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Self-assembly of cholesterol-hydrotropic dendrimer conjugates into micelle-like structure: Preparation and hydrotropic solubilization of paclitaxel

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Abstract

A hydrotropic dendrimer, made of polyglycerol dendrimer of generation 4 (PGD-G4), was conjugated with cholesterol. The self-assembled structure of the conjugate in water was characterized by ^{1}H-NMR, dynamic light scattering (DLS) and atomic force microscopy (AFM). One cholesterol molecule was conjugated to PGD-G4, which was confirmed by MALDI-TOF mass spectroscopy. The DLS analysis showed that the conjugate formed self-assembly with a diameter of 49.9–59.9 nm. The AFM image suggests that the self-assembly is a micelle-like sphere. The level of paclitaxel solubilization by the conjugate in water was similar to that of PGD-G4 itself, and this suggests that the PGD-G4 is located on the outer part of the self-assembly to function as a hydrotrope.

Keywords: Dendrimer; Hydrotrope; Cholesterol; Self-assembly; Paclitaxel; Atomic force microscopy

1. Introduction

Dendrimers are nano-sized, highly branched macromolecules with monodispersed characteristics. The name was derived from the Greek words dendron (tree) and meros (part). Due to the nano-sized spherical shape and multivalent surface functionalities, they have been used as a host of biomimetic and nanotechnological applications [1]. Solubilization of hydrophobic compounds by dendrimers has been extensively studied in the drug delivery area [2–4]. Biocompatible and/or biodegradable dendrimers have been applied to developing controlled-release systems and tissue scaffolds. Although many studies have focused on encapsulation of small guest molecules into the dendritic core, controlling self-assembly of dendrimers in solution has remained a challenge. Self-assembly of dendrimers or dendritic polymers has been studied since 1990's. V. Hest et al. prepared amphiphilic poly(propylene imine) dendrimer-polystyrene conjugates and achieved generation-dependent aggregation of the conjugates into spherical micelles, micellar rods and vesicular structure[5]. V. Percec et al. have reported controlling shape of supramolecular assembly of dendritic side-groups [6]. Dendritic structure also can change the self-assembled structure into nanofibers by designing dendritic molecules itself [7] and dendritic derivatives [8]. These reports suggest that the dendritic architectures play a crucial role for determining nano- and micro-scaled self-assembled structure. However, self-assembled dendrimers as drug carriers have not been designed and studied yet.

In our previous studies, polyglycerol dendrimers (PGDs) were examined for their ability as 'hydrotropic dendrimers'.[9,10] The term 'hydrotropic agents' refers to a diverse class of water-soluble compounds that, at high concentrations, enhance the water-solubility of poorly soluble solutes. It was found that dendritic structure of PGDs with 4–5 generations significantly increased aqueous solubility of paclitaxel (PTX), a poorly water-soluble drug. ^{1}H NMR spectra of PTX before and after mixing with PGDs in D_2O suggested that the aromatic rings and some methyne

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groups of PTX were surrounded by PGDs [10]. The solubilizing mechanism of PGDs is quite different from conventional polymeric micelles and unimolecularlly dendrimertic miclles [4]. There is no need to have a hydrophobic core as a drug reservoir in PGDs. Those results and reports have led us to the hypothesis that PGDs can act as self-assembled micellar shell with hydrotropic solubilization of PTX.

In this study, cholesterol was conjugated with PGD (generation 4, G4), and its self-assembled structure was evaluated by dynamic light scattering (DLS) and atomic force microscopy (AFM). Cholesterol was selected as a hydrophobic moiety of the conjugate because cholesterol conjugated with water-soluble polymers is known to form stable aggregates in aqueous solution [11,12]. The effect of the self-assembled outer shell on PTX solubility was evaluated by hydrotropic solubilization test.

2. Methods

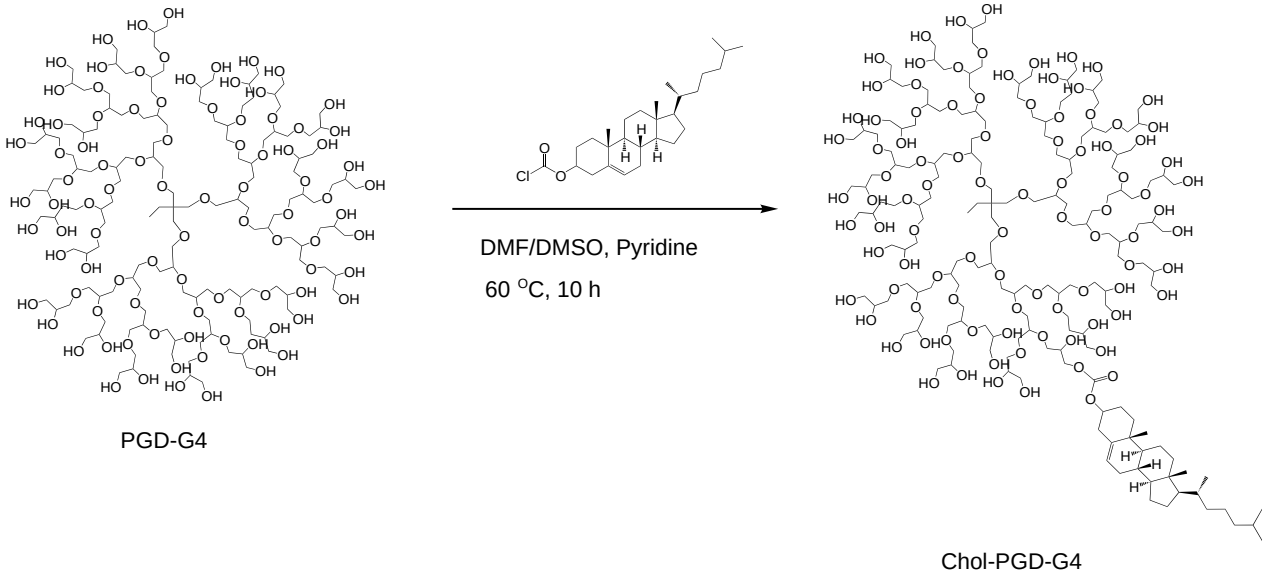
2.1. Materials

PGD-G4 (M_w: 3508) was prepared by the method described in our previous report [10]. Cholesterol chloroformate was purchased from Tokyo Kasei Kogyo. Co., Ltd, Tokyo, Japan. Paclitaxel was obtained from Samyang Genex Corp. (Taejeon, South Korea). Benzylated cellulose membrane (MWCO 1000) was purchased from Sigma (St Louis, MO). Dimethylsulfoxide (DMSO) and dimethylformamide (DMF) were purchased from Nakarai Tesque, Inc., Kyoto, Japan and was distilled under vacuum. Mica for AFM measurements was purchased from Furuuchi Chemical Co., Shinagawa, Tokyo, Japan. Other reagents were used as received without other purification.

2.2. Preparation of cholesterol-conjugated PGD-G4 (Chol-PGDG4) (Scheme 1)

A Chol-PGDG4 was synthesized by conjugating cholesterol chloroformate and PGD-G4 (Scheme 1). The PGD-G4 (0.308 g, 0.088 mmol) was dissolved in 3 ml of DMSO/DMF (1:1) at 80 °C. It took 1–2 h to dissolve the cholesterol chloroformate. Cholesterol chloroformate (43.4 mg, 0.097 mmol) and pyridine (0.1 ml) was added to the solution, and stirred for 1 h at 80 °C and then 9 h at 60 °C. The obtained crude product was purified by dialysis against DMSO and then water using the benzylated cellulose membrane, and then lyophilized to obtain the Chol-PGDG4 as pale yellow oil. The molecular weight of the obtained product was estimated by MALDI-TOF mass spectroscopy using Kompact MALDIβ (Kratos Analytical Japan, Hadano, Japan) in linear mode. Samples for mass spectrum were prepared by casting the matrix compound (*trans*-3-indole acrylic acid) with Chol-PGDG4 (1 mM in 50% methanol/water) onto the slide and the solvent was evaporated. The chemical structure was characterized by ^{1}H-NMR spectroscopy using a 750 MHz FT-NMR spectrometer (Varian, Unity plus, CA).

Yield: 63% ^{1}H-NMR (750 MHz, DMF-d_7): 5.62 (brm, H6 of chol), 3.99 (brm, H3 of chol), 4.79−4.58 (m, OH of PGD-G4), 3.85−3.55 (m, CH and CH_2 of PGD-G4), 3.47 (s, $C(CH_2)_3$ of PGD-G4), 2.22 (s, H4 of chol), 2.05 (s, H7 of chol), 1.54 (brq, CH_2CH_3), 1.48 (brm, H11, H14 of chol), 1.39 (brm, H8, H9 of chol), 1.31 (m, H12 of chol), 1.26−1.10 (br, H1, H18, H19, H22, H23, H24 of chol), 1.02 (t, CH_3 of PGD-G4), 0.97 (brd, H26, H27 of chol), 0.81 (brs, H18 of chol); MALDI-TOF MS calcd. for $C_{172}H_{334}O_{95}$ 3920.13 found 3974.0 + $3H_2O$.



Scheme 1. Preparation of cholesterol-conjugated PGD-G4 (Chol-PGDG4).

2.3. Preparation and analysis of self-assembled Chol-PGD-G4

After lyophilization of Chol-PGD-G4, 1 wt% of aqueous solution was prepared by adding distilled water to Chol-PGD-G4 at room temperature. Chol-PGD-G4 was immediately dissolved in this condition, and the solution was stirred for 10 min. The self-assembled structure was characterized by dynamic light scattering (DLS) and atomic force microscope (AFM) measurements. Hydrodynamic radius (R_h) of the Chol-PGD-G4 was measured by using a PD2000DLS (Protein Detectors, Inc., MA) at a fixed angle (90°). The membrane filter with a 0.2 μm nylon membrane was used, and 0.5 ml of PGD-dissolved aqueous solution was injected to a cell. Solution concentration was 2.6×10^{-6} M (1 wt%). Samples for AFM were prepared by diluting the Chol-PGD-G4 solution with ultrapure water to achieve the concentration of 1×10^{-5} and 10^{-7} M. A drop of the diluted solution (50 μL) was deposited on the face of a freshly cleaved mica disk. After 2 min, the mica disk was dried up by nitrogen gas. All AFM images were obtained in air with a commercial AFM unit (SPA400-SPI3800, Seiko Instruments Inc., Chiba, Japan) equipped with a calibrated 20 μm *xy*-scan and 10 μm *z*-scan range PZT-scanner. Silicon cantilever (SI-DF20, spring constant: 15 N/m, resonance frequency: 132 kHz, Seiko Instruments Inc.) was used, and images were taken in a dynamic force mode (DFM mode) at the optimal force.

2.4. Solubility test of paclitaxel

Excess paclitaxel (PTX, ~10 mg/ml) was added to screw-capped vials containing fixed volume of Chol-PGD-G4 solutions. This mixture in the vials was stirred using a magnetic stirring bar at 37 °C. The samples were taken after 24 h, filtered through a 0.2 μm nylon membrane and analyzed for paclitaxel using HPLC. The concentration of PTX was determined by an isocratic reverse-phase HPLC

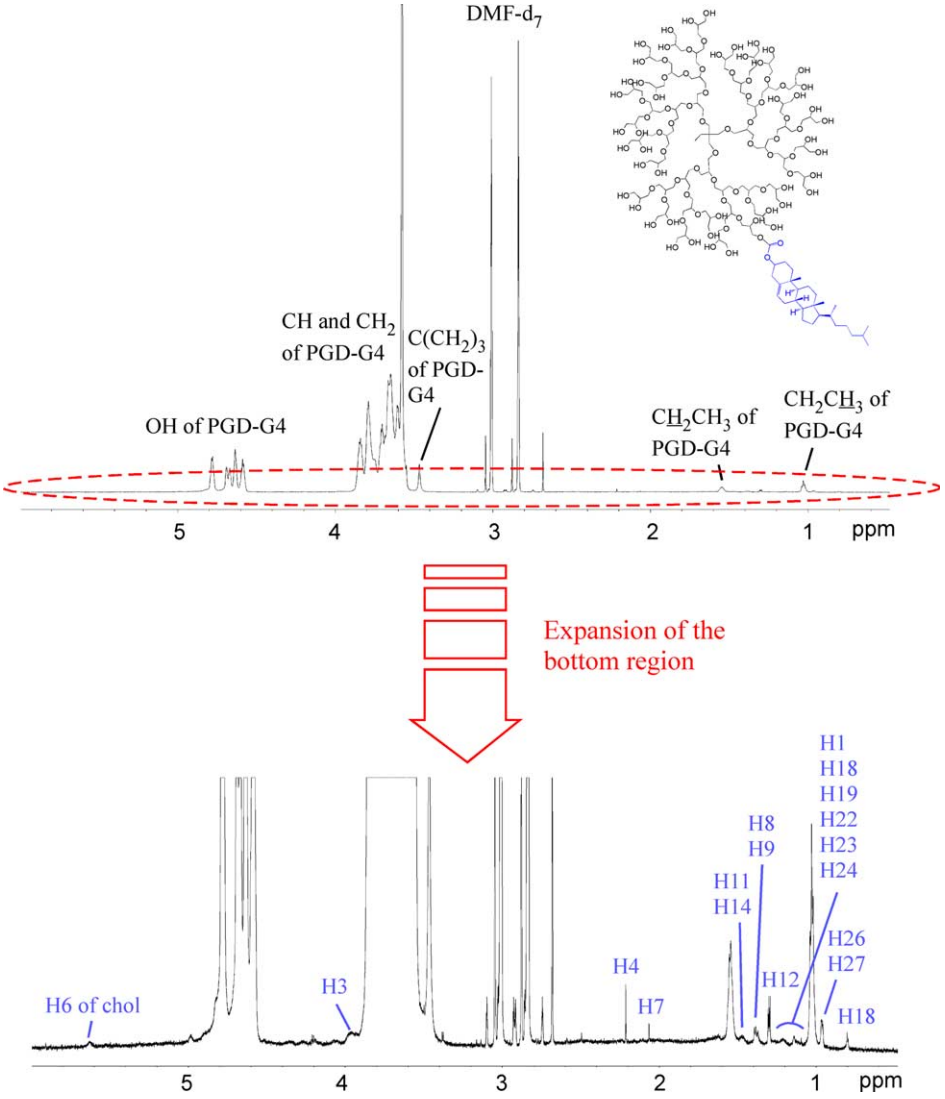


Fig. 1. ^{1}H-NMR spectrum of Chol-PGD-G4 in DMF-d_7.

(HITACHI LaChrom, Interface D-7000, Column oven L-7300, Autosampler L-7200, Pump L-7100) using a Symmetry column (Water Corp., Milford, MA) at 25 °C. The mobile phase consisted of acetonitrile-water (45:55 v/v) with a flow rate of 1.0 ml/min. A diode array detector was set at 227 nm. The PTX concentrations in the samples were obtained from a calibration curve.

3. Results and discussion

3.1. Preparation and self-assembled structure of Chol-PGD-G4

The cholesterol group was introduced to PGD-G4 via carbonate linkage that is a biodegradable linkage and releases carbon dioxide after the degradation [13]. This degradation process is likely to be advantageous in terms of no production of acids like poly(lactic acid). The PGD G-4 and cholesterol chloroformate was conjugated at the molar ratio of 1: 1.3 to introduce one cholesterol group per PGD-G4 molecule. From the ^{1}H-NMR spectrum, all the peaks attributed to PGD-G4 and cholesterol group were observed (Fig. 1). However, the peak intensities of cholesterol group were relatively smaller than expected even in DMF-d_7 and DMSO-d_6, and the peaks were difficult to observe in D_2O. These results suggest that the cholesterol groups are strongly associated in the polar organic solvents and aqueous conditions as seen in the case of hydrophobic part of polymeric micelles [14]. In the MALDI-TOF mass spectrum for Chol-PGD-G4, a single peak was observed at 3974 ($+3H_2O$) *m/z* although original peak of PGD-G4 at 3507 ($+H$) *m/z* was also observed. This means that one cholesterol group was introduced to each PGD molecule, and some carbonate linkages in Chol-PGD-G4 was cleaved during the laser irradiation of MALDI TOF mass measurements.

In order to characterize self-assembled structure of Chol-PGD-G4, DLS and AFM of Chol-PGD-G4 were conducted. From histogram analysis of the DLS, mean diameter of Chol-PGD-G4 at the concentration of 2.6×10^{-6} M was calculated to be 49.9–59.9 nm (Fig. 2(a)). Although small intensity (%) of lager diameter was observed, 81% of the scattering intensity was come from the diameter of 49.9–59.9 nm. On the other hand, the diameter of PGD G-4 itself was observed at

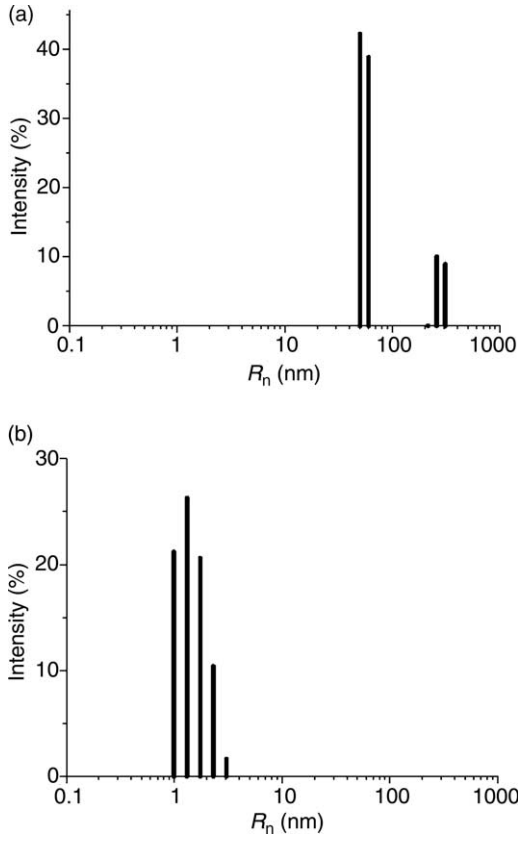


Fig. 2. Histgrams of (a) Chol-PGD-G4 and (b) PGD-G4 in water on DLS. The concentration was 2.6×10^{-6} M for both.

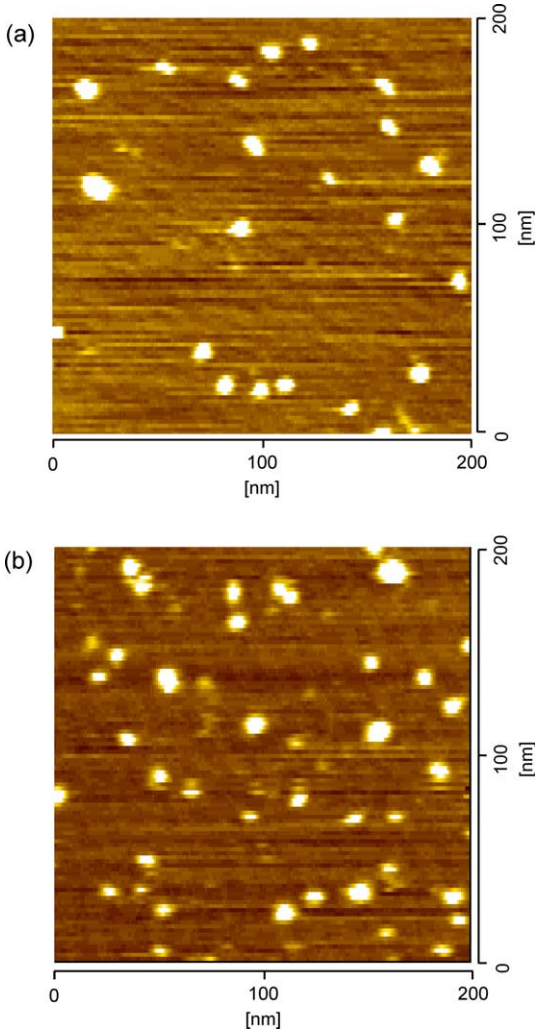


Fig. 3. AFM images of Chol-PGD-G4 at the concentrations of (a) 1×10^{-5} and (b) 1×10^{-7} M.

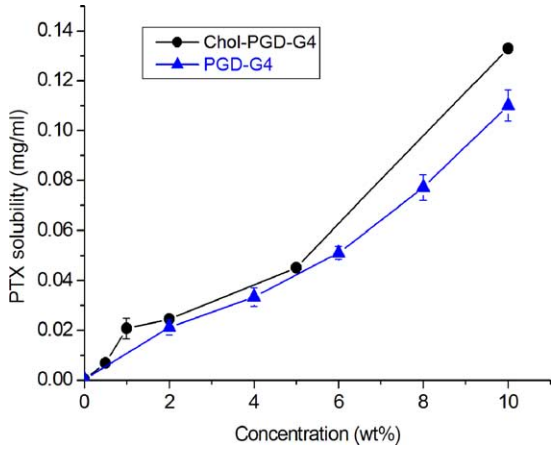


Fig. 4. Aqueous paclitaxel solubility as a function of the concentration of Chol-PGD-G4 (black circle) and PGD-G4 (blue triangle). (Mean ± SD, $n = 3$).

0.99–2.3 nm (Fig. 2(b)). These results suggest that the conjugation of one cholesterol group to PGD-G4 induces formation of self-assembly in water. In addition, the self-assembled Chol-PGD-G4 showed spherical-like structure both at 1×10^{-7} and 10^{-5} M in the dry state (Fig. 3(a) and (b)). The AFM results indicate that the mean diameter of Chol-PGD-G4 was around 20 nm. The inconsistency of the results between the DLS and the AFM was due to dehydration of Chol-PGD-G4 on the mica surface for the AFM measurements. Thus, the self-assembly of Chol-PGD-G4 is likely to swell in water.

3.2. Solubilization of PTX in water

Our previous study suggested that PGD-G4 functioned as a good hydrotrope to solubilize PTX without any hydrophobic region. From the viewpoint of the solubilizing effect of PGD-G4, association of the PGD-G4 molecules may affect the solubilizing ability. After making the self-assembly of Chol-PGD-G4, the solubilization test was carried out using PGD-G4 as a control. As shown in Fig. 4, PTX solubility of Chol-PGD-G4 was almost similar to that of PGD-G4 at the concentration ranging from 0.5 to 10 wt%. This result suggests that the PGD-G4 molecules exist on outer parts of the self-assembly and act as the hydrotrope. The cholesterol group does not seem to participate in the solubilization of PTX, because hydrophobic core of self-assembly can generally solubilize PTX very well [15]. Presumably, once the self-assembled structure was formed, the assembly was stable in aqueous conditions due to strong hydrophobic interaction of cholesterol groups, and therefore, the outer parts consisting PGD-G4 could interact with PTX.

4. Conclusion

A cholesterol group was introduced to polyglycerol dendrimer generation 4 (PGD-G4) via carbonate linkage. The obtained conjugate (Chol-PGD-G4) was spontaneously assembled in water to from micelle-like structure, which was confirmed by ^{1}H-NMR, DLS and AFM analysis. The Chol-PGD-G4 solubilized paclitaxel (PTX) as well as PGD-G4 itself. The self-assembled formation of Chol-PGD-G4 in nano-scale provides new drug formulations with hydrotropic solubilization.

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