Supporting Information

for

Synthesis and Function of Amphiphilic Glucan Dendrimers as Nanocarrier for Protein Delivery

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Materials and methods

Materials.

Phosphate-Buffered Saline (PBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dodecyl isocyanate was purchased from Tokyo chemical industry (Japan). Pyrene, dibutyltin dilaurate, carbonic anhydrase (CA) from bovine, bovine serum albumin (BSA) from bovine, malate dehydrogenase (MDH) from pig heart, citrate synthase (CS) from porcine heart, and β-Galactosidase (β-Gal) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TokyoGreen-β-Gal was purchased from Sekisui Medical (Tokyo, Japan).

Characterization of the glucan dendrimers

The glucans were synthesized according to the previous literature. [*Biocatalysis and Biotransformation*, 2003, **21**, 167-172.] The glucans were dissolved in D_2O at a concentration of 10 mg mL^{-1}. NMR spectra were obtained with a Bruker Avance III 400 MHz spectrometer. Chemical shifts (δ) are expressed in parts per million and are reported relative to 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) as an internal standard.

Synthesis of amphiphilic glucan dendrimers.

Scheme S1 synthetic scheme of amphiphilic glucan dendrimer

Glucan dendrimers (0.70 g, 4.32 mmol as anhydrous glucose unit) was dissolved in 32 ml of dry dimethylsulfoxide (DMSO). Then, dodecyl isocynate (27 mg, 1.29×10^{-1} mmol) and dibutyltin dilaurate (0.21 g, 3.3×10^{-1} mmol) as a catalyst was added to the solution under nitrogen atmosphere at 45 °C. After stirring for 24 h, the reaction mixture was poured into a mixture solution (1 L) of ethanol/diethylether (v/v: 9/1). The precipitate was obtained, washed with a mixture solution of ethanol/diethylether (v/v:

9/1), and dried overnight under reduced pressure. The crude product was dissolved in DMSO, was dialyzed against distilled water (MWCO 3.5K; Spectrum Laboratories), and was lyophilized. The degree of substitution of dodecyl group was determined by ^{1}H-NMR spectroscopy based on the integrated areas of anomeric proton in the glucopyranosyl rings at 5.05 ppm and the methyl proton in dodecyl groups at 0.85 ppm.

Characterization of amphiphilic glucan dendrimers

Dynamic light scattering (DLS)

The size distributions of glucan dendrimers and amphiphilic glucan dendrimers were measured with dynamic light scattering (DLS, Zetasizer nano ZS, Malvern Instruments, U.K.). The concentration of polymers was 1 mg mL^{-1} in PBS.

Size exclusion chromatography equipped with multi-angle light scattering (SEC-MALS)

Size exclusion chromatography equipped with multi-angle light scattering (SEC-MALS) was performed on a chromatography system with a refractive index detector (Optilab T-rEX, Wyatt technology) connected to MALS (DAWN HELEOS II, Wyatt Technology) in 0.05 M $NaNO_3$ aqueous solution. Two linear-type silica-based columns (WTC-100S5, Wyatt technology) was used as the column for SEC-MALS measurements. The densities of glucan dendrimers (Φ_H) were calculated from Equation (1) using hydrodynamic radius (R_H), avogadro number (N_A) and weight-average molecular weight (M_w).

$$\Phi_H = \frac{3M_w}{4\pi N_A R_H^3} \quad (1)$$

Fluorescence measurements

The solution of amphiphilic glucans was mixed with 1 μM pyrene with stirring for 24 hours. The hydrophobicity of hydrophobic domain in amphiphilic glucans can be

estimated by the I_1/I_3 ratio of the intensity of the emission at 374 nm (I_1) to that of the emission at 385 nm (I_3) for pyrene spectra.

Small-Angle X-ray Scattering (SAXS)

SAXS measurements were performed at SPring-8 BL40B2. A 30 cm × 30 cm imaging plate (Rigaku R-AXIS VII) detector was placed at 2.1 m away from the sample. The wavelength (λ) of the X-ray was 1.0 Å. The set-ups provided a q range of 0.06–3.5 nm^{-1}, where q is the magnitude of the scattering vector defined by $q = 4\pi\sin\theta/\lambda$ with the scattering angle of 2θ. The X-ray transmittance of the sample was measured with ion chambers located in front of and behind the sample. The glucan solutions (1 mg/ml) or PBS buffer was poured into a quartz capillary (Diameter: 2 mm, Hilgenberg GmbH). SAXS from sample solutions and solvent was measured at an exposure time of 180 sec. The resulting 2D SAXS images were converted to one dimensional I(q) versus q profiles by circular averaging with FIT2D program. The corresponding background intensity of the capillary filled with PBS buffer was subsequently subtracted. The pair distribution function P(r) of glucan dendrimers and amphiphilic glucan dendrimers were estimated from the corresponding SAXS profiles by using SCATTER program (http://www.bioisis.net/tutorial/9).

Interaction of amphiphilic glucan dendrimers with proteins.

The binding constants of rhodamine-labeled proteins with the amphiphilic glucan dendrimers were estimated with fluorescence correlation spectroscopy (FCS). FCS measurements were performed using FluoroPoint-Light (Olympus Co., Tokyo, Japan). Various concentrations of the amphiphilic glucan solutions were mixed with rhodamine-modified insulin or rhodamine-modified BSA (final concentration 2.5 nM). The resulting mixture solutions were incubated at room temperature for 24 h. All experiments were performed triplicate with an acquisition time of 10 s for each measurement. The FCS data were analyzed using FluoroPoint-Light software. The obtained curves were fitted with the following equation to calculate the binding constants for the proteins:

$$K = \frac{1}{K_d} \qquad (2)$$

$$K_d = \frac{[A][B]}{[AB]} \quad (3)$$

$$Y = \frac{K_d + [A] + [B] - \sqrt{(K_d + [A] + [B])^2 - 4[A][B]}}{2[A]} \quad (4)$$

, where K_d is the dissociation constant, [A] and [B] are the molar concentrations of rhodamine-modified proteins, amphiphilic glucan dendrimers, and Y is the degree of complexations of proteins and amphiphilic glucan dendrimers in the solution.

Cell culture and fluorescence imaging

HeLa cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS). HeLa cells were seeded in a multi-well glass bottom dish (4 wells, Matsunami Glass Ind., Ltd., Japan) at a density of 1.0×10^4 cells/well and incubated for 24 h at 37 ºC in 5% CO_2. After removal of the medium, 1 mL of fresh DMEM containing rhodamine labeled C12G15-3.2 was added, and the cells were incubated at 37 ºC. After each time points, they were observed by confocal laser scanning microscopy (CLSM; Carl Zeiss LSM 780) at a magnification of 40× with excitation wavelength of 561 nm for rhodamine.

For lysosome staining, HeLa cells (1×10^4 cells) were pre-cultured in the multi-well glass bottom dish, and rhodamine labeled C12G15-3.2 (0.5 mg/ml) were added to the medium and were incubated for 4 h, followed by the addition of LysoTracker™ Green DND-26 (Thermo Ficher Scientific, Waltham, MA, USA) at a concentration of 1 μM. The fluorescence images of the cells were observed by the CLSM.

Cellular uptake mechanism of the glucan dendrimer

HeLa cells were plated in a cultured 12-well dish at a density of 8.0×10^4 cells/well and incubated for 24 h at 37 ºC in 5% CO_2. After the medium was removed, 1 mL of fresh DMEM containing rhodamine functionalized C12G15-3.2 (0.5 mg/mL) was added and HeLa cells were incubated at 4 ºC or 37 ºC. After incubation for 4 h, the cells were washed with the fresh medium three times, trypsinized, and suspended in the stain buffer. Flow cytometry (LSR Fortessa cell analyzer, BD Biosciences, San Jose, CA, USA) was performed with a 543nm He-Ne laser.

To determine the endocytic pathway of C12G15-3.2, various endocytosis inhibitors were mixed with cells. Before the addition of rhodamine functionalized

C12G15-3.2, HeLa cells (8.0×10^4 cells/well) were cultured with medium containing cytochalasin D (5 μM), methyl-β-cyclodextrin (2.5 mM), chloroquine (50 μM), chlorpromazine (25 μM), or 5-(*N*-ethyl-N-isopropyl) amirolide (EIPA, 50 μM). After incubation for 30 min, the medium was removed and 1 mL of fresh medium containing C12G15-Rho (0.5 mg/mL). After HeLa cells incubated with the C12G15-3.2 for 4 h, cells were washed with medium three times, trypsinized, and suspended in the stain buffer. Flow cytometry was then performed.

Cytotoxity

The cytotoxicity of amphiphilic glucan dendrimers with different concentrations was evaluated with Cell Counting Kit-8 (CCK-8, DOJINDO Japan). HeLa cells were seeded in 96-well dish at a density of 1.0×10^3 cells/well and were cultured for 24 h at 37 ºC in 5% CO_2. After the medium was removed, 50 μL of fresh medium containing glucan dendrimers or amphiphilic glucan dendrimers were added. Cells were cultured at 37 ºC in 5% CO_2. After cells were incubated for 4 h, CCK-8 solution was added to cells. After 90 min, the absorbance at 450 nm was measured using a microplate reader (SH-1000, CORONA electric, Japan).

Intracellular protein delivery

HeLa cells were seeded in a glass-bottom dish at 1.0×10^4 cells/dish and cultured for 24 h at 37 ºC in 5% CO_2. After the culture medium was removed, 1 mL of fresh medium containing C12G15-3.2 (0.5 mg mL^{-1})–β-Galactosidase (10 nM) complex was added to the cells. After incubation for 4 h at 37 ºC in 5% CO_2, the cells were washed with fresh medium. TokyoGreen-β-Gal (10 μM) was added to the medium. After 30 min, the cells were observed by CLSM with excitation by an argon laser (488 nm). The emitted fluorescence was detected through a 503–530 nm bandpass filter.

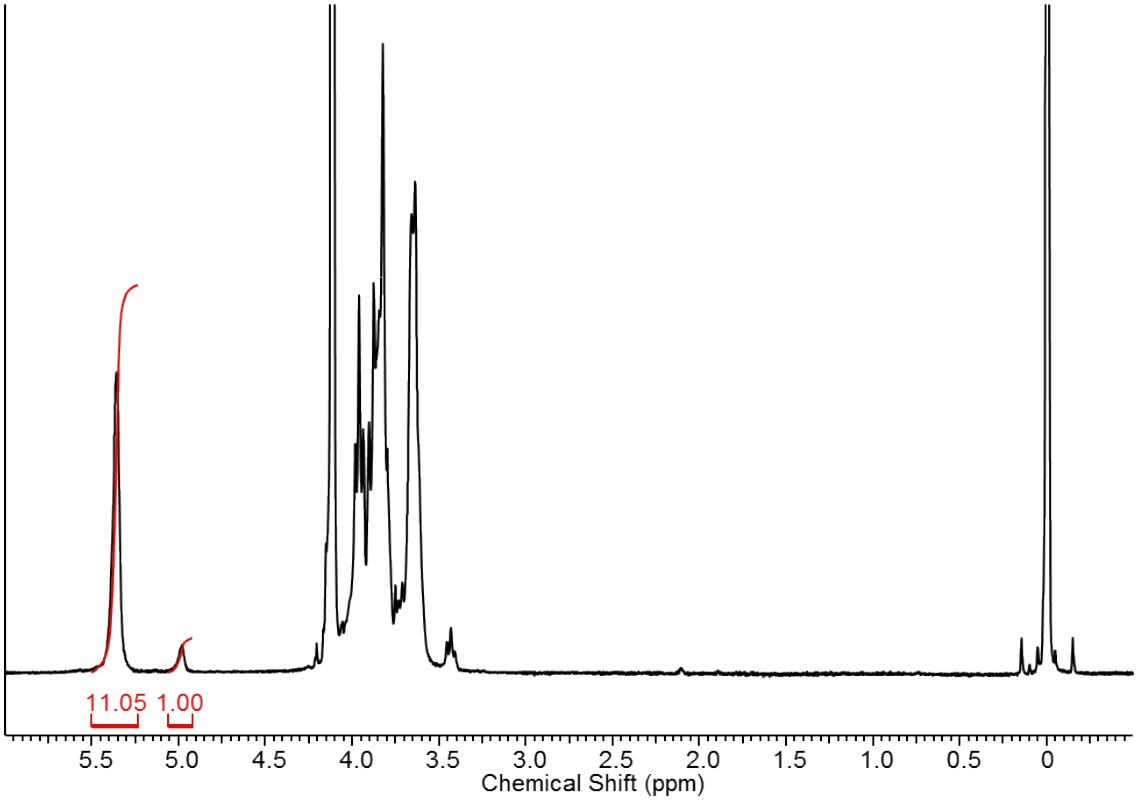

Figure S1 ^{1}H-NMR spectrum of G20 in D_2O at 80°C

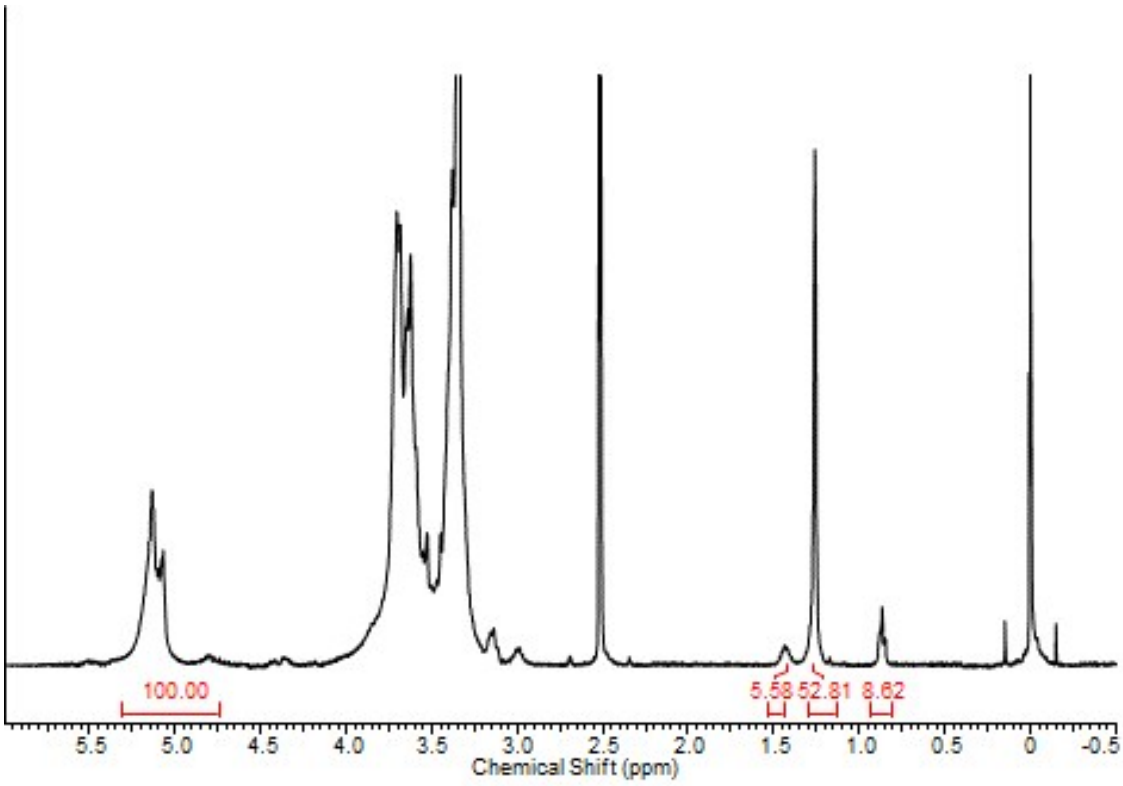

Figure S2 ^{1}H-NMR spectrum of C12G20-0.6 in a mixed solvent (DMSO-d_6 : D_2O = 9 : 1) at room temperature.

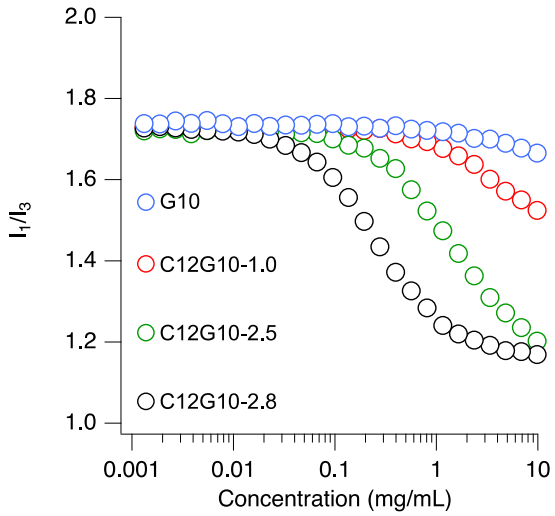

Figure S3 The plots of the I_1/I_3 values as a function of the concentration of G10 and amphiphilic G10s

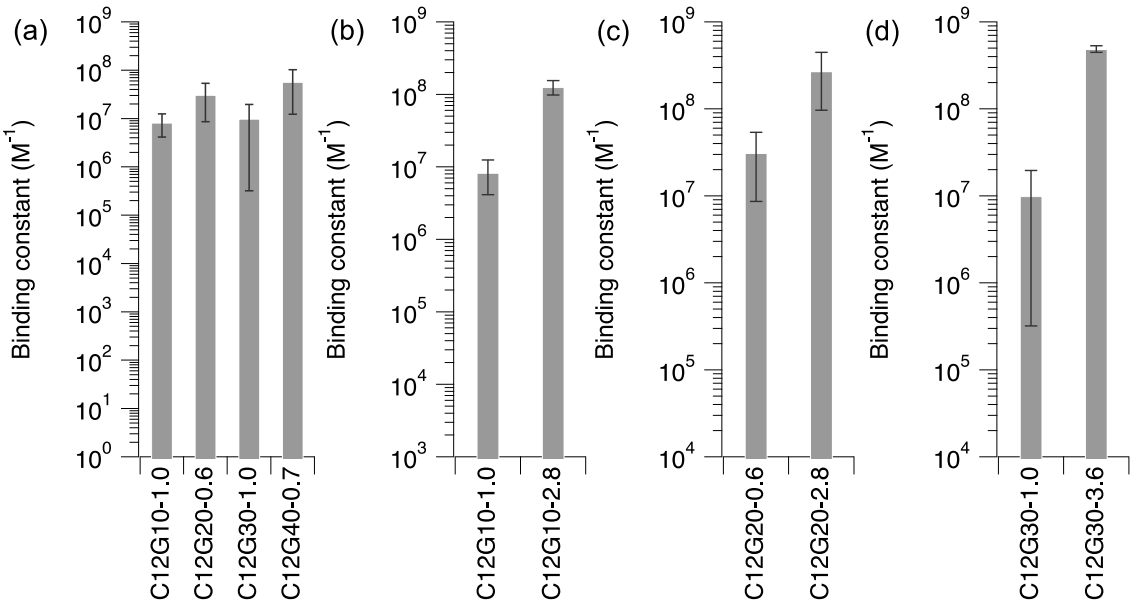

Figure S4 The binding constants (K_a) of various amphiphilic glucan dendrimers with TAMRA-BSA (2.5 μM). a) The effect of molecular weight of glucan dendrimers on the binding constants, b-d) The effect of the degree of substitution of dodecyl group on the binding constants.