synthesis of Hydrophobized Pullulans

Pullulan acetate as hydrophobized pullulans was synthesized by the method reported by Motozato et al. (31) and Na, Jeong, and Lee (32), with 2 g of pullulan suspended in 20 ml of formamide and dissolved by vigorous stirring at 54°C. Then, 6 ml of pyridine and 15 ml of acetic anhydride were added, and the mixture was stirred at 54°C for 48 hr. After 48 hr, dark-brown precipitant was obtained and then purified by reprecipitation with 1000 ml of distilled water and 500 ml of methanol. The resultant precipitant was vacuum-dried for 3 days, and white powder was obtained.

Preparation of Clonazepam-Loaded Nanospheres

Nanospheres of hydrophobized pullulans were prepared by the dialysis method (33–36) as follows. Hydrophobized pullulans (40 mg) were dissolved in 10 ml of DMSO. The solution was stirred at room temperature and solubilized entirely. To form nanospheres, the solution was dialyzed using a molecular cutoff 12,000-g/mol dialysis tube (Sigma Chemical Co., St. Louis, MO) against 1.0 L of distilled water times three for 3 hr, and then distilled water was exchanged at intervals of 3–6 hr for 2 days. The solution was used for analysis and then freeze-dried.

Preparation of drug-loaded nanospheres was carried out as follows. Hydrophobized pullulans (40 mg) was dissolved in 10 ml of DMSO, and subsequently 10–40 mg of CNZ was added. Then, the solution was stirred at room temperature and solubilized entirely. To form nanospheres and remove free drug, the solution was dialyzed using a molecular cutoff 12,000-g/mol dialysis tube against 1.0 L times three of distilled water for 3 hr, and then distilled water was exchanged at intervals of 3–4 hr for 24 hr. The solution was used for analysis and then freeze-dried.

For measurement of drug-loading content, freeze-dried samples of nanospheres were suspended in methanol, vigorously stirred for 2 hr, and sonicated for 15 min. The resulting solution was centrifuged at 20,000 *g* for 30 min, and the supernatant was taken for measurement of the drug concentration using an ultraviolet (UV) spectrophotometer (Shimadzu UV-1201, Shimadzu Co. Ltd., Tokyo, Japan) at 309 nm.

Measurement of Fluorescence Spectroscopy

To investigate the fluorescence spectroscopy characteristics, hydrophobized pullulan nanospheres without drug were prepared as follows. Hydrophobized pullulans (40 mg) was dissolved in 10 ml of DMSO and dialyzed for up to 2 days using the method described above. The resultant solution was adjusted to the various concentrations of hydrophobized pullulans.

The critical assembling concentrations (CACs) of the hydrophobized pullulans were estimated by fluorescence spectroscopy (Shimadzu RF-5301 PC spectrofluorophotometer, Shimadzu Co. Ltd.) using pyrene as a hydrophobic probe (8,37,38) to prove the potential for assembly formation. To get 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 amount was adjusted to give a pyrene concentration in the final solution of either 6.0×10^{-7} M. To each vial, 10 ml of various concentrations of hydrophobized pullulan solutions was added, and then the solutions were heated for 3 hr at 65°C to equilibrate the pyrene and the hydrophobized pullulan nanospheres; the solutions were left to cool overnight at room temperature. The fluorescence spectra were measured at an excitation wavelength of 339 nm. The emission wavelength was 390 nm for the excitation spectra. Excitation and emission bandwidths were 1.5 nm and 1.5 nm, respectively.

Fourier Transform Infrared Spectroscopy Measurement

Fourier transform infrared (FTIR) spectroscopy (Nicolet, Magna IR 550) was used to confirm the structure of the hydrophobized pullulans.

Transmission Electron Microscopy Measurements

A drop of nanosphere suspension containing 0.01% phosphotungstic acid was placed on a carbon film coated on a copper grid for TEM and freeze-dried. Observation was done at 80 kV in a JEOL JEM-2000 FX II, Japan.

Photon Correlation Spectroscopy Measurements

PCS measurements were made with a Zetasizer 3000 (Malvern Instruments, England) with a helium-neon laser beam at a wavelength of 633 nm at 25°C (scattering angle of 90°). A nanosphere solution was used for particle size measurement (concentration 1 mg/ml), and it was measured without filtering.

In Vitro Release Studies

The release experiment in vitro was carried out as follows. CNZ-loaded hydrophobized pullulan nanospheres (7 mg) and 1 ml PBS (0.1 M, pH 7.4) were put into dialysis tubes (molecular cutoff 12,000 g/mol), and the tubes were introduced into a 100-ml bottle with 50 ml PBS; the media were stirred at 100 rpm at 37°C. At specific time intervals, whole medium was taken and replaced with fresh PBS to prevent saturation of the drug. The concentration of the released CNZ into the PBS was determined by a UV spectrophotometer (Shimadzu UV-1201) at 309 nm ($\eta = 10{,}500\ M^{-1}cm^{-1}$) (35).

RESULTS AND DISCUSSION

Pullulan is a water-soluble, neutral, linear polysaccharide consisting of α-1,4 and α-1,6 glycosidic linkages (Fig. 1). Pullulans cannot self-associate in aqueous solutions due to their water solubility. Therefore, hydrophobized pullulans were prepared by introducing an acetate group into hydroxyl groups of the glucose unit of pullulans, producing pullulan acetate. Pullulan acetate (as the hydrophobized pullulans) was analyzed by FTIR measurements (Fig. 2). The spectrum showed the introduction of the acetate group, indicated by C═O stretching at 1752 cm^{-1}, CH_3 deformation at 1375 cm^{-1}, and O − C═O bonds at 604 cm^{-1}. Hydrophobized pullulan (pullulan acetate) was not soluble in water. However, pullulan acetate was easily soluble in various solvents, such as DMSO, dimethylformamide, tetrahydrofuran, dichloromethane, chloroform, 1,4-dioxane, and so on.

Among these solvents, one of the water-miscible solvents (DMSO) was selected for preparing self-assembling nanospheres by the dialysis method. The dialysis method has been used frequently for preparing polymeric micelles, liposomes, and micelle-forming polymeric drugs and for the preparation of drug-targeting carriers. Also, the dialysis method is an acceptable, simple, and effective preparation method for small and narrow size distributed colloidal carriers using amphiphilic materials such as block and graft copolymers.

Particle Size Measurements and Morphological Observations

To prove self-assembly in water, hydrophobized pullulans were dialyzed against water, and the dialyzed resultant solution was viewed to be a milk-like colloidal suspension. Then, formation of self-assembling nanospheres of hydrophobized pullulans was analyzed by PCS (Fig. 3). The particle size distribution of hydrophobized pullulans based on number average was 74.3 ± 38.2 nm, and it showed a narrowed unimodal pattern without any aggregated precipitants. This result was similar to behavior with amphiphiles such as block or graft copolymer micelles and liposomes. In a previous study with block copolymers composed of poly(γ-benzyl L-glutamate) (PBLG) and poly(ethylene oxide) (PEG), we obtained a micellar size value of about 20–60 nm (39). Also, Yokoyama et al. reported that PEG-P(Asp(ADR)) block copolymer micelles self-assembled in water have a diameter of 50–60 nm (14,40).

Of course, although particle size was slightly larger than that of the block copolymer micelles described above, hydrophobized pullulans have acceptable characteristic size distributions of self-assembly as injectable drug carriers. To investigate the stability of hydrophobized pullulan nanospheres, particle size as a function of time was measured; it is summarized in Table 1. After 21 days, the hydrophobized pullulan nanospheres maintained their initial milk-like colloidal suspensions without any precipitants, and the particle size was 84.0 ± 43.2 nm, which is almost same as the initial size distribution value. Also, after 45 days, the particle size distribution was not significantly changed. These results indicated that hydrophobized pullulan nanospheres have superior stability for use as colloidal carriers of drug molecules.

The morphology of hydrophobized pullulan nanospheres observed by TEM is shown in Fig. 4. Just after dialysis of the hydrophobized pullulans, one drop of the resultant suspension with 0.01% phosphotungstic acid

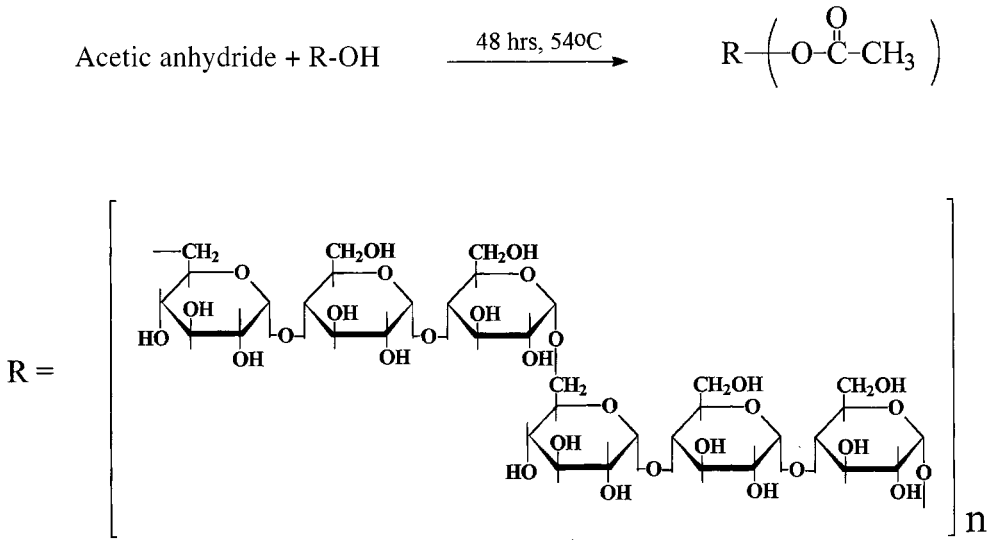

Figure 1. Synthesis scheme of pullulan acetate as hydrophobized pullulans.

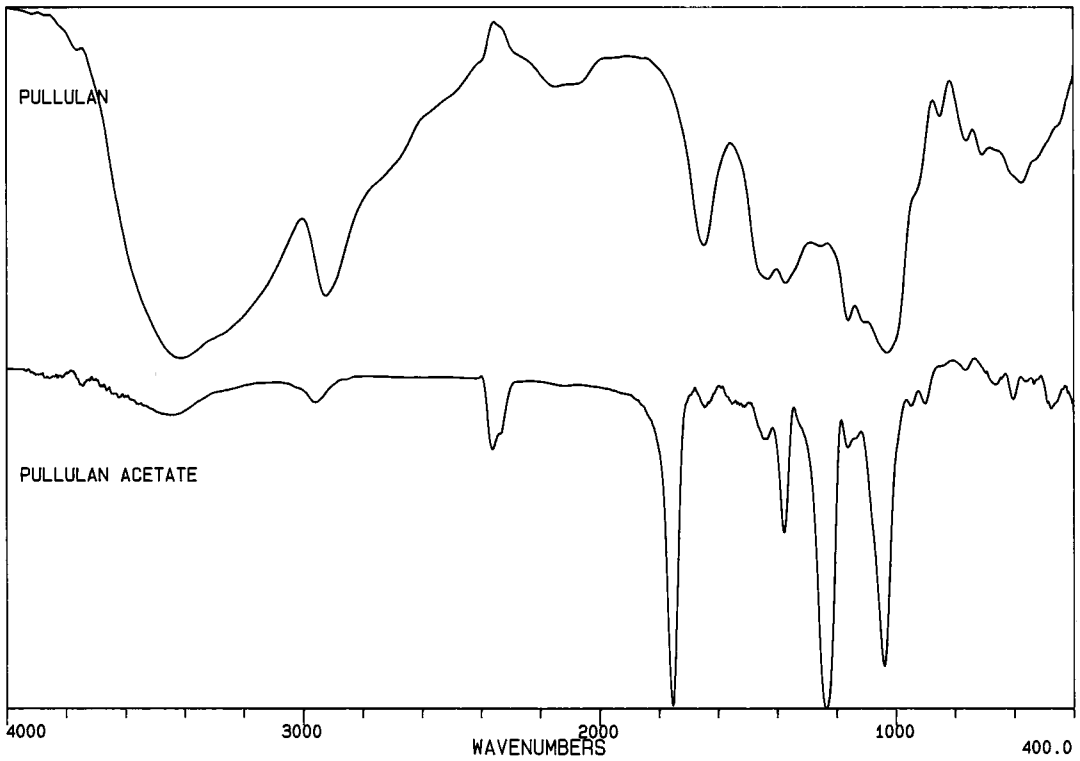

Figure 2. Fourier transform infrared spectroscopy of pullulan and pullulan acetate.

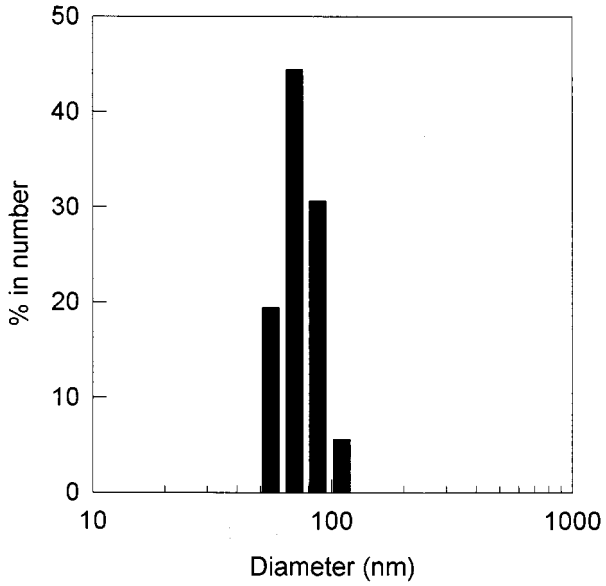

Figure 3. Particle size distribution of hydrophobized pullulans(concentration 1 mg/ml) measured by photon correlation spectroscopy at 25°C.

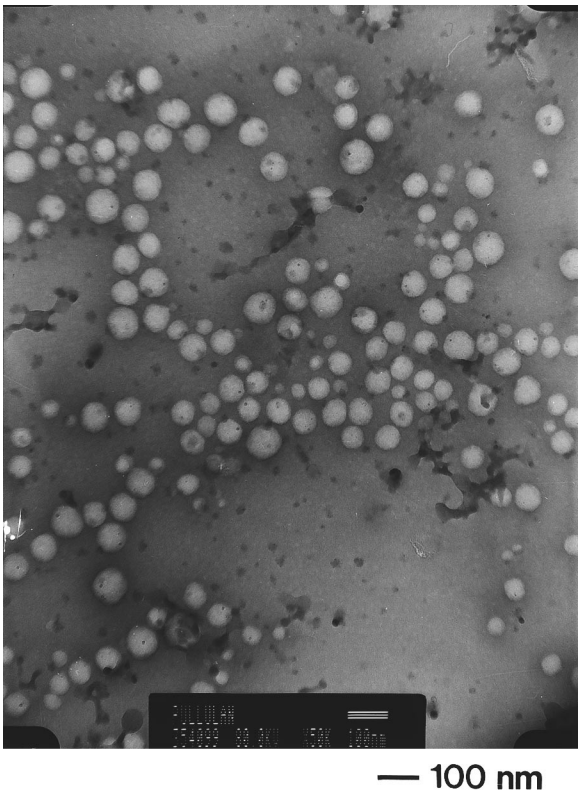

Figure 4. Morphology of self-assembling nanospheres of hydrophobized pullulans observed by transmission electron microscope. Nanospheres were negatively stained with 0.01 wt% phosphotungstic acid.

was placed on a copper grid and then freeze-dried. It is well defined that hydrophobized pullulans have nice spherical shapes that range from about 50 to 100 nm by TEM observations; this size is almost the same as with particle size measurements. These results indicate that hydrophobized pullulans have a potential for self-assembling and characteristics of colloidal carriers for injectable drug delivery.

From the results of particle size measurements and TEM observations, hydrophobized pullulans exist as self-assembling nanospheres with sizes in the same range as polymeric micelles or viruses, which are much larger than proteins. Viruses can be regarded as natural models of drug delivery carriers considering their ability to penetrate to specific cells. Also, the virus size is smaller than the threshold of recognition of the RES. Reticuloendothelial clearance generally increases with increasing size of particulate system. Due to their reduced size (less than 100 nm), the nanospheres of hydrophobized pullulans may also penetrate through the sinusoidal and fenestrated capillaries, which have diameter of about 100 nm; the nanospheres then may have a potential for prolonged

Table 1

Particle Size Distribution of Hydrophobized Pullulans (Concentration 1 mg/ml) Measured by Photon Correlation Spectroscopy at 25°C

	Particle Size (nm)		
	Intensity Average	Volume Average	Number Average
0 days	78.8 ± 35.5	76.9 ± 42.0	74.3 ± 38.2
After 21 days	90.8 ± 42.1	87.5 ± 48.2	84.0 ± 43.2

blood circulation, resulting in a longer half-lives for drugs or targeting to specific organs and cells (14,15,40).

Fluorescence Spectroscopy Study

To investigate whether or not hydrophobized pullulans actually revealed self-association behavior, such as block copolymer micelles or other amphiphilic systems do, the fluorescence probe technique, using pyrene as a hydrophobic probe, was used to determine the CAC.

Wilhelm et al. reported the determination, by the fluorescence technique using pyrene as a hydrophobic probe, of micelle formation by poly(styrene) (PS) and poly(ethylene oxide) (PEO) di- or triblock copolymers in water, and they determined the critical micelle concentration (CMC) from the fluorescence and excitation spectra as pyrene partitions between aqueous and micellar environments (38). This method was also used by Kwon et al. to prove the polymeric micelle formation of poly(β-benzyl L-aspartate) (PBLA) and PEO diblock copolymer in water (8). Also, in our previous report, it was reported that PBLG and PEO block copolymer formed polymeric micelles, and the CMC was relatively lower than that typical for poloxamers (CMC = 1–24 g/L) (41). If hydrophobized pullulans can be self-assembled in water, formation of the self-assembly should be proved by this fluorescence probe technique in the same manner as block copolymer micelles. Also, the CAC can be estimated to prove the potential of self-assembly formation on critical concentrations using pyrene as a hydrophobic probe (37,38,42).

Figure 5a shows the fluorescence emission spectra of pyrene at a fixed excitation wavelength of 339 nm in the presence of varying concentrations of hydrophobized pullulans. The higher the concentrations of hydrophobized pullulans, the higher the fluorescence intensity, which indicates the formation of self-assembly of hydrophobized pullulans in water as for block copolymeric micelles (8,38). Pyrene will preferentially partition into hydrophobic microdomains with a concurrent change in the molecule's photophysical properties (8,43–45). Total fluorescence intensity of a fluorescence probe increases on micellization by self-assembly; this has been utilized to determine the CMC for a host of surfactants (46) and PEO-PS (44). Changes of total fluorescence intensity were negligible at the low concentrations of hydrophobized pullulans. As the concentration of hydrophobized pullulans increased, the total fluorescence intensity increased logarithmically at a critical concentration of hydrophobized pullulans, that is, the CAC. These results

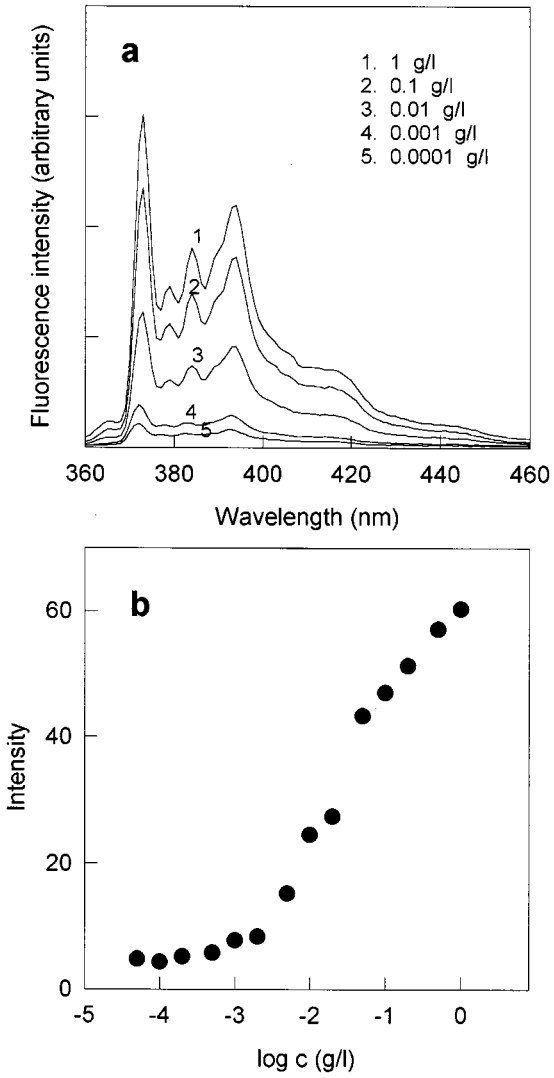

Figure 5. (a) Fluorescence spectra of pyrene (6.0×10^{-7} M)/hydrophobized pullulans (HP) against HP concentration in distilled water (excitation wavelength 339 nm); and (b) plots of the fluorescence intensity of pyrene versus log *c* of the HP in the distilled water.

were plotted as the intensity versus log *c* to a sigmoidal curve (Fig. 5b). The CAC is taken as the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low hydrophobized pullulan concentrations.

Figure 6a shows the excitation spectra of pyrene in the various concentrations of hydrophobized pullulans. In the excitation spectrum, a red shift was observed with

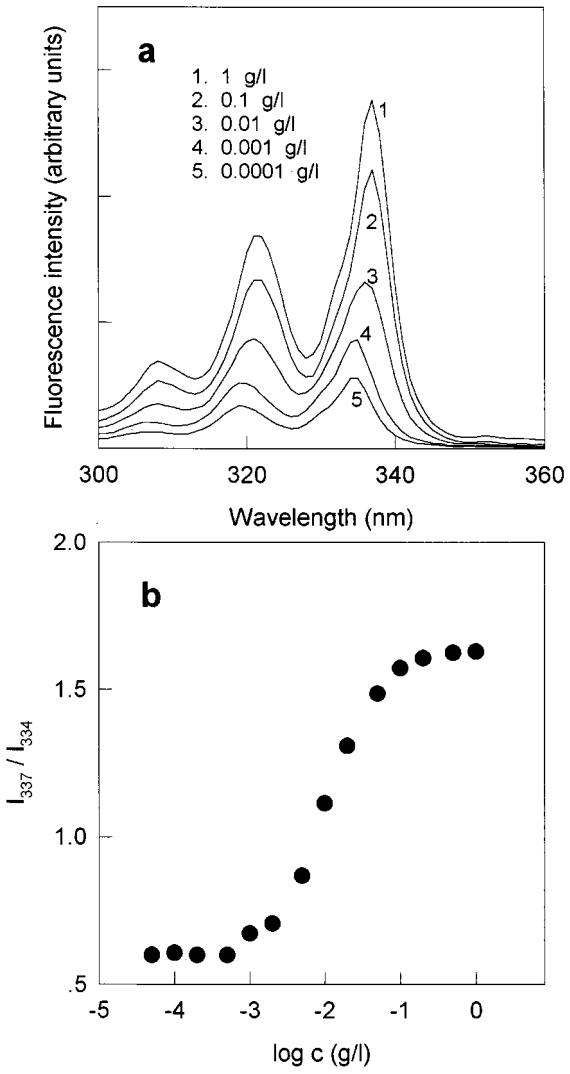

Figure 6. (a) Fluorescence excitation spectra of pyrene (6.0×10^{-7} M)/hydrophobized pullulans (HP) against HP concentration in distilled water (emission wavelength 390 nm); and (b) plots of the intensity ratio I_{337}/I_{334} from pyrene excitation spectra versus log *c* of the HP in the distilled water.

increasing concentration of hydrophobized pullulans. A red shift of pyrene in the excitation spectrum was observed in the study of micelle formation of PS-PEO block copolymers (38). The (0,0) bands in the pyrene excitation spectra were examined and compared with the intensity ratio I_{337}/I_{334}; this ratio takes the value characteristic of pyrene in water at low concentrations and the value of pyrene entirely in the hydrophobic domain. A plot of I_{337}/I_{334} versus log *c* is shown in Fig. 6b. A flat region in the low concentration extreme and a sigmoidal region in the crossover region were noted. This result indicated that a signal change in the region of 0.0022 g/L could be evaluated for the CAC values of hydrophobized pullulans. Practically, the values for CAC from intensity versus log *c* (Fig. 5b) and from I_{337}/I_{334} versus log *c* are quite similar.

From the study of fluorescence probe measurements, it was clear that hydrophobized pullulans could be self-assembled in water on critical concentrations (i.e., CAC), and that they have an amphiphilic nature such as block copolymer micelles.

Drug Loading and Release Study

Table 2 shows the effect of the feeding amount of drug on the drug-loading contents, loading efficiency, and the changes of particle size of nanospheres of hydrophobized pullulans. We observed that the higher the feeding amount of drug was, the more the drug-loading contents and the lower the drug loading efficiency were. After dialysis, the resultant CNZ-loaded nanospheres of hydrophobized pullulans were observed as a milk-like suspension without any other precipitants. Similar results of these phenomena were shown with CNZ-loaded polymeric micelles or core-shell type nanoparticles (35,39). Also, the particle size of nanospheres of hydrophobized pullulans against drug-loading contents was slightly increased with increased drug-loading contents.

To study the drug release behavior, CNZ-loaded nanospheres of hydrophobized pullulans were simply redistributed in PBS (pH 7.4, 0.1 M) without surfactant. Figure 7 shows the release kinetics of CNZ from the nanospheres of hydrophobized pullulans as a function of drug-loading contents. CNZ is continually released in vitro over 2 days (10 wt%) and 4 days (16.7 wt%), and the release pattern was revealed in pseudo-zero-order kinetics. Although direct comparison cannot be performed, the drug release rate from the nanospheres is relatively faster than that of other microsphere systems because of the high surface area and small size of the nanospheres.

It was observed that the higher the drug loading contents were, the slower the drug release was. This phenomenon was reported by several authors (35,36,47). Gref et al. reported that crystallization of hydrophobic drug occurred inside the nanospheres, and, especially, at higher loading contents of drug in the nanospheres, a phase separation occurs, leading to the crystallization of part of the drug (47). Then, hydrophobic drug loaded into nanospheres has a slower release at higher drug loading contents, different from hydrophilic water-soluble drugs. Also, our group observed that CNZ release had slower

Table 2

Effect of Drug Loading Contents on Particle Size Distribution

Hydrophobized Pullulans	Feeding Amount of Drug	Loading Contents (wt%)	Loading Efficiency (wt%)	Particle Size (nm)		
				Intensity Average	Volume Average	Number Average
40	0	—	—	78.8 ± 35.5	76.9 ± 42.0	74.3 ± 38.2
40	10	10.2	45.4	96.1 ± 41.4	91.1 ± 53.4	90.5 ± 37.5
40	20	16.7	40.1	104.0 ± 32.1	101.6 ± 43.7	98.7 ± 40.5

rate kinetics from the nanospheres with higher drug-loading contents. On the other hand, at low drug loading, CNZ is relatively present as a molecular dispersion inside the nanospheres (47). The crystallized drug should dissolve and diffuse more slowly into the outer aqueous phase than that of molecular dispersion. These characteristics of drug release behavior were supported by calorimetric analysis (data not shown), as reported previously (35).

Also, because of differences in the diffusivity of drug molecules to the outer aqueous phase, drug release kinetics are affected not only by drug-loading contents, but also by the size of nanospheres and polymer degradation rates. For the same drug-loading contents, the drug release rates in large nanospheres were slower than that for small-size nanospheres, as reported elsewhere (21,48).

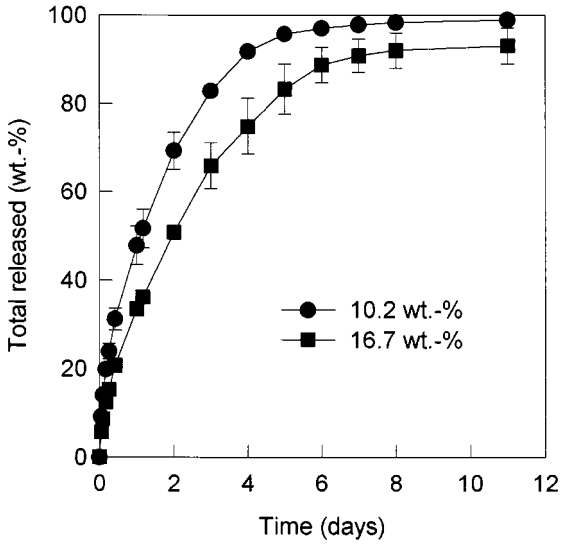

Figure 7. Release of clonazepam from nanospheres of hydrophobized pullulans in PBS (0.1 M, pH 7.4) at 37°C with a shaking speed of 100 rpm.

But, at present, direct comparison of drug release rates with different sizes of nanospheres cannot be performed because different drug loading leads to different particle sizes. Of course, further investigations on the drug release characteristics are needed for small-size nanospheres as a function of drug-loading contents, the size of nanospheres, and the polymer used. Therefore, control of the drug release kinetics can be achieved by optimizing the chemical nature of the polymers used, the drug-loading contents, and the particle size of the nanospheres (47).

CONCLUSION

In conclusion, we prepared self-assembling nanospheres of hydrophobized pullulans. Pullulan acetate (as hydrophobized pullulans) was synthesized by acetylation of pullulan, and it was characterized by FTIR measurement. From the results of PCS, hydrophobized pullulans could be self-assembled in water as nanospherical aggregates, and their number-average particle size was 74.3 ± 38.2 nm with a unimodal distribution. Also, morphological studies by TEM showed the self-assembly of hydrophobized pullulans had nice spherical shapes, and their size range was about 50–100 nm, which was in accordance with PCS measurements. Their size and morphology have acceptable properties for intravenous injectable drug-targeting carriers. A fluorescence probe technique was used for self-association of hydrophobized pullulans in water using pyrene as a hydrophobic probe. From the fluorescence measurements, the fluorescence intensity of pyrene increased with increasing concentration of hydrophobized pullulans, which indicates self-assembly formation of hydrophobized pullulans in water. Also, in the fluorescence excitation spectrum, a red shift was observed with increasing concentration of hydrophobized pullulans. These results also revealed that hydrophobized pullulans could be self-assembled in water, and from the

plot of I_{337}/I_{334} versus log *c* of hydrophobized pullulans, the CAC was evaluated as 0.0022 g/l, which was considerably lower than that of low molecular weight surfactants or poloxamer. A drug-loading study was performed using CNZ as a hydrophobic model drug. We observed that the higher the feeding amount of drug was, the more the drug-loading contents, the lower the drug loading efficiency, and the larger the particle size were. Clonazepam was released from nanospheres by pseudo-zero-order kinetics, and the increased drug loading contents led to slower release of drug.

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