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Carboxylated Hyperbranched Poly(glycidol)s for Preparation of pH-Sensitive Liposomes

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Abstract

Previous reports by the authors described intracellular delivery using liposomes modified with various carboxylated poly(glycidol) derivatives. These linear polymer-modified liposomes exhibited pH-dependent membrane fusion behavior in cellular acidic compartments. However, the effect of the backbone structure on membrane fusion activity remains unknown. Therefore, this study specifically investigated the backbone structure to obtain pH-sensitive polymers with much higher fusogenic activity and to reveal the effect of the polymer backbone structure on interaction with the membrane. Hyperbranched poly(glycidol) (HPG) derivatives were prepared as a new type of pH-sensitive polymers and modified liposomes using these polymers. The resultant HPG derivatives exhibited high hydrophobicity and intensive interaction with the membrane concomitantly with the increasing degree of polymerization. Furthermore, HPG derivatives showed stronger interaction with the membrane than linear polymers show. Liposomes modified with HPG derivatives of high DP delivered contents into cytosol of DC2.4 cells, a dendritic cell line, more effectively than the linear polymer-modified liposomes do. Results show that the backbone structure of pH-sensitive polymers affected their pH-sensitivity and interaction with liposomal and cellular membranes.

Keywords: pH-sensitive liposome / cytoplasmic delivery / hyperbranched polymer / membrane fusion / dendritic cell

Introduction

Cytoplasmic delivery of bioactive molecules such as proteins and nucleic acids, which are unable to permeate a cellular membrane themselves, is important to establish therapies—such as immunotherapy and gene therapy—based on these molecules. Although various systems have been attempted for application to cytoplasmic delivery, one of promising systems is pH-sensitive liposome, which induces destabilized and/or fusogenic activity under mildly acidic conditions. Various methods

have been applied to produce pH-sensitive liposomes. For example, pH-sensitive amphiphiles, such as oleic acid and cholesteryl hemisuccinate, have been mixed with non-bilayer-forming phospholipid dioleoylphosphatidylethanolamine (DOPE) to yield pH-sensitive liposomes [1,2]. Another efficient method for pH-sensitization of liposome is modification of stable liposomes with pH-sensitive membrane active molecules such as fusion peptides derived from viral fusogenic proteins, or synthetic polymers with carboxyl groups such as poly(alkyl acrylic acid)s [3,4]. Earlier studies by the authors developed a series of carboxylated poly(glycidol) derivatives for pH-sensitization of liposomes [5–7]. These polymers have a linear backbone structure similar to that of poly(ethylene glycol) (PEG) and carboxyl groups on the side chains, which control interaction of the polymer backbone with lipid membranes in a pH-dependent manner. Earlier studies showed that these polymer-modified liposomes are stable at neutral pH, but that they exhibit considerable destabilization under mildly acidic conditions and deliver contents into cytosol by membrane fusion with endosome/lysosome membranes [5–7].

Generally, membrane fusion as a biological function is mediated by fusogenic proteins. For example, enveloped viruses of various kinds have proteins that promote fusion of their envelope with cellular membranes to invade target cells. A very well studied viral fusion protein is influenza virus hemagglutinin (HA), which forms a fusion-active trimeric structure in the intracellular acidic compartment endosome and causes membrane fusion [8]. Considering these protein-mediated fusion processes, it might be important that fusogenic proteins having a bulky steric structure interact with a membrane for efficient membrane fusion because such interaction might generate a defective area and initiate membrane fusion.

Synthetic polymers of various kinds reportedly interact with membranes and induce membrane fusion [4,5,7,9,10]. Considering that these synthetic polymers generally have a linear structure, it is presumed that their interaction with membranes might not be so effective to generate defective regions for initiation of membrane fusion as sterically bulky proteins do. To date, the influence of the backbone structure of fusogenic polymers on their membrane fusion activity remains unknown. Hyperbranched polymers tend to take on a three-dimensional and spherical structure, which differs from those of linear polymers taking on a random coil structure [11–15]. Recently, 3-methyl-glutarylated poly(glycidol) was synthesized by the authors. It destabilizes phospholipid membranes under a weakly acidic environment and causes membrane fusion [7]. For the present study, its analogous polymers were prepared using hyperbranched poly(glycidol)s (HPGs) with different degrees of polymerization (DP), 3-methyl-glutarylated HPGs (MGlu-HPGs). Results described herein demonstrate that the DP and backbone structure of the pH-sensitive polymers affected their pH-sensitive fusion properties and their performance as intracellular delivery vehicles.

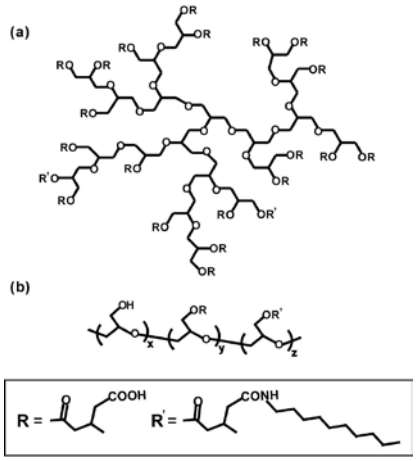

Figure 1. Structures of MGlu-HPG20-C_{10} (a) and linear MGluPG-C_{10} (b).

Materials and methods

Materials.

HPGs with DPs of 10, 20, 40 and 60, which are respectively designated as HPG10, HPG20, HPG40 and HPG60, were provided by Daicel Chemical Industries, Ltd. (Osaka, Japan). Egg yolk phosphatidylcholine (EYPC) and L-dioleoyl phosphatidylethanolamine (DOPE) were kindly donated by NOF Co. (Tokyo, Japan). Pyrene, pyranine, 1-aminodecane and Triton X-100 were obtained from Tokyo Chemical Industries Ltd. (Tokyo, Japan). *p*-Xylene-bis-pyridinium bromide (DPX) was from Molecular Probes (Oregon, USA). *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)dioleoyl phosphatidylethanolamine (NBD-PE) and lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). 3-Methylglutaric anhydride was obtained from Aldrich (Milwaukee, WI). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ovalbumin (OVA) and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO.). 3-Methylglutarylated linear poly(glycidol) (MGluPG) (Fig. 1) was prepared as previously reported using two kinds of poly(glycidol)s with different molecular weights: PG76 with number average molecular weight (Mn) of 5.6×10^3 and weight average molecular weight (Mw) of 8.7×10^3, and PG222 with Mn of 1.6×10^4 and Mw of 2.5×10^4, which were evaluated using gel permeation chromatography with Shodex KD-803 and KF805L columns (Showa Denko) and poly(ethylene glycol)s as the standard. Obtained polymers were designated as MGluPG76 and MGluPG222, respectively[7]. FITC-OVA was prepared by reacting OVA (10 mg) with FITC (11.8 mg) in 0.5 M $NaHCO_3$ (4 mL, pH 9) at 4 °C for three days and subsequent dialysis.

Synthesis of Hyperbranched Poly(glycidol) Derivatives.

3-Methyl-glutarylated hyperbranched poly(glycidol) (MGlu-HPG) was prepared by reaction of HPG with varying DP with 3-methylglutaric anhydride. HPG10 (0.765 g) and LiCl (0.765 g) were dissolved in pyridine (18 mL) and 3.0 equiv. of 3-methylglutaric anhydride (3.98 g) was added to the solution. The mixed solution was kept at 115 °C for 24 h with stirring. Then, the reaction mixture was evaporated and dialyzed against water for 3 days. The product was recovered by freeze-drying. HPG20, HPG40 and HPG60 were also reacted with 3-methylglutaric anhydride by the same procedure. As anchor moieties for fixation of MGlu-HPG onto liposome membranes, 1-aminodecane was combined with carboxyl groups of MGlu-HPG. Each polymer was dissolved in water around pH 7.4, and 1-aminodecane (0.18 equiv. to carboxyl group of polymer) was reacted to carboxyl groups of the polymer using DMT-MM (0.18 equiv. to carboxyl groups of polymers) at room temperature for three days with stirring. The obtained polymers were purified by dialysis in water.

Cell Culture.

DC2.4 cells, which were an immature murine DC line, were provided from Dr. K. L. Rock (Harvard Medical School, USA) and were grown in RPMI 1640 supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine, 100 μM non-essential amino acids (Gibco, Inc.), 50 μM 2-mercaptoethanol (2-ME) and antibiotics at 37 °C [16].

Precipitation pH.

Precipitation pH of polymers was determined by measuring the optical density of aqueous polymer solutions (0.25 mg/mL) at various pH. Polymers were dissolved in 30 mM sodium acetate and 120 mM NaCl aqueous solution of various pH. After 5 min incubation at 25 °C, optical densities of the polymer solutions at 500 nm were measured by using a spectrophotometer (Jasco V-520). Precipitation pH was determined using optical density-pH profiles as the pH at which OD drastically rose.

Pyrene Fluorescence.

A given amount of pyrene in acetone solution was added to an empty flask, and acetone was removed under vacuum. Polymer (0.2 mg/mL) dissolving in 25 mM MES and 125 mM NaCl solution of a given pH was added to the flask, yielding 1 μM concentration of pyrene. The sample solution was stirred overnight at room temperature, and emission spectra with excitation at 337 nm were recorded. The fluorescence intensity ratio of the first band at 373 nm to the third band at 384 nm (I_1/I_3) was analyzed as a function of pH of the solution.

Preparation of Pyranine-Loaded Liposomes.

To a dry, thin membrane of EYPC (7 mg) was added 500 μL of aqueous 35 mM pyranine, 50 mM DPX, and 25 mM MES solution (pH 7.4), and the mixture was sonicated for 2 min using a bath-type sonicator. The liposome suspension was further hydrated by freeze and thaw, and was extruded through a polycarbonate membrane with a pore size of 100 nm. The liposome suspension was applied to a sepharose4B column to remove free pyranine from the pyranine-loaded liposomes. Polymer-modified liposomes were also prepared according to the above procedure using dry membranes of mixtures of EYPC and various polymers (EYPC/polymer = 7/3, w/w).

Release of Pyranine from Liposome.

Release of pyranine from liposome was measured as previously reported [7,17]. Pyranine fluorescence was quenched by DPX inside of the liposomes, but this molecule exhibits intense fluorescence when released from the liposome [17]. For study of interaction of polymers with lipid membranes, to a suspension of pyranine-loaded liposomes (lipid concentration: 2.0×10^{-5} M) in 25 mM MES and 125 mM NaCl buffer of varying pH was added a given amount of the polymer dissolved in the same buffer (final concentration: 0.013 mg/mL) at 25 °C, and fluorescence intensity (512 nm) of the mixed suspension was followed with excitation at 416 nm using a spectrofluorometer (Jasco FP-6500). For study of release behavior of polymer-modified liposomes, polymer-modified liposomes encapsulating pyranine were added to 25 mM MES and 125 mM NaCl buffer of varying pHs at 37 °C and fluorescence intensity of the suspension was monitored (lipid concentration: 2.0×10^{-5} M). The percent release of pyranine from liposomes was defined as

$$\text{Release (\%)} = (F_t - F_i) / (F_f - F_i) \times 100$$

where F_i and F_t mean the initial and intermediary fluorescence intensities of the liposome suspension, respectively. F_f is the fluorescent intensity of the liposome suspension after the addition of TritonX-100 (final concentration: 0.1%).

Liposome Size Change

EYPC liposomes were prepared as described above without pyranine and DPX. EYPC liposomes (4.1 mM, 103.4 μL, pH 7.4) were added to 25 mM MES and 125 mM NaCl buffer of various

pHs (2370 μL). And then, 26.6 μL of polymer solution of the same buffer (10 mg/mL, pH 7.4) was added. The mixed solutions were incubated overnight. pH of the mixed solution was measured and liposome diameters were evaluated using a Nicomp 380 ZLS dynamic light scattering instrument (Particle Sizing Systems, Santa Barbara, CA) equipped with a 35 mW laser (λ=632.8 nm). Data was obtained as an average of more than three measurements on different samples.

Intracellular Behavior of Liposomes.

The FITC-OVA-loaded liposomes containing Rh-PE were prepared as described above except that mixtures of polymers and EYPC containing Rh-PE (0.1 mol%) was dispersed in phosphate-buffered saline containing FITC-OVA (4 mg/mL). The DC2.4 cells (2×10^5 cells) cultured for 2 days in 35-mm glass-bottom dishes were washed with Hank's balanced salt solution (HBSS, Sigma), and then incubated in serum-free RPMI medium (500 μL). The FITC-OVA-loaded liposomes (100 μg/mL of FITC-OVA, 500 μL) were added gently to the cells and incubated for 4 h at 37 °C. After the incubation, the cells were washed with HBSS three times. Confocal laser scanning microscopic (CLSM) analysis of these cells was performed using LSM 5 EXCITER (Carl Zeiss Co. Ltd.). Fluorescence intensity of these cells was also determined using a Coulter Epics XL Flow Cytometer (Coulter Corporation, Florida, USA) [18].

Fusion of Liposomes in Cell.

Liposomes containing NBD-PE and Rh-PE (each 0.6 mol%) were prepared as described above using DOPE as an additional lipid component and suspended in PBS. The DC2.4 cells (2×10^5 cells) cultured for 2 days in 35-mm glass-bottom dishes were washed with HBSS, and then incubated in serum-free RPMI medium (500 μL). Then, the liposomes suspension (1.0 mM of liposomal lipid, 500 μL) were added gently to the medium of the cells and incubated for 4 h at 37 °C. After the incubation, the cells were washed with HBSS three times and analyzed by CLSM. Fluorescence of NBD-PE and Rh-PE was observed through specific path filters (λ_{em} = 500–530nm for NBD-PE and λ_{em} > 560nm for Rh-PE) with excitation at 488 nm. Fluorescence intensities of these cells were also determined by flow cytometry with excitation at 488nm. The fluorescence intensity ratio of NBD-PE to Rh-PE was defined as

$$\mathrm{NBD/Rh} = (I_{\mathrm{NBD}} - I_{\mathrm{NBD},0}) / (I_{\mathrm{Rh}} - I_{\mathrm{Rh},0})$$

where I_{NBD} and I_{Rh} are the fluorescence intensities of the liposome-treated cells detected by FL1 (λ_{em} = 505–545 nm) and FL2 channel (λ_{em} = 560–590 nm), respectively. $I_{\mathrm{NBD},0}$ and $I_{\mathrm{Rh},0}$ are the fluorescence intensities of untreated cells detected by FL1 and FL2 channel, respectively.

Table 1. Compositions of Various Hyperbranched and Linear Poly(glycidol) Derivatives

Polymer	Hydroxyl unit/%	Carboxylated unit/%	Anchor unit/%
MGlu-HPG10	0	100	-
MGlu-HPG20	0	100	-
MGlu-HPG40	0	100	-
MGlu-HPG60	5	95	-
Linear MGluPG76	10	90	-
Linear MGluPG222	6	94	-
MGlu-HPG10-C_{10}	0	88	12
MGlu-HPG20-C_{10}	0	90	10
MGlu-HPG40-C_{10}	0	90	10
MGlu-HPG60-C_{10}	5	85	10
Linear MGluPG76-C_{10}	10	75	15
Linear MGluPG222-C_{10}	9	81	10

Estimated by ^{1}H NMR.

Results and discussion

Characterization of HPG Derivatives.

Previous studies developed and assessed a series of carboxylated linear poly(glycidol) derivatives

for pH-sensitization of liposome [5–7]. Especially, MGluPG-modified liposomes were stable at neutral pH and showed strong fusogenic activity under weakly acidic conditions [7]. Considering the function of viral fusogenic proteins for viral membrane fusion, it was assumed that a polymer with a three-dimensional backbone structure can interact with membranes more effectively and intensively than a linear polymer. Therefore, a hyperbranched polymer was selected as a backbone structure. We prepared four kinds of MGlu-HPGs with different molecular sizes, namely MGlu-HPG10, MGlu-HPG20, MGlu-HPG40, and MGlu-HPG60, using HPGs with DPs of 10, 20, 40, and 60, as pH-sensitive polymers with a hyperbranched structure. Also, two kinds of MGluPGs with different chain lengths, namely MGluPG76 and MGluPG222, were prepared as pH-sensitive polymers with a linear structure, using PG76 and PG222. Hydrodynamic diameters of HPG10, HPG20, HPG40, HPG60, PG76, and PG222 were estimated to be 2.0, 2.6, 3.2, 3.6, 4.8, and 8.6 nm, respectively, according to the method of Hester and Mitchell using GPC with PEG standards [19].

Compositions of these polymers, which were estimated using ^{1}H NMR, are resented in Table 1. For all polymers, only low percentages of unreacted glycidol units remained on the polymer backbone after the reaction of HPG with 3-methylglutaric anhydride, demonstrating the high efficiency of these reactions, which is consistent with a previous report [7]. Fundamentally, every repeating unit possesses a carboxyl group in the resultant polymers. Results of an earlier study showed that attachment of 1-aminodecane to about 8 unit% of a carboxylated poly(glycidol) chain is sufficient to fix the polymer chain onto an EYPC liposome membrane [5,7]. Based on previous studies, 1-aminodecane was combined to about 10 unit% of the polymer chains in this study (Table 1).

Acid-base titration of these polymers was performed to estimate the p*K*a values. As presented in Table 2, MGlu-HPGs had p*K*a values around 5.9–6.5 and p*K*a slightly increased concomitantly with increasing DP. For polymers with a hyperbranched structure, their chain density increases concomitantly with increasing DP. Therefore, hydrophobic interactions among these crowded polymer chains of HPG with higher DP might engender a more compact

Table 2. p*K*a and Precipitation pH of Various Hyperbranched and Linear Poly(glycidol) Derivatives

Polymer	pKa	Precipitation pH	Degree of protonation at precipitation pH
MGlu-HPG10	5.9	4.4	0.98
MGlu-HPG20	6.2	4.6	0.98
MGlu-HPG40	6.5	4.7	0.99
MGlu-HPG60	6.5	4.9	0.94
Linear MGluPG76	6.2	4.9	0.99
Linear MGluPG222	6.1	4.8	0.99

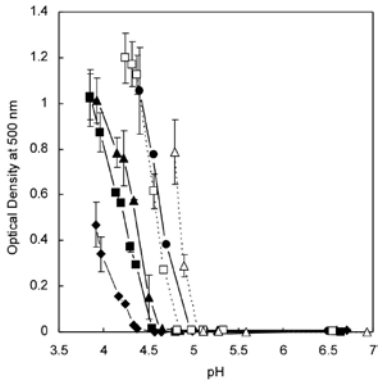

Figure 2. Optical densities at 500 nm for solutions of MGlu-HPG10 (closed diamonds), MGlu-HPG20 (closed squares), MGlu-HPG40 (closed triangles), MGlu-HPG60 (closed circles), linear MGluPG76 (open triangles), and linear MGluPG222 (open squares) dissolved in 30 mM sodium acetate and 120 mM NaCl of various pH (0.25 mg/mL) at 25 °C. Each point is the mean ± SD (*n* = 3).

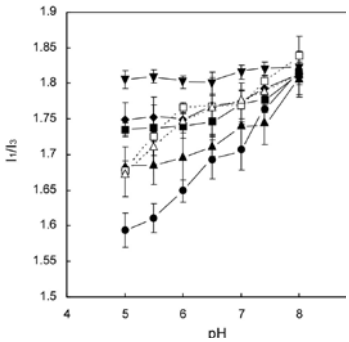

Figure 3. pH-Dependence of I_1/I_3 of pyrene fluorescence in the absence (closed inverted triangles) or presence of MGlu-HPG10 (closed diamonds), MGlu-HPG20 (closed squares), MGlu-HPG40 (closed triangles), MGlu-HPG60 (closed circles), linear MGluPG76 (open triangles), and linear MGluPG222 (open squares) dissolving in 25 mM MES and 125 mM NaCl solution. Concentration of polymers and pyrene were 0.2 mg/mL and 1 μM, respectively. I_1/I_3 was defined as the fluorescence intensity ratio of the first band at 373 nm to the third band at 384 nm.

conformation. Such a compact conformation of HPGs increases spatial density of carboxyl groups in the polymer chains, which might suppress dissociation of carboxyl groups to avoid repulsive electrostatic forces among carboxylate anions. In addition, hydrophobic environment of the crowded polymer chains may induce suppression of dissociation of carboxyl groups. As a result, pKa of the MGlu-HPGs might increase with increasing DP.

Hydrophobicity of carboxylated polymers affects their precipitation behaviors [20]. For that reason, it was estimated that the pH at which the polymers precipitate using the following optical densities of these polymer solutions as the solution pH was decreased (Fig. 2). These polymers were soluble in water at neutral pH; their solutions were transparent. However, the polymer solutions suddenly became turbid at a specific pH, which was defined as the precipitation pH. The precipitation pH thresholds for MGlu-HPGs were estimated as presented in Table 2. The precipitation pH shifted to slightly higher pH values with increasing DP of MGlu-HPGs, indicating that hydrophobicity of MGlu-HPG increases concomitantly with increasing DP, consistent with previous observations for methacrylic acid copolymers with varying hydrophobicities and a previous report by the authors [7,20]. Acid–base titration for these polymers revealed that the degree of protonation for their carboxyl groups was around 0.94–0.99 at the precipitation pH: most carboxyl groups must be protonated to elicit precipitation of these polymers.

Hydrophobicity of the polymers was further investigated using a fluorescence probe pyrene. An emission intensity ratio of the first (373 nm) to the third (384 nm) peaks of pyrene, I_1/I_3, is known to be sensitive to the micro-environmental polarity surrounding the pyrene molecule [21]. Consequently, this ratio has been widely used to estimate the hydrophobic nature of polymers [22,23]. Figure 3 depicts the I_1/I_3 ratio of pyrene fluorescence in the buffer dissolving various polymers as a function of pH. In buffers dissolving MGlu-HPG10 or MGlu-HPG20, the I_1/I_3 ratios of pyrene were around 1.75 at pH 5, suggesting that these polymers formed few domains with a hydrophobic nature, even after protonation of carboxyl groups of the polymer chain. On the other hand, a significant decrease in the I_1/I_3 ratio is seen in the presence of MGlu-HPG40 or MGlu-HPG60 under weakly acidic conditions. These results suggest that MGlu-HPGs with higher DP formed more hydrophobic domains probably because of their globular structure. The presence of linear MGluPGs also affected the I_1/I_3 ratio, which tends to decrease below pH 6.0. However, their I_1/I_3 values were generally higher than those of MGlu-HPGs with similar DP.

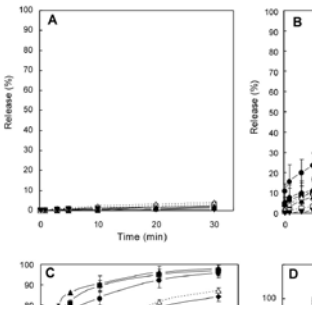

Figure 4. Pyranine release from EYPC liposomes induced by various hyperbranched poly(glycidol) derivatives. Time courses at pH 7.4 (A), pH 6.5 (B), and pH 6.0 (C), and pH-dependence (D) of pyranine release induced by MGlu-HPG10 (closed diamonds), MGlu-HPG20 (closed squares), MGlu-HPG40 (closed triangles), MGlu-HPG60 (closed circles), linear MGluPG76 (open triangles), and linear MGluPG222 (open squares) or without polymer (closed and inverted triangles). Percent release of pyranine after 10 min-incubation was shown (D). Polymer and lipid concentrations were 0.013 mg/mL and 2.0×10^{-5} M, respectively. Each point is the mean ± SD (n = 3).

Linear MGluPGs might be unable to form hydrophobic domains as much as MGlu-HPGs because of their linear backbone structure.

Interaction of HPG Derivatives with Lipid Membrane.

We have shown that anchoring moiety into liposomes was necessary for poly(glycidol) derivatives to interact with liposomal membrane [7]. MGlu-HPG with anchor moieties (MGlu-HPG-C_{10}) were added to liposomes encapsulating both pyranine and its quencher DPX, and fluorescence of the released pyranine was monitored (Fig. 4). At neutral pH, no polymer showed a content release (Fig. 4A), indicating that these polymers did not disrupt liposome membrane under this condition. On the other hand, release of the contents was observed for all MGlu-HPG-C_{10} at pH 6.5 (Fig. 4B). Complete release was achieved below pH 6.0 (Fig. 4C), indicating that protonated polymers disrupt the liposome membrane. As portrayed in Fig. 4D, the content release in the weakly acidic region increased in the order of DP of MGlu-HPGs-C_{10}, indicating that the polymer with higher hydrophobicity triggers release more strongly. In addition, MGlu-HPGs-C_{10} triggered the content release more strongly than linear MGluPG-C_{10} under the weakly acidic condition, demonstrating that the hyperbranched polymers can destabilize the liposome membrane more strongly than linear polymers.

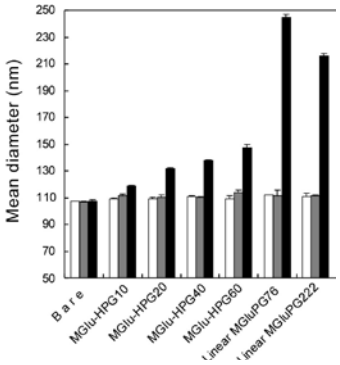

Figure 5. Mean diameters of EYPC liposomes after overnight incubation with various polymers or without polymer at pH 7.4 (open bars), pH 6.5 (gray bars) and pH 5.5 (closed bars). Polymer and lipid concentrations were 0.11 mg/mL and 1.7×10^{-4} M, respectively. Each point is the mean ± SD (*n* = 3).

Interaction of the polymers with the liposomes was also investigated through inspection of the liposome size change. EYPC liposomes were incubated with MGlu-HPGs-C_{10} or linear MGluPGs-C_{10} at various pHs overnight; their diameters were evaluated by DLS (Fig. 5). The liposome size changed only slightly after incubation with MGlu-HPGs-C_{10} at pHs 7.4 and 6.5, but incubation at pH 5.5 increased their diameter to some extent. This range of increase rose with increasing DP. On the other hand, incubation with linear MGluPGs-C_{10} induced remarkable liposome size change at pH 5.5. Because linear MGluPG-C_{10} has a high degree of freedom on their conformation, they might promote intervesicular interaction, engendering aggregation of liposomes. In contrast, MGlu-HPGs-C_{10} might interact with the membrane in single liposome because of their compact conformation.

Preparation of pH-Sensitive Liposomes Using HPG.

Figure 6 depicts pH-sensitive content release behaviors of liposomes modified with MGlu-HPGs-C_{10} or linear MGluPGs-C_{10}. All liposomes retained pyranine at pH 7.4 (Fig. 6A). However, MGlu-HPGs-C_{10}-modified liposomes enhanced their content release below pH 6.0; almost complete release was achieved at pH 5.5 (Figs. 6B and 6C). As portrayed in Fig. 6D, liposomes modified with MGlu-HPGs-C_{10} of low DP exhibited higher content release in acidic pH than MGlu-HPGs-C_{10} of high DP. These observations differ from the case of content release induced by addition of these polymers into the liposome suspensions (Fig. 4). These might be resulted from the difference of protonation behavior of polymers between on the liposome surface and in an aqueous medium. It is possible that the protonation of MGlu-HPG-C_{10} with low DP is enhanced on the liposome membrane because carboxylate anions of the small-size polymer might exist in close vicinity of the

membrane. Comparison of liposomes modified with linear and hyperbranched polymers shows that MGlu-HPG-C_{10}-modified liposomes induced the content release at a higher pH region than linear MGluPG-C_{10}-modified liposomes. Therefore, the liposomes having the hyperbranched polymers might destabilize the endosome in the early stage of endocytic pathway after their uptake by a cell.

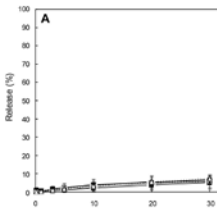

Figure 6. Pyranine release from EYPC liposomes modified with various hyperbranched and linear poly(glycidol) derivatives. Time courses at pH 7.4 (A), pH 6.0 (B), and pH 5.5 (C), and pH-dependence (D) of pyranine release from EYPC liposomes modified with MGlu-HPG10 (closed diamonds), MGlu-HPG20 (closed squares), MGlu-HPG40 (closed triangles), MGlu-HPG60 (closed circles), linear MGluPG76 (open triangles), linear MGluPG222 (open squares), and unmodified EYPC liposomes (closed and inverted triangles). Percent release of pyranine after 10 min-incubation was shown (D). Lipid concentrations were 2.0×10^{-5} M. Each point is the mean ± SD (n = 3).

Cytoplasmic Delivery by Polymer-Modified Liposomes.

Previously, we have shown that liposomes modified with linear MGluPG can be used for cytoplasmic delivery of antigenic proteins, such as OVA, into dendritic cells for induction of antigen-specific immune responses [24]. Therefore, we compared the performance of the MGluHPG-modified liposomes as antigenic protein delivery vehicles with that of the linear MGluPG modified liposomes.

We prepared the MGlu-HPGs-C_{10}-modified liposomes labeled with Rh-PE and loaded with FITC-OVA, and their interaction with DC2.4 cells was compared with that for the linear MGluPG-C_{10}-modified EYPC liposomes or bare EYPC liposomes labeled with Rh-PE and loaded with FITC-OVA. As presented in Fig. 7A, cells treated with the bare liposome displayed weak and punctate fluorescence of Rh-PE and FITC-OVA. Considering that the liposomes were generally taken up by a cell *via* endocytosis, it is highly likely that FITC-OVA molecules were still trapped in endosome and/or lysosome. In contrast, cells treated with MGlu-HPG-C_{10}-modified liposomes showed punctate fluorescence of Rh-PE but diffuse fluorescence of FITC-OVA (Figs. 7B and 7C), indicating that lipid molecules existed in endosome and lysosome but that FITC-OVA molecules existed in cytoplasm. These liposomes have the capability of destabilizing lipid membranes under a weakly acidic environment. Therefore, it is likely that FITC-OVA molecules were transferred from endosome into cytoplasm. Furthermore, the fluorescence from cells treated with MGlu-HPG60-C_{10}-modified liposomes was much brighter than that from cells treated with MGlu-HPG20-C_{10}. Although diffuse fluorescence of FITC-OVA was also observed for cells treated with the linear MGluPG-C_{10}-modified liposomes (Figs. 7D and 7E), their fluorescence was weaker than the case of MGlu-HPG60-C_{10}-modified liposomes.

The Rh-fluorescence intensity of the liposome-treated cells was evaluated by flow cytometry (Fig. 7F). The Rh-fluorescence intensity increased concomitantly with increasing DP of MGlu-HPGs-C_{10}, indicating that liposomes modified with MGlu-HPG-C_{10} of higher DP were taken up more efficiently. In addition, cells treated with the MGlu-HPG60-C_{10}–modified liposomes showed higher intensity than those treated with the linear MGluPGs-C_{10}–modified liposomes. This result suggests that liposomes having polymers of a hyperbranched structure were taken up by cells more efficiently than those with

the polymers of a linear structure. We have shown that linear MGluPG-modified liposomes are taken up by DC2.4 cells through their interaction with the cellular scavenger receptors, which recognize carboxylate anions of polymers [18, 24]. Negatively charged carboxylate groups of the hyperbranched polymer tend to locate in the peripheral region of the polymer. Therefore, these groups might be recognized by scavenger receptors efficiently, thereby promoting their uptake by the cells. These results demonstrate that modification of liposomes with MGlu-HPG-C_{10} can produce pH-sensitive liposomes that achieve efficient cytoplasmic delivery of proteins.

Fusion of Polymer-Modified Liposomes within Cell.

Finally, we attempted to verify the fusion of MGlu-HPG-C_{10}-modified liposomes in the cells. The polymer-modified liposomes containing NBD-PE and Rh-PE were prepared to detect fusion of the liposomes with intracellular membranes [6,25]. Fusion of the labeled liposomes with endosomal membranes causes dilution of these fluorescent lipids in the membrane, resulting in decrease of energy transfer efficiency between these fluorescent lipids.

The fluorescent lipid-labeled liposomes with or without polymers were applied to DC2.4 cells and incubated for 4 h. Then cellular fluorescence was observed using a CLSM under irradiation of light with wavelength of 488 nm, which is for excitation of NBD-PE (Fig. 8). As Fig. 8A shows, cells treated with the bare liposomes displayed only fluorescence of Rh-PE, suggesting that fluorescence of NBD-PE was quenched by energy transfer to Rh-PE and hence the bare liposomes did not fuse with the endosomal membrane. In contrast, the cells treated with MGlu-HPG60-C_{10}-modified or linear MGluPG76-C_{10}-modified liposomes exhibited not only Rh-PE-fluorescence but also NBD-PE-fluorescence, indicating that fusion between these liposomes and the endosomal membranes occurred (Figs. 8B and 8C). However, the fluorescence of NBD-PE was very weak, suggesting that their fusion was not efficient.

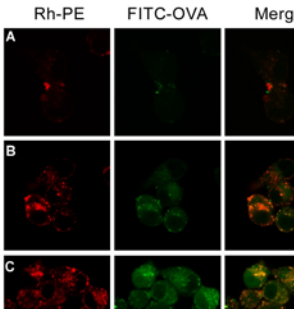

Figure 7. Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with Rh-PE-labeled and FITC-OVA-loaded EYPC liposomes of various types: plain liposomes (A); liposomes modified with MGlu-HPG20-C_{10} (B), MGlu-HPG60-C_{10} (C), linear MGluPG76-C_{10} (D), or linear MGluPG222-C_{10} (E). FITC-OVA concentration was 50 μg/mL. Intracellular localization of Rh-PE (red) and FITC-OVA (green) was observed using a CLSM. Scale bar represents 10 μm. (F) Fluorescence intensities of DC2.4 cells treated with Rh-PE-labeled EYPC liposomes modified with or without polymers of various types. The fluorescence intensities of Rh-PE were determined by flow cytometry.

The intracellular fusion behavior of the liposomes containing a non-bilayer-forming lipid DOPE, which is known to enhance membrane fusion, was also examined (Figs. 8D–F). For cells treated with the bare EYPC/DOPE liposomes, fluorescence of NBD-PE remained very weak (Fig. 8D). However, intensive fluorescence of NBD-PE was detected from cells treated with the DOPE-containing liposomes having either MGlu-HPG60-C_{10} or linear MGluPG76-C_{10}, indicating that these polymer-modified liposomes fused efficiently with endosomal membranes (Figs. 8E and 8F).

The fluorescence intensity ratios of NBD-PE to Rh-PE for the liposome-treated cells were evaluated by flow cytometry and were expressed as relative values using the ratio of the bare EYPC liposome-treated cells as the standard (Fig. 8G). The cells treated with either polymer-modified EYPC liposomes showed a 2–3 times increase in the NBD/Rh ratio compared with those treated with the base EYPC liposomes. Furthermore, no significant difference was found between the cells treated with any MGlu-HPG-C_{10}-modified and linear MGluPG-C_{10}-modified EYPC liposomes. These results indicate that these polymer-modified EYPC liposomes possess similar abilities to fuse with the endosomal membrane. Indeed, the EYPC/DOPE liposomes modified with these polymers caused a more significant increase in the NBD/Rh ratio than EYPC liposomes having the same polymers (Fig. 8G). In particular, the EYPC/DOPE liposomes modified with the hyperbranched polymers showed a higher NBD/Rh ratio than linear polymers, indicating that the backbone structure of the polymer affects their ability to generate the fusion ability of EYPC/DOPE liposomes.

The performance of MGlu-HPG-modified liposomes as a cytoplasmic delivery system was shown to increase as MGlu-HPG with higher DP was used for liposome modification. There might be various modes of interaction between MGlu-HPG with lipid membranes, such as absorption onto the membrane, penetration into the membrane, and solubilization lipid molecules. Therefore, their ability to destabilize lipid membranes might be influenced by their size. Optimization of molecular size may generate MGlu-HPG-modified liposomes with even higher performance.

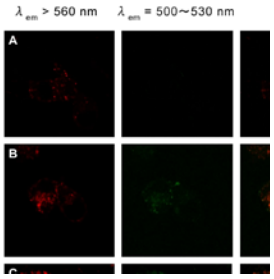

Figure 8. CLSM images of DC2.4 cells treated with EYPC (A-C) or EYPC/DOPE (1/1, mol/mol) (D-F) plain liposomes (A, D) or liposomes modified with MGlu-HPG60-C_{10} (B, E), and linear MGluPG76-C_{10} (C, F). Liposomal lipid concentration was 0.5 mM. Fluorescence of NBD-PE and Rh-PE upon excitation at 488 nm was observed using a CLSM. Scale bar represents 10 μm. (G) Fluorescence intensity ratios of NBD-PE to Rh-PE for DC2.4 cells treated with EYPC (closed symbols) or EYPC/DOPE (open symbols) liposomes modified with or without polymers. Fluorescence intensity ratios were evaluated by flow cytometry and were expressed as relative values using the ratio of the plain EYPC liposome-treated cells as the standard.

Conclusion

A new type of pH-sensitive polymer with a hyperbranched backbone—MGlu-HPG-C_{10}—was synthesized. Its feasibility for production of pH-sensitive liposomes was then investigated. Their ability for pH-sensitization of liposomes was enhanced with increasing DP. Modification of liposomes with MGlu-HPG-C_{10} produced highly pH-sensitive liposomes that undergo content release at mildly acidic pH. The MGlu-HPG-C_{10}-modified liposomes encapsulating OVA delivered their contents efficiently into the cytosol of DC2.4 cells. Especially, liposomes having MGlu-HPG-C_{10} with high DP exhibited higher fusion ability and more efficient cellular internalization property than the liposomes modified with the counterpart polymers with a linear

backbone structure. This is the first report describing the importance of the polymer backbone structure for polymer-based functionalization of liposomes. The MGlu-HPG-C_{10}-modified liposomes showed excellent ability to deliver the loaded proteins into cytosol of dendritic cell-derived cells. Therefore, they might have potential usefulness for the delivery of antigenic proteins.

Acknowledgments

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Figure captions

Figure 1. Structures of MGlu-HPG20-C_{10} (a) and linear MGluPG-C_{10} (b).

Figure 2. Optical densities at 500 nm for solutions of MGlu-HPG10 (closed diamonds), MGlu-HPG20 (closed squares), MGlu-HPG40 (closed triangles), MGlu-HPG60 (closed circles), linear MGluPG76 (open triangles), and linear MGluPG222 (open squares) dissolved in 30 mM sodium acetate and 120 mM NaCl of various pH (0.25 mg/mL) at 25 °C. Each point is the mean ± SD (n = 3).

Figure 3. pH-Dependence of I_1/I_3 of pyrene fluorescence in the absence (closed inverted triangles) or presence of MGlu-HPG10 (closed diamonds), MGlu-HPG20 (closed squares), MGlu-HPG40 (closed triangles), MGlu-HPG60 (closed circles), linear MGluPG76 (open triangles), and linear MGluPG222 (open squares) dissolving in 25 mM MES and 125 mM NaCl solution. Concentration of polymers and pyrene were 0.2 mg/mL and 1 μM, respectively. I_1/I_3 was defined as the fluorescence intensity ratio of the first band at 373 nm to the third band at 384 nm.

Figure 4. Pyranine release from EYPC liposomes induced by various hyperbranched poly(glycidol) derivatives. Time courses at pH 7.4 (A), pH 6.5 (B), and pH 6.0 (C), and pH-dependence (D) of pyranine release induced by MGlu-HPG10 (closed diamonds), MGlu-HPG20 (closed squares), MGlu-HPG40 (closed triangles), MGlu-HPG60 (closed circles), linear MGluPG76 (open triangles), and linear MGluPG222 (open squares) or without polymer (closed and inverted triangles). Percent release of pyranine after 10 min-incubation was shown (D). Polymer and lipid concentrations were 0.013 mg/mL and 2.0×10^{-5} M, respectively. Each point is the mean ± SD (n = 3).

Figure 5. Mean diameters of EYPC liposomes after overnight incubation with various polymers or without polymer at pH 7.4 (open bars), pH 6.5 (gray bars) and pH 5.5 (closed bars). Polymer and lipid concentrations were 0.11 mg/mL and 1.7×10^{-4} M, respectively. Each point is the mean ± SD (n = 3).

Figure 6. Pyranine release from EYPC liposomes modified with various hyperbranched and linear poly(glycidol) derivatives. Time courses at pH 7.4 (A), pH 6.0 (B), and pH 5.5 (C), and pH-dependence (D) of pyranine release from EYPC liposomes modified with MGlu-HPG10 (closed diamonds), MGlu-HPG20 (closed squares), MGlu-HPG40 (closed triangles), MGlu-HPG60 (closed circles), linear MGluPG76 (open triangles), linear MGluPG222 (open squares), and unmodified EYPC liposomes (closed and inverted triangles). Percent release of pyranine after 10 min-incubation was shown (D). Lipid concentrations were 2.0×10^{-5} M. Each point is the mean ± SD (n = 3).

Figure 7. Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with Rh-PE-labeled and FITC-OVA-loaded EYPC liposomes of various types: plain liposomes (A); liposomes modified with MGlu-HPG20-C_{10} (B), MGlu-HPG60-C_{10} (C), linear MGluPG76-C_{10} (D), or linear MGluPG222-C_{10} (E). FITC-OVA concentration was 50 μg/mL. Intracellular localization of Rh-PE (red) and FITC-OVA (green) was observed using a CLSM. Scale bar represents 10 μm. (F) Fluorescence intensities of DC2.4 cells treated with Rh-PE-labeled EYPC liposomes modified with or without polymers of various types. The fluorescence intensities of Rh-PE were determined by flow cytometry.

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