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Nanoparticles of hydrophobized cluster dextrin as biodegradable drug carriers: solubilization and encapsulation of amphotericin B

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Cluster dextrin (CDex), a highly branched cyclic dextrin, is a novel glucose polymer that is produced from a waxy corn starch by a branching enzyme. Despite its large molecular weight (462 kDa), CDex is highly water-soluble and is easily digested by enzymes, such as α-amylase. Amphotericin B (AmB) is a broad-spectrum fungicidal antibiotic used primarily in the treatment of life-threatening systemic fungal infections. Unfortunately, AmB is insoluble in water; therefore, we have attempted to render it soluble and transportable by encapsulating it in hydrophobized CDex nanoparticles. The amphiphilic polymers of CDex were prepared with hydrophobic groups bonded through ester bonds, and the degrees of substitution were 1.00 mol/mol % for n-octanoyl CDex, 1.80 mol/mol % for stearoyl CDex, 1.07 mol/mol % for oleoyl CDex, and 1.07 mol/mol % for cholesteryl CDex. To study the formation of the monodispersed nanoparticles of the hydrophobized CDexs by self-assembly and their complex formations with AmB, size-exclusion chromatography and dynamic light scattering were utilized. AmB encapsulated in the cholesteryl CDex nanoparticle was found to be non-hemolytic, even at 30 μg/mL, which suggested that it had a much higher concentration than the minimum inhibitory concentration of 0.78 μg/mL required to inhibit Saccharomyces cerevisiae. Therefore, the biodistributions of the AmB-loaded cholesteryl CDex nanoparticles and AmBisome (liposomal AmB) were further compared in mice. After intravenous injection of the AmB-loaded cholesteryl CDex nanoparticle, prolonged persistence of AmB in the circulating blood was observed.

Key words: Cluster dextrin - Nanoparticle - Amphotericin B - Biodegradation - Biodisposition.

Cluster dextrin (CDex), a highly branched cyclic dextrin, is a novel glucose polymer that is produced from a waxy corn starch by the cyclization reaction of a branching enzyme [1-3]. Despite its large molecular weight (462 kDa), CDex is highly soluble in water and has a relatively low propensity for retrogradation compared to that of commercial dextrins [4]. As CDex is a safe substance consisting of glucose, a variety of applications, such as food additives for sports beverages or taste improvement, a spray drying aid for extracts of fruits and vegetables, and the powderization of fish oil, are proposed for the use of CDex.

Amphotericin B (AmB) is an antifungal agent; it was isolated in 1956 from a soil actinomycete, Streptomyces nodosus. It is used primarily as a broad-spectrum fungicidal antibiotic in the treatment of life-threatening systemic fungal infections [5, 6]. For many years, the classic antifungal drug Fungizone (AmB-desoxycholate) has been the mainstay of antifungal therapy [7, 8].

The strong lipophilic properties of AmB prompted investigations of its encapsulation in liposomes and its binding to lipid complexes in an effort to increase both its efficacy and safety. Subsequently, three lipid formulations, Amphotec (AmB colloidal dispersion), Abelcet (AmB lipid complex), and AmBisome (liposomal AmB), were developed and licensed. Of these formulations, AmBisome is the most widely used; however, it is associated with some toxicity issues and is not universally effective. Therefore, alternative formulations need to be developed [9-11].

One of the approaches for improving the performance of a drug and reducing its toxicity involves the use of a macromolecular carrier system [12-15]. In particular, polysaccharide-based nanoparticles have been recognized as promising drug carriers because of their hydrophobic domain, which is surrounded by a hydrophilic outer shell, that serves as a preserver for various hydrophobic drugs [16-18]. Examples of hydrophobically modified polysaccharides used to prepare nanoparticles include pullulan, dextran, starch, dextrin, and cellulose [18-25].

Most biodegradable polymers used in drug delivery are specifically intended for parenteral administration. Above all, the biodisposition of dextran has been widely investigated [26-29]. Moreover, we have reported that certain neutral polysaccharides, such as dextran and pullulan, can accumulate in the liver for long periods after their injections because these materials are scarcely metabolized enzymatically in the circulating blood [30-33]. Therefore, the higher molecular weight glucans are taken up into the hepatocytes by non-specific fluid-phase endocytosis.

CDex is as digestible as commercial dextrins to form glucose by α-amylase and α-glucosidase, which are both present in the small intestine. However, we have also reported that intravenously administered FITC-labeled CDex is rapidly eliminated from the blood, followed by an appreciable excretion into the urine [34]. High-performance size exclusion chromatographic analysis showed that the FITC-labeled CDex was quickly degraded into small molecules (- 6 kDa) in the blood. Therefore, CDex alone would be expected to avoid long-term hepatic accumulation.

Because AmB is insoluble in water, we synthesized hydrophobized CDex and attempted to render AmB soluble and transportable by encapsulating it in the CDex nanoparticles. Furthermore, we examined the biodegradabilities of the AmB-loaded CDex nanoparticles.

I. MATERIALS AND METHODS

1. Materials

Cluster dextrin (CDex) (MW = 462 kDa) was kindly supplied by Ezaki Glico Co., Ltd. (Osaka, Japan). Cholesterol chloroformate, stearoyl chloride, oleoyl chloride and n-octanoyl chloride were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Amphotericin B (AmB) and porcine pancreatic α-amylase were obtained from Sigma-Aldrich Co., Ltd. (St. Louis, MO, United States). Fungizone was purchased from Bristol-Myers Squibb Co., Ltd. (Tokyo, Japan), and AmBisome was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). All other chemicals and reagents were of the highest grade commercially available.

2. Animals

Male ddY mice aged 5 weeks were purchased from Shimizu Labo-

ratory Supplies (Shizuoka, Japan), housed under standard conditions, and given commercial food and tap water. All animal experiments were conducted in accordance with the institutional guideline for the care and use of laboratory animals for research, which in turn conforms to the guidelines of the Science Council of Japan.

3. Synthesis of hydrophobized CDex

To CDex (250 mg) dissolved in 10 mL of N-methylpyrrolidone (NMP) containing triethylamine (50 mmol) at 80 °C, cholesterol chloroformate (207.9 mg), stearoyl chloride (140.2 mg), oleoyl chloride (139.3 mg), or n-octanoyl chloride (75.3 mg) was added. The molar ratio between the glucose unit and the reagent in the reaction was 1:0.3. The reaction proceeded at room temperature for 24 h after which diethyl ether was added to precipitate the substituted CDex. The solid obtained was dried in vacuo overnight to a constant weight, dissolved in 300 mL of water and then dialyzed against water extensively for 72 h. The aqueous solution for each polymer was lyophilized, and four separate hydrophobized CDexs were obtained: CDex-cho, CDex-stearoyl, CDex-oleoyl and CDex-octanoyl. The recovery yield of hydrophobized CDexs on synthesis was above 85 % each. These solid products were stored in a desiccator, and the degrees of substitution in the hydrophobized CDexs were determined by ^{1}H-NMR.

^{1}H NMR spectra was recorded at 35°C on a Jeol JMN-LA500 (Tokyo, Japan) operating at 500 MHz, respectively, using a 5 mm ϕ sample tube. ^{1}H NMR experiments were typically acquired with 32K data points covering a spectral width of 10000 Hz and with a ca. 3.7 s pluse delay time and 16 scans. DMSO-d_6 was used as a solvent (99.9 atom % D, Wako Pure Chemical Industries, Ltd.). Chemical shift values are expressed in ppm downfield using tetramethylsilane (TMS) as an internal standard.

4. Preparation of the AmB-loaded nanoparticles

AmB (20 mg) and a hydrophobized CDex (100 mg) were dissolved in 10 mL of dimethyl sulfoxide (DMSO), and then the solution was dialyzed against distilled water for 72 h using a dialysis tube with a 6,000-8,000-Da cut-off. Distilled water was exchanged every 4 h; afterward, the sample solution in the tube was lyophilized.

5. Dynamic light scatterings measurements

Zetasizer Nano ZS (Malvern Instrument, United Kingdom) equipped with a 4.0-mW He-Ne laser (633 nm), was used to measure the dynamic light scatterings (DLS) of the aqueous polymer solutions, 1 mg/mL, at 25 °C. The scattering angle was fixed at 173°, and the results were taken from three 3-min runs for a total accumulation correlation function (ACF) time of 9 min.

6. Critical aggregation concentrations measurements

The critical aggregation concentrations (CAC) of hydrophobized CDex was determined by fluorescence spectroscopy (Hitachi 650-10S, Japan) with N-phenyl-1-naphthylamine (PNA) as the hydrophobic probe [35]. A known amount of PNA in acetone was added individually to a series of 10-mL vials, and the acetone was evaporated. Next, 2-mL aliquots of hydrophobized CDex solutions at concentrations from 1.8 to 4000 μg/mL were added to each vial, giving a final concentration for PNA of 1×10^{-7} M. The sample solutions were incubated at 40 °C for 1 h to allow for PNA inclusion into the nanoparticles to equilibrate, and then the samples were left to cool at room temperature. The fluorescence emission spectra were scanned between 350 and 600 nm at an excitation wavelength of 340 nm.

7. Scanning electron microscopy (SEM)

The morphologies of the particles were observed by scanning electron microscopy (SEM). A dilute solution of nanoparticle was smeared on the specimen stub and dried. The samples were sputter-coated with a thin platinum layer using a platinum sputter module in a high-vacuum evaporator. The coated samples were then scanned and photomicrographs were taken with SEM (Hitachi S-530, Japan).

8. High performance size-exclusion chromatography analysis

High performance size-exclusion chromatography (HPSEC) was performed using a liquid chromatography apparatus (LC-9A, Shimadzu, Japan) equipped with a differential refractometer (RI-8000, Tosoh, Japan). A 7.8 × 300-mm TSKgel G4000PWXL column (Tosoh, Japan) was used at 40 °C, where the mobile phase was water, the flow rate was 1.0 mL/min, and the injection volume was 50 μL. Three-dimensional analysis was performed using the HPSEC system equipped with a photodiode array detector (Waters, United States).

9. AmB analysis

Chromatography was performed using an HPLC system (LC-10AD, Shimadzu, Kyoto, Japan) equipped with a variable-wavelength UV detector (SPD-20A, Shimadzu, Kyoto, Japan). The detection wavelength was 405 nm, and a 4.6 × 150-mm C18 reversed phase column (TSKgel ODS 80TM, Tosoh, Tokyo, Japan) was used at ambient temperature. The mobile phase was a mixture of sodium acetate buffer (10 mM, pH 7.0) and acetonitrile (60:40 (v/v)), and the flow rate was 1.0 mL/min. The injection volume was 20 μL [36].

10. Enzymatic degradation of the modified CDex

To 2 mg of CDex or CDex-cho dissolved in 2 mL of water at 37 °C, α-amylase (0.2 units) was added. At 0, 0.5 and 24 h, a solution of 50 μL was injected into the HPSEC.

The amount of reducing terminals was determined by the dinitrosalicylic acid (DNS) method. To 10 mg of CDex, CDex-cho, or AmB-loaded CDex-cho dissolved in 1 mL of 0.1 M phosphate buffer (pH 6.0) containing 0.02 % sodium azide at 37°C, α-amylase (1 unit) was added. At 0, 0.5, 1, 2, 4 and 24 h, 50-μL samples were collected and incubated with 1 mL of the DNS reagent for 15 min at 95 °C. After cooling, the absorbance was measured at 640 nm.

11. Hemolytic activity of the AmB preparation [37]

Blood from the *vena cava* of male ddY mice were collected and centrifuged at 2,000 rpm. The supernatant and buffy coats were pipetted, and the red blood cells (RBCs) were diluted with an isotonic phosphate buffer, pH 7.4. The proper dilution factor was estimated from the UV absorbance of the hemoglobin in the supernatant at 576 nm after the RBCs had been completely lysed with added water. A properly diluted sample of the RBCs gave an absorbance of 0.4-0.5. Solutions of the diluted RBCs with different concentrations of AmB were incubated at 37 °C for 30 min and then placed in ice to arrest hemolysis. The unlysed RBCs were immediately removed by centrifugation at 10,000 rpm for 30 s. The supernatant was collected and analyzed for hemoglobin with a UV spectrometer at 576 nm. The percentage of hemolyzed RBCs was determined using the following equation: % hemolysis = (Abs-Abs0)/(Abs100-Abs0) × 100, where Abs, Abs0, and Abs100 are the absorbance values for the sample, the control with no hemolysis, and the 100 % lysed sample, respectively.

12. Assay of the antimicrobial activity [38]

The minimum growth inhibitory concentrations (MIC) was measured by the two-fold serial broth dilution method. *Saccharomyces cerevisiae* (IFO:0203), as the model fungus, was cultured in 2.5 % malt extract at 25 ºC for 48 h. The MIC was defined as the lowest concentration of AmB loaded into the hydrophobized CDex nanoparticles at which growth was below 0.03 in optical density or not visible.

13. Animal experiment

Male ddY mice were injected through the tail vein with AmB-loaded CDex-cho, AmBisome, or Fungizone at a dose of 5 mg/kg