Self-organized Pullulan/Deoxycholic Acid Nanogels: Physicochemical Characterization and Anti-cancer Drug-releasing Behavior

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Abstract The objective of this study was to develop new self-organized nanogels as a means of drug delivery in patients with cancer. Pullulan (PUL) and deoxycholic acid (DOCA) were conjugated through an ester linkage between the hydroxyl group in PUL and the carboxyl group in DOCA. Three types of PUL/DOCA conjugates were obtained, differing in the number of DOCA substitutions (DS; 5, 8, or 11) per 100 PUL anhydroglucose units. The physicochemical properties of the resulting nanogels were characterized by dynamic light scattering, transmission electron microscopy, and fluorescence spectroscopy. The mean diameter of DS 11 was the smallest (approx. 100 nm), and the size distribution was unimodal. To determine the organizing behavior of these conjugates, we calculated their critical aggregation concentrations (CACs) in a 0.01-M phosphate buffered saline solution. They were 10.5×10^{-4} mg/mL, 7.2×10^{-4} mg/mL, and 5.6×10^{-4} mg/mL for DS 5, 8, and 11, respectively. This indicates that DOCA can serve as a hydrophobic moiety to create self-organized nanogels. To monitor the drug-releasing behavior of these nanogels, we loaded doxorubicin (DOX) onto the conjugates. The DOX-loading efficiency increased with the degree of DOCA substitution. The release rates of DOX from PUL/DOCA nanogels varied inversely with the DS. We concluded that the PUL/DOCA nanogel has some potential for use as an anti-cancer drug carrier because of its low CAC and satisfactory drug-loading capacity.

Keywords: self-organized nanogels, anti-cancer drug, pullulan, deoxycholic acid

INTRODUCTION

Many studies of self-organized nanogels derived from polysaccharides have been carried out because of their potential use in the biomedical field and pharmaceutical industry. In particular, because of their enhanced permeation and retention characteristics–which improve their potential to be localized in tumor sites–they have been investigated for their potential usefulness in anti-cancer drug delivery [1-6]. Each nanogel is composed of a polycore that can contain a water-insoluble drug and an outer hydrophilic shell that serves as a barrier against interactions with surrounding biological materials [7,8]. Na *et al.* reported their findings on self-organizing nanogels consisting of curdlan conjugated to sulfonylurea [7]. The sulfonylurea groups contained noncovalent cross-linkages that resulted in the formation of nanoaggregates with a polycore structure, which is suitable for anti-cancer drug delivery. They also reported a nanogel system prepared from pullulan acetate and its derivatives [8-11] that demonstrated excellent tumor-targeting efficiency [8] and pH-sensitivity to responsive in tumor acidity [9,11].

We used pullulan/deoxycholic acid (PUL/DOCA) conjugates to prepare a self-organized nanogel for anti-cancer drug delivery. PUL (Fig. 1A) is a linear polysaccharide that contains an α-(1-6)-linked maltotriosyl repeating unit. Its derivatives have been investigated for their potential use in the pharmaceutical industry and in the biomedical field. The Sunamoto group reported a cholesterol-bearing PUL that forms monodispersed self-aggregating molecules through intra- or intermolecular linkages in a dilute aqueous solution [12,13]. These aggregates help to maintain thermal stability for the proteins in the resulting conjugates. DOCA (Fig. 1B), which is bile acid, is a hydrophobic moiety that has also been used to make self-assembling nanoparticles [14,15] to facilitate the oral administration of hydrophilic polymers [16]. When DOCA is conjugated to chitosan, it can function as a good noncovalent cross-linking moiety, but it cannot escape from chitosan easily because of the amide bond that forms between them. The result is a prolonged retention time for these nanoparticles, which may result in inflammation of local blood vessels or embolization. In our study, DOCA was conjugated to PUL through an ester linkage, which is one of easiest to cleave enzymatically or by hydrolysis *in vivo*. This characteristic would allow us to avoid the problems associated with chito-

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san/DOCA conjugates.

We investigated the efficacy of 3 types of nanogels that differed in terms of the number of DOCA substitutions (DSs). They were physicochemically characterized by particle size, fluorescence intensity, and critical aggregation concentration (CAC) in order to evaluate their pharmacological properties, *i.e.*, drug-loading efficiency and drug-releasing patterns.

MATERIALS AND METHODS

Materials

PUL (MW: 100,000 kDa) was obtained from Hayashibara (Okayama, Japan). DOCA, dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), doxorubicin (DOX), and pyrene were purchased from the Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used in this study were of reagent grade.

Synthesis of PUL/DOCA Conjugates

DOCA was conjugated to PUL through DCC- and DMAP-mediated ester formation. When DOCA was placed in dried dimethyl sulfoxide (DMSO), its carboxyl groups were activated by the addition of DCC and DMAP (Fig. 1). Activated DOCA was then added in 50-, 150-, and 250-mg portions to 50 mL of dried DMSO containing 1 g of PUL and left at room temperature for 24 h. The reactant mixture was then filtered and dialyzed against distilled water for 2 days using a dialysis tube (molecular cut-off: 12,000 kDa). The distilled water was changed every 3 to 6 h. The PUL/DOCA conjugate was recovered by filtration and washed thoroughly with diethyl ether and distilled water then lyophilized. To completely remove the nonreactants, the lyophilized sample was again dissolved in DMSO and dialyzed against distilled water. This process was repeated 3 times. The degree of substitution (DS), defined as the number of DOCA groups per 100 anhydroglucose units of PUL, was determined by nuclear magnetic resonance (^{1}H-NMR). The NMR measurements were taken with all samples dissolved in DMSO-d_6 (Aldrich, Milwakee, WI, USA); tetramethylsilane (TMS; Aldrich) was used as a reference.

Preparation of Self-organizing Nanogels from PUL/DOCA Conjugates

Self-organizing nanogels were prepared using a diafiltration method (molecular cut-off: 2,000 kDa). Each 50-mg portion of PUL/DOCA with a different DS was dissolved in 20 mL of DMSO. Each solution was stirred at room temperature and dialyzed against distilled water for 3 days. The solution was filtered using a 0.45-μm filter to remove the precipitated material.

Particle Size Analysis

The particle size was determined by dynamic light scattering (DLS; Zetasizer 3000, Malvern Instruments Ltd., UK) using an argon ion laser beam at a wavelength of 488 nm and a scattering angle of 90°. Each sample was filtered through a 0.45-μm filter directly into a precleaned cylindrical cell (diameter: 10 mm). The sample concentration was kept at 1.0 mg/mL.

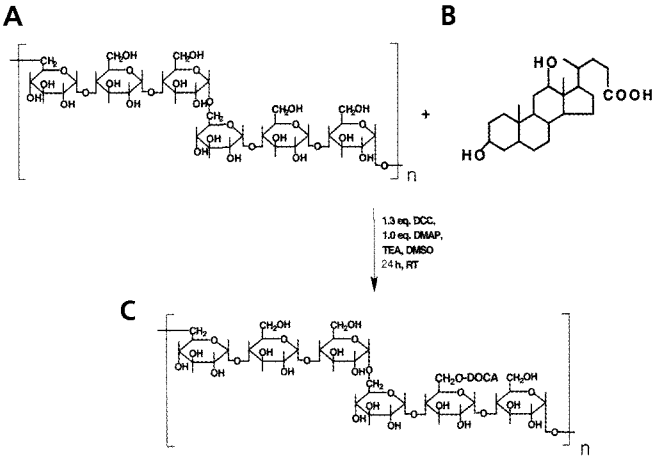

Fig. 1. Chemical structure of (A) PUL, (B) DOCA, and (C) the PUL/DOCA conjugate.

Morphological Observations of the PUL/DOCA Nanogels

To observe the nanoparticles in a dried state, a drop of the self-organized nanogels was dissolved in water and placed on a carbon-coated copper grid then dried under a vacuum at 30°C. Samples were observed without staining by transmission electron microscopy (TEM; Philips CM 30, Philips Electron Optics, Eindhoven, the Netherlands) at 80 kV.

Measurement of Fluorescence (Pyrene)

A stock solution of pyrene (6.0×10^{-2} M) in acetone was prepared and stored at 5°C until used. To measure steady-state fluorescence spectra, this solution was added to distilled water to produce a pyrene concentration of 12.0×10^{-7} M. The solution was then distilled under a vacuum at 60°C for 1 h to remove the acetone from the solution. The acetone-free pyrene solution was mixed together with solutions containing self-aggregated nanoparticles in concentrations of 1×10^{-4} to 1.0 mg/mL. The final concentration of pyrene in each sample solution was 6.0×10^{-7} M, which is nearly equal to its solubility in water at 25°C.

DOX Loading and Release

DOX·HCl (20 mg) and 1.3 Eq triethylamine were allowed to react in *N,N*-dimethyl acetamide (DMAc) (3 mL) to form the DOX basic adduct. PUL/DOCA (50 mg) was added to the DOX solution, and the mixture was stirred overnight at 4°C in the dark. The polymer/DOX

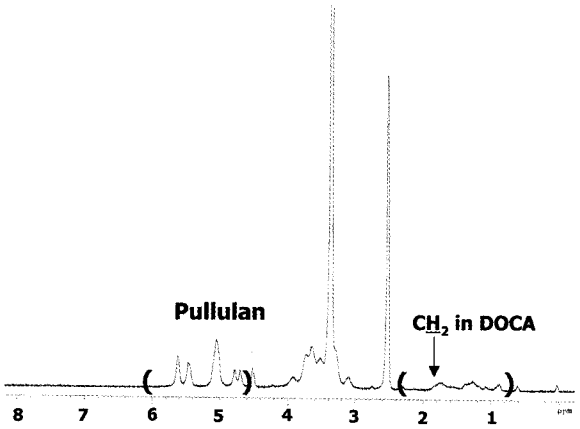

Fig. 2. ^{1}H-NMR of DS 5. The sample was dissolved in DMSO-d_6, and tetramethylsilane (TMS) was used as a reference.

solution was then transferred to dialysis tubing (Spectra/Por; molecular weight cut-off: 15,000 kDa) and dialyzed against a 0.01-M phosphate buffered saline (PBS) solution (pH = 7.4) for 3 days at 25°C. The buffer was exchanged with a fresh solution every 3 h for the first 24 h, then daily. The solution was then filtered through a 0.45-μm filter to remove any polymer or drug that had precipitated out of solution and was freeze-dried to obtain the DOX-loaded nanogels. To determine the drug-loading capacity of these nanogels, a freeze-dried sample was placed in DMAc, stirred vigorously for 2 h, and then sonicated for 3 min. The resulting solution was centrifuged at 20,000 g for 30 min, and the supernatant was analyzed using an ultraviolet (UV) spectrophotometer at 490 nm.

The release rate was measured *in vitro*, as follows: 2 mL of the drug-loaded nanogel solution (1 g/L) was transferred in 10 mL of PBS to a dialysis tube. The release medium was stirred at 100 rpm at 37°C. At predetermined sampling times, all of the medium was removed and replaced with fresh PBS to maintain a sink condition. The amount of DOX in the solution was determined by UV spectroscopy at 490 nm.

RESULTS AND DISCUSSION

Physicochemical Characterization of PUL/DOCA Nanogels

Bonding between the PUL hydroxyl group and the DOCA carboxyl group was induced by means of a conventional carbodiimide reaction, in which carbonyl compounds bind preferably to the hydroxyl group at C6 in polysaccharides. ^{1}H-NMR spectra showed that DOCA had bonded successfully with PUL. Specifically, ^{1}H-NMR (300 MHz, DMSO-d_6) presented δ 0.6 to δ 1.8 (2H, CH_2 in methylene; -C, -O, -O-C) for DOCA and broad multiple peaks in the range of 4.5 to 5.8 ppm for PUL (Fig. 2). The degree of substitution calculated for each PUL-DOCA bond (*i.e.*, the number of DOCA units per 100 PUL glucose units) was 4.86, 8.12, and 11.26, respectively, for DS 5, 8, and 11.

Table 1. Properties of PUL/DOCA nanogels in aqueous media

Sample	Particle size[a] ± SD (nm)	CAC[b] × 10^{-4} (mg/mL)	DOX-loaded particle size[a] ± SD (nm)	Drug-loading efficiency[c] ± SD (wt%)
DS 5	147 ± 34	10.5	110 ± 34	18.5 ± 3.6
DS 8	121 ± 24	7.2	102 ± 29	21.4 ± 4.2
DS 11	99 ± 22	5.6	87 ± 16	25.2 ± 3.1

[a]Mean diameter (intensity average) measured by dynamic light scattering.
[b]Critical aggregation concentration determined from I_{338}:I_{335} data.
[c]The loading efficiencies were measured as total loaded drug weight/initial drug weight × 100.

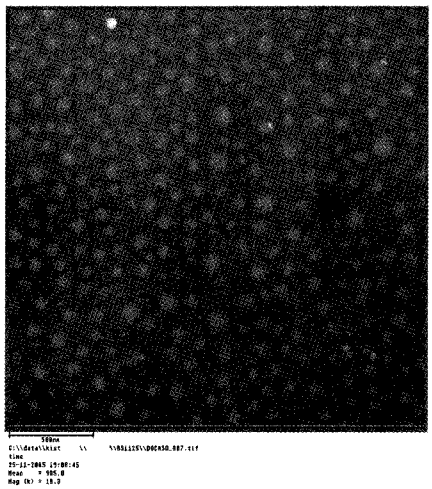

Fig. 3. Transmission electron microscopy (TEM) photograph (scale: bar = 500 nm) of DS 5 nanogels.

Self-organized nanogels were prepared by dialysis, which prevented the rapid, uncontrolled precipitation of polymer during the self-assembly process. The size and size distribution of the nanogels were measured by DLS. The nanogels prepared from the PUL/DOCA conjugates were less than 200 nm in diameter, with a unimodal size distribution. The diameter of DS 11 was approximately 100 nm, which is smaller than the critical size required for a particle to be recognized by the reticuloendothelial system (Table 1). These results indicate that a high concentration of DOCA groups in the PUL molecule increase the chance for hydrophobic interactions to occur and result in particle squeezing, which results in a smaller particle size. The morphology of the DS 5 nanogel, as seen by TEM, is shown in Fig. 3. It is spherical, with a diameter ranging from less than 100 nm.

The self-organizing conjugates that form in aqueous media were monitored during a pyrene study by fluorescence spectroscopy (Fig. 4). At low DS-5 concentrations, the change in total fluorescence intensity and the shift in the (0,0) band at 335 nm were negligible. As the DS-5

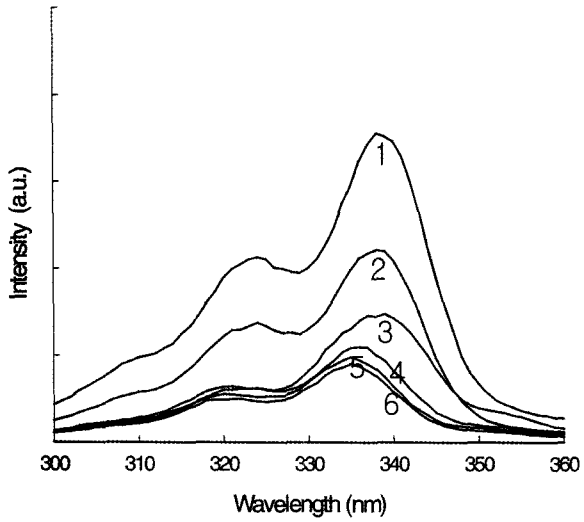

Fig. 4. Excitation spectra of pyrene (6.0×10^{-7} M) in distilled water in the presence of DS 5 as a function of polymer concentration (emission wavelength: 390 nm).

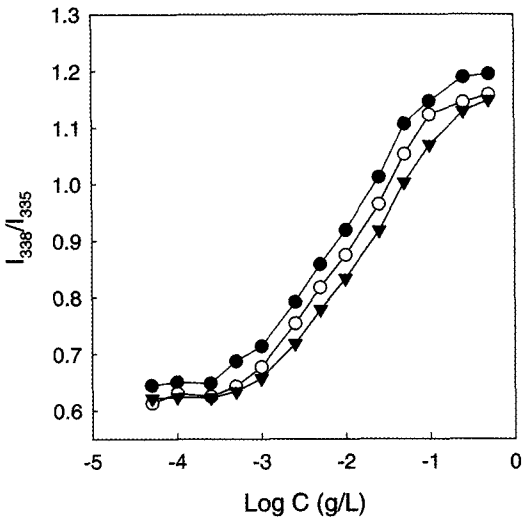

Fig. 5. Plot of intensity ratio I_{338}:I_{335} from excitation spectra versus log C of DS 5 (▼), 8 (O), and 11 (●) self-organized nanogels.

concentration increased, however, the total fluorescence intensity also increased and a red shift in the (0,0) band was observed, indicating that pyrene had been transferred from the aqueous media to the less polar microdomains (interior) of the nanoparticles. The (0,0) band for pyrene at 335 nm shifts to 338 nm when DS 5 is added. The CAC (*i.e.*, the threshold polymer concentration necessary for the self-assembly of nanogels through intra- or intermolecular associations) was determined by monitoring the change in the intensity ratio (I_{338}:I_{335}) of the pyrene in the presence of the polymer. Fig. 5 shows the intensity ratios (I_{338}/I_{335}) for DS 5, 8, and 11 conjugates at various concentrations. The CAC values were determined by the number of crossover points when the concentration was low. The CACs of self-organized nanogels at pH 7.4 are listed in Table 1. The calculated CAC was lower than the typical critical micelle concentration (CMC) for low-molecular-weight surfactants, such as sodium dodecyl sulfate and deoxycholic acid in water [14,17]. The lower CAC values for the conjugates may comprise one of the important characteristics of the polymeric amphiphiles examined in this study; *i.e.*, a small amount of the conjugate self-organizes and maintains stability under dilute conditions.

DOX Loading and Release Test

Recently, various agents with anti-cancer effect have been investigated and commercialized [18,19]. DOX-an anthracycline glycoside antibiotic produced by *Streptomyces peucetius* var. *caesius*-was selected as the model anti-cancer drug. This drug, which is available commercially as a hydrochloride salt, has shown efficacy in the treatment of individuals with solid tumors, including breast cancer, ovarian carcinoma, transitional cell bladder carcinoma, and thyroid carcinoma [20]. However, it is cardiotoxic. Thus, in the field of drug-delivery, the development

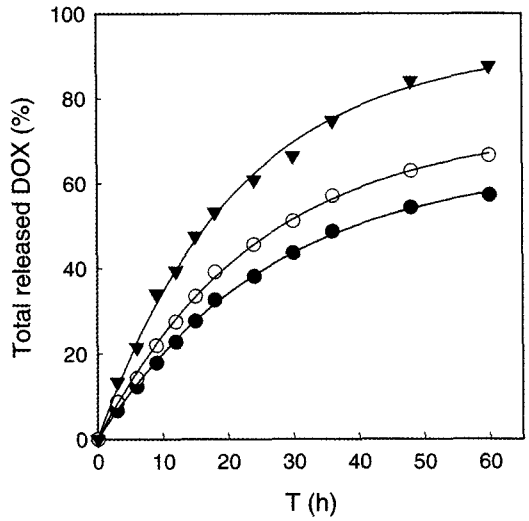

Fig. 6. DOX release behaviors of PUL/DOCA nanogels. DS 5 (▼), 8 (O), and 11 (●) self-organized nanogels.

of safe and effective carrier system for DOX is particularly challenging.

The mean diameter of drug-loaded DS 5 nanogels at pH 7.4 is 110 nm; this is smaller than the diameter of drug-free DS 5 nanogels. This result was observed with all samples. The addition of the hydrophobic drug may reduce the swelling capacity of the nanogel by increasing of the hydrophobic interactions between the DOCA groups and the hydrophobic drugs. The hydrophobic interactions affected the drug-loading efficiency and content of the self-organized nanogels. While the drug-loading efficiency and the loading content of PUL/DOCA increased as the DS increased, the particle size of the nanogels decreased under the same conditions (Table 1).

To study DOX-release kinetics, we dispersed DOX-loaded PUL/DOCA nanogels in a dialysis tube containing PBS buffer (pH = 7.4). The total amount of drug released from DS 5, 8, and 11 in 24 h was 61, 46, and 38%, respectively (Fig. 6). DOX was released from the nanogels in a manner resembling first-order kinetics; thus, the increase in drug-loading and DS of self-organized nanogels resulted in a slower release of DOX. The regression equation for each group was $y = 64.9248(1-e^{-0.0374x})$, $y = 73.6340(1-e^{-0.0402x})$, and $y = 92.5329(1-e^{-0.0465x})$ for DS 5, 8, and 11, respectively. This finding is consistent with those of other researchers [21-23]. Gref *et al.* [22] used differential scanning calorimetry to show that a hydrophobic drug in nanospheres becomes partially crystallized as the amount of drug increases, but disperses in the nanoparticles in smaller amounts. The crystallized drug is expected to dissolve and diffuse more slowly into the outer aqueous phase relative to the molecular dispersion. As a result, the release rate for the hydrophobic drug from nanoparticles containing more of the drug was slower than that from nanoparticles with less of the drug [22]. Therefore, the drug-release kinetics can be controlled by regulating the drug-loading content of the nanoparticles or by changing the DS.

CONCLUSION

Self-organized nanogels made from PUL/DOCA conjugates were prepared to determine their effectiveness as anti-cancer drug delivery vehicles. The average size of the PUL/DOCA nanogels was less than 150 nm in diameter, with a unimodal size distribution. The nanogels have a low CAC and can control the drug release rate. Thus, PUL/DOCA nanogels may have potential to serve as carriers for anti-cancer drugs.

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