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Carboxylated polyamidoamine dendron-bearing lipid-based assemblies for precise control of intracellular fate of cargo and induction of antigen-specific immune responses

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Abstract

For establishment of advanced medicines such as cancer immunotherapy, high performance carriers that precisely deliver biologically active molecules must be developed to target organelles of the cells and to release their contents there. From the viewpoint of antigen delivery, endosomes are important target organelles because they contain immune-response-related receptors and proteins of various types. To obtain carriers for precision endosome delivery, a novel type of polyamidoamine dendron-based lipid having pH-sensitive terminal groups was synthesized for this study. Liposomes were prepared using these pH-sensitive dendron-based lipids and egg yolk phosphatidylcholine. Their pH-responsive properties and performance as an endosome delivery carrier were investigated. pH-Sensitive dendron lipid-based liposomes retained water-soluble molecules at neutral pH but released them under weakly acidic conditions. Especially, liposomes containing CHexDL-G1U exhibited highly sensitive property responding to very weakly acidic pH. These dendron lipid-based liposomes released the contents specifically in endosome. The timing of content release can be controlled by selecting pH-sensitive dendron lipids for liposome preparation. Significant tumor regression was induced in tumor-bearing mice by administration of CHexDL-G1U-modified liposomes containing model antigenic protein. Furthermore,

CHexDL-G1U-modified liposomes induced WT1 tumor antigenic peptide-specific helper T cell proliferation. Results demonstrate that dendron lipid-based liposomes are useful as potent vaccine for cancer immunotherapy.

Keywords: dendron; liposome; pH-sensitive; cellular immunity; peptide vaccine

1. Introduction

Since the discovery of liposomes by Bangham, liposomes have been regarded as an ideal drug carrier. Many efforts have been made to improve the accuracy of drug delivery using liposomes. An effective approach is to give stimulus-sensitive properties to liposomes. Therefore, liposomes of various kinds have been developed to have sensitivity to stimuli such as temperature, pH, light, ultrasound, and magnetic fields[1–4]. These liposomes retain drug molecules under physiological conditions, whereas they become destabilized at target sites of the body, which have environments different from physiological environments or which are applied with the stimuli, resulting in drug release there.

pH-Sensitive liposomes are regarded as important stimuli-sensitive liposomes from the perspectives of tumor tissue-selective drug delivery and intracellular drug delivery. Tumor tissues are known to have a weakly acidic environment of around pH 7.0–6.5[5, 6], which is lower than the pH of normal tissues. Therefore, liposomes must have very high pH-sensitivity to respond to such tiny pH decreases for this purpose. Some liposome systems have achieved tumor microenvironmental pH-responsive detachment of hydrophilic layer such as PEG to promote association of liposomes to tumor cells[7, 8]. However, widely used phosphatidylethanolamine-based pH-sensitive liposomes need

56 acidic environments of pH below 6 to induce their pH-triggered collapses[9–11], implying
57 some difficulty of producing liposomes to release pH-responsive contents efficiently in
58 such a weakly acidic environment. To use pH-sensitive liposomes for intracellular
59 delivery, precise control of pH-sensitive region is necessary to induce drug release at a
60 specified organelle. Liposomes are generally taken up by cells through endocytosis and
61 are trapped in early endosomes, of which the interior is very weakly acidic pH (pH 6.5).
62 Endosome interior pH decreases during maturation to lysosomes, which have somewhat
63 more acidic pH (pH 4.5)[12]. To date, various molecular designs to achieve drug release at
64 late endosomes or lysosomes have been reported[13, 14], although selective release of
65 contents at early endosomes persists as a challenge for future research.

66 Release of contents at specific intracellular organelles is especially effective for
67 antigen delivery because endosomes contain many target receptors or proteins to
68 modulate immune responses. More importantly, the intracellular fate of antigens in
69 antigen-presenting cells strictly determines the mode of antigen-specific immunity[15, 16].
70 When an antigen is transferred into lysosomes and is hydrolyzed there, degraded antigenic
71 peptides are carried onto major histocompatibility complex (MHC) class II molecules.
72 Presentation of antigenic peptide via MHC II molecules promotes the differentiation of
73 CD4+ T lymphocytes into antigen-specific helper T (Th) cells to activate humoral

74 immunity. When an antigen is transferred to cytosol and is processed via proteasomes,
75 these degraded peptides are presented via MHC I molecules to promote the differentiation
76 of CD8+ T cells into cytotoxic T lymphocytes (CTLs). Actually, CTL-based immune
77 response (cellular immunity) plays a crucially important role in eliminating virus-infected
78 cells or attacking immunogenic cancer cells. Another route to activate cellular immunity
79 is known as vacuolar pathway of cross-presentation, which is achieved through antigen
80 released in early endosomes[17, 18]. Therefore, control of the intracellular fate of antigen
81 using pH-sensitive liposomes is expected to provide on-demand immunity-inducing
82 systems for the treatment of immunity-relating diseases including cancer, tolerance,
83 autoimmunity-relating diseases, and infectious diseases.

84 We earlier developed pH-sensitive liposomes modified with polyglycidols or
85 polysaccharides having 3-methyl glutarylated (MGlu) units or 2-carboxycyclohexane-1-
86 carboxylated (CHex) units to induce membrane fusion of liposomes with endosomal
87 membrane responding to acidic pH in late endosomes or lysosomes[19–21]. These pH-
88 sensitive polymer-modified liposomes achieved efficient cytosolic transfer of antigenic
89 proteins or peptides, leading to induction of antigen-specific cellular immunity[22, 23]. We
90 also developed cytosolic antigen delivery liposomes using polyamidoamine dendron-
91 bearing lipid assemblies, which changes the assembling structure at weakly acidic pH

92 from vesicle to micelle-like morphology[24]. The pH-responsive region of polyamidoamine
93 dendron-bearing lipid is determined by the protonation behavior of primary and tertiary
94 amines in a dendron moiety. Indeed, polyamidoamine dendron-bearing lipid assemblies
95 induce sharp pH-sensitive membrane destabilization activity below pH 6.3, which
96 corresponds to the late endosome pH region[24]. Consequently, cytosolic antigen delivery
97 can be achieved using conventional pH-sensitive liposomes. Nevertheless, early
98 endosome-specific antigen release towards induction of cross-presentation remains
99 challenging.

100 For this study, carboxylated polyamidoamine dendron-bearing lipids were designed
101 to control pH-sensitivity precisely for selective antigen release at specific intracellular
102 organelles (Fig. 1). MGlu units or CHex units were introduced to polyamidoamine
103 dendron-bearing lipids as a pH-sensitive moiety. These dendron lipids have both carboxy
104 groups and tertiary amines to be protonated in the very weakly acidic pH region. For that
105 reason, these dendron lipid-containing liposomes are expected to induce content release
106 responding to a slight pH decrease. Here, syntheses of carboxylated polyamidoamine
107 dendron-bearing lipids were investigated along with their pH-sensitivity, intracellular
108 behavior and induction of antigen-specific immune responses using both model antigen
109 and WT1-derived natural tumor antigenic peptides.

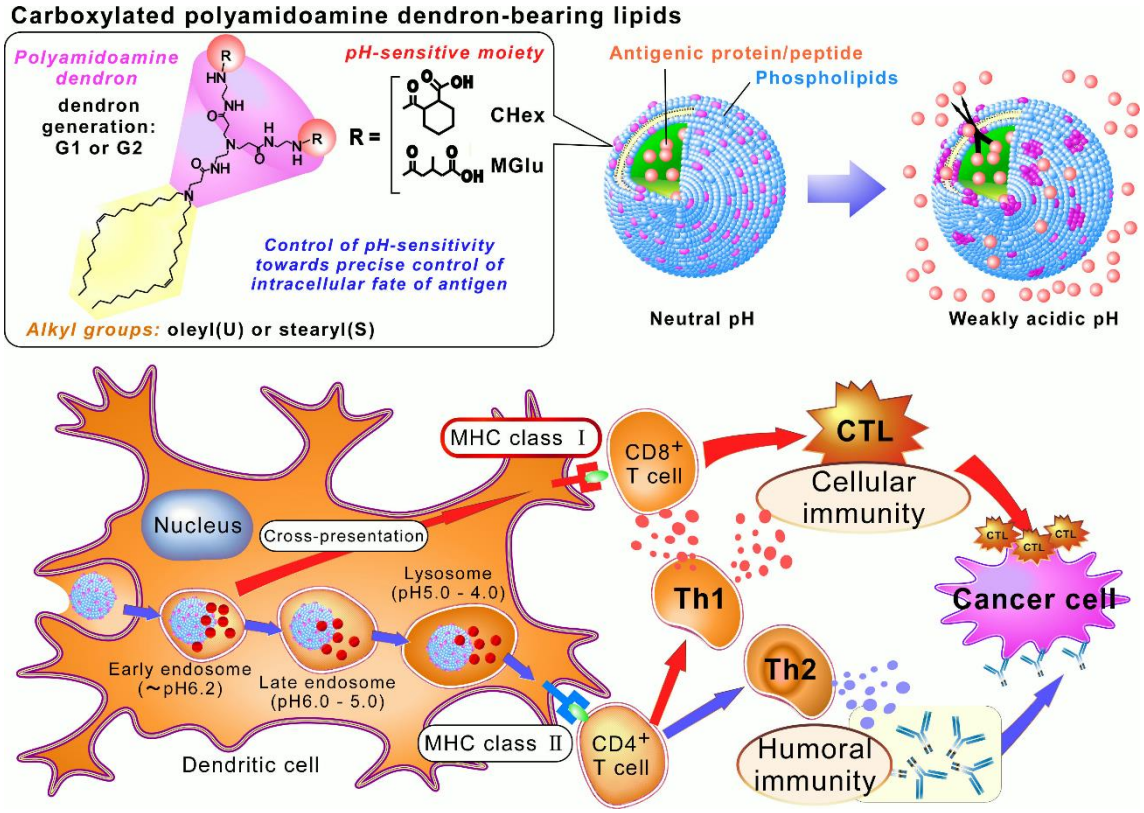


Figure 1. Design of carboxylated polyamidoamine dendron-bearing lipids for precise control of intracellular fate of cargo and induction of antigen-specific immune responses.

2. Materials and Methods

2.1. Materials

Egg yolk phosphatidylcholine (EYPC) was kindly donated by NOF Co. (Tokyo, Japan). 3-Methylglutaric anhydride, 1,2-cyclohexanedicarboxylic anhydride, ovalbumin (OVA), fetal bovine serum (FBS), *p*-xylene-bis-pyridinium bromide (DPX), calcein,

120 triethylamine and monophosphoryl lipid A (MPLA) were purchased from Sigma (St.
121 Louis, MO.). Pyranine and Triton X-100 were obtained from Tokyo Chemical
122 Industries Ltd. (Tokyo, Japan). Lissamine rhodamine B-sulfonyl
123 phosphatidylethanolamine (Rh-PE) was purchased from Avanti Polar Lipids
124 (Birmingham, AL, USA). FITC-OVA was prepared by reacting OVA (10 mg) with
125 FITC (11.8 mg) in 0.5 M $NaHCO_3$ (4 mL, pH 9.0) at 4°C for three days and subsequent
126 dialysis. Polyamidoamine dendron-bearing lipids (DL-G1S, DL-G1U and DL-G2S)
127 were synthesized as previously reported[25, 26].

128 2.2. Synthesis of carboxylated polyamidoamine dendron-bearing lipids

129 Carboxylated dendron lipids were synthesized by reaction of dendron lipids
130 with acid anhydrides, such as 3-methylglutaric anhydride and 1,2-
131 cyclohexanedicarboxylic anhydride (Scheme S1).

132 For the synthesis of MGluDL-G1U, DL-G1U (430 mg, 0.909 mmol) dissolved
133 in DMF (4 mL) was mixed with 3-methylglutaric anhydride (618 mg, 4.23 mmol)
134 dissolved in DMF (3 mL) and triethylamine (0.37 mL, 5 mmol). The mixed solution
135 was kept at 50°C under argon atomosphere for 7 days with stirring. Solvent was
136 removed under vacuum, and the residue was chromatographed on LH-20 using

137 methanol as an eluent and was subsequently chromatographed on silica gel using
138 chloroform-methanol-water (60/35/5, v/v/v) as an eluent. The yield was 208 mg
139 (20.5%). ^{1}H NMR for MGluDL-G1U ($CDCl_3$, 400 MHz): δ (ppm) 0.88 (m, C*H*$_3$(CH_2)$_6$-
140), 1.03 (s, -CH(C*H*$_3$)-), 1.2-1.4 (m, CH_3(C*H*$_2$)$_6$-, -CH_2(CH_2)$_5$$CH_2$-), 1.60 (s, -C*H*$_2$$CH_2$N-
141), 2.0 (m, -CH_2C*H*$_2$CH-), 2.03-2.45 (m, -CH_2C*H*$_2$N-, -CH_2C*H*$_2$CO, -C*H* (CH_3)-), 2.46-
142 2.95 (m, -C*H*$_2$$CH_2$CO, -$CH_2$C*H*$_2$NH-), 3.2-3.4 (m, -C*H*$_2$$CH_2$N-, -
143 COC*H*$_2$CH(CH_3)C*H*$_2$COOH), 5.35 (m, -CH_2C*H*CH*H*CH_2-). Calc [M]+ ($C_{63}H_{117}N_7O_9$)
144 m/z 1115.9. Found ESI-MS [M+ H]+ m/z 1114.8.

145 For the synthesis of CHexDL-G1U, DL-G1U (207.7 mg, 0.234 mmol)
146 dissolved in DMF (3 mL) was mixed with 1,2-cyclohexanedicarboxylic anhydride
147 (390.5 mg, 2.53 mmol) dissolved in DMF (5 mL) and triethylamine (0.17 mL, 2.4
148 mmol). The mixed solution was kept at 50°C under argon atomosphere for 4 days with
149 stirring. Solvent was removed under vacuum, and the residue was chromatographed on
150 LH-20 using methanol as an eluent. The yield was 80.9 mg (29.3%). ^{1}H NMR for
151 CHexDL-G1U ($CDCl_3$, 400 MHz): δ (ppm) 0.88 (m, C*H*$_3$(CH_2)$_6$-), 1.2-1.4 (m,
152 CH_3(C*H*$_2$)$_6$-, -CH_2(C*H*$_2$)$_5$$CH_2$-, -C*H*$_2$$CH_2$N-), 1.4-1.9 (m, -COCH-(C*H*$_2$)$_4$-CHCOOH),
153 2.0 (m, -CH_2C*H*$_2$CH-), 2.3-2.7 (m, -CH_2C*H*$_2$N-, -CH_2C*H*$_2$COO), 2.6-2.8 (m, -
154 C*H*$_2$$CH_2$COO, -$CH_2$C*H*$_2$$NH_2$, -COC*H*-$CH_2$), 3.25 (m, -C*H*$_2$$CH_2$N-), 5.35 (m, -

155 $CH_2CHCHCH_2$-), Calc $[M]^+$ ($C_{67}H_{121}N_7O_9$) m/z 1167.9. Found ESI-MS $[M]^+$ m/z

156 1166.6.

157 For the synthesis of CHexDL-G1S, DL-G1S (145.3 mg, 0.307 mmol) dissolved

158 in DMF (5 mL) was mixed with 1,2-cyclohexanedicarboxylic anhydride (265 mg, 1.63

159 mmol) dissolved in DMF (5 mL) and triethylamine (0.125 mL, 1.7 mmol). The mixed

160 solution was kept at 50°C under argon atomosphere for 25 h with stirring. Solvent was

161 removed under vacuum, and the residue was chromatographed on LH-20 using

162 methanol as an eluent and was subsequently chromatographed on silica gel using

163 chloroform-methanol (4/1, v/v) as an eluent. The yield was 80.3 mg (22.3%). ^{1}H NMR

164 for CHexDL-G1S ($CDCl_3$, 400 MHz): δ (ppm) 0.88 (m, $CH_3(CH_2)_{15}$-), 1.26 (s,

165 $CH_3(CH_2)_{15}$-), 1.42 (m, -CH_2CH_2N-), 1.4-1.9 (m, -COCH-(CH_2)$_4$-CHCOOH), 2.32 (m, -

166 $CH_2CONHCH_2CH_2NH_2$), 2.36 (m, -CH_2CONH-), 2.42 (m, -CH_2N-), 2.50 (t, -

167 $CONHCH_2CH_2$-), 2.6-2.8 (m, -COCH-CH_2), 2.67 (t, -CH_2CH_2CONH-), 2.74 (t, -

168 $CH_2CH_2CONHCH_2CH_2NH_2$), 2.83 (t, -CH_2NH_2), 3.22 (m, -CONHCH_2-), 3.29 (m, -

169 $CH_2CH_2NH_2$). Calc $[M]^+$ ($C_{67}H_{121}N_7O_9$) m/z 1172.0. Found ESI-MS $[M]^+$ m/z 1170.8.

170 For the synthesis of CHexDL-G2S, DL-G2S (594 mg, 0.45 mmol) dissolved in

171 DMF (5 mL) was mixed with 1,2-cyclohexanedicarboxylic anhydride (8.13 g, 46.2

mmol) dissolved in DMF (5 mL) and triethylamine (1.0 mL, 13.6 mmol). The mixed solution was kept at 50°C under argon atomosphere for 38 h with stirring. Solvent was removed under vacuum, and the residue was chromatographed on LH-20 using methanol as an eluent and was subsequently chromatographed on silica gel using chloroform-methanol (4/1, v/v) as an eluent. The yield was 204 mg (23.5%). ^{1}H NMR for CHexDL-G2S ($CDCl_3$, 400 MHz): δ (ppm) 0.88 (m, C*H*$_3$(CH$_2$)$_{15}$-), 1.26 (s, CH$_3$(C*H*$_2$)$_{15}$-), 1.42 (m, -C*H*$_2$CH$_2$N-), 1.4-1.9 (m, -COCH-(C*H*$_2$)$_4$-CHCOOH), 2.32 (m, -C*H*$_2$CONHCH$_2$CH$_2$NH$_2$), 2.36 (m, -C*H*$_2$CONH-), 2.42 (m, -C*H*$_2$N-), 2.50 (m, -CONHCH$_2$C*H*$_2$-), 2.6-2.8 (m, -COC*H*-CH$_2$), 2.67 (m, -C*H*$_2$CH$_2$CONH-), 2.74 (m, -C*H*$_2$CH$_2$CONHCH$_2$CH$_2$NH$_2$), 2.83 (m, -C*H*$_2$NH$_2$), 3.22 (m, -CONHC*H*$_2$-), 3.29 (m, -C*H*$_2$CH$_2$NH$_2$).

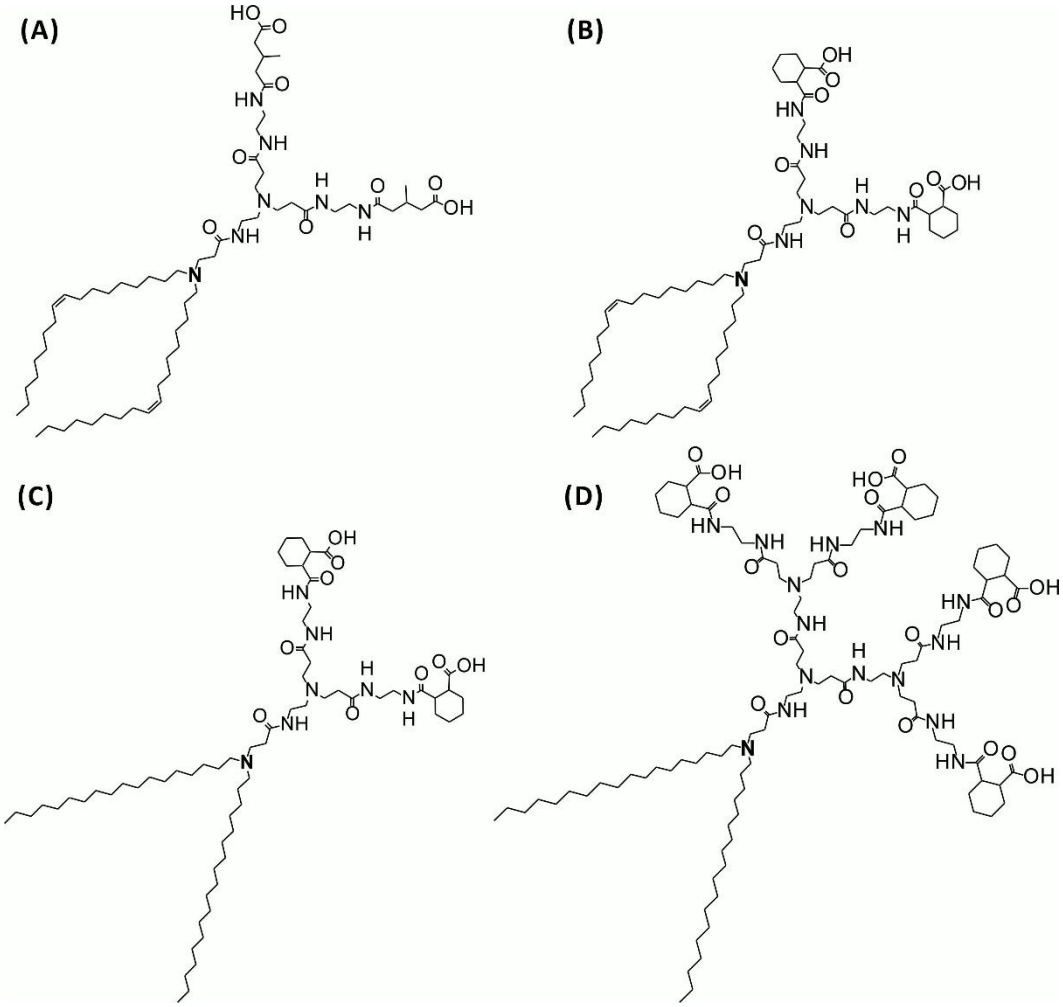


Figure 2. Chemical structures of carboxylated dendron-bearing lipids: (A) MGluDL-G1U, (B) CHexDL-G1U, (C) CHexDL-G1S, and (D) CHexDL-G2S.

2.3. Surface pressure measurement

Surface pressures were measured with a tensiometer USI-3-22 (USI System, Fukuoka, Japan). A small aliquot (4 μL) of dendron lipid dissolved in chloroform was spred onto 10 mM phosphate and 150 mM NaCl aqueous solution at a given pH (100 ×

191 400 mm^2), which was used as the subphase, in the trough. The monolayer was
192 compressed at a rate of 20 mm/sec after the initial delay period of 20 min for
193 evaporation of organic solvent. The subphase temperature was controlled to be 37°C.

194 2.4. Preparation of carboxylated dendron lipid-containing liposomes

195 A dry, thin membrane composed of a mixture of EYPC and dendron lipid (10
196 mol% or 25 mol%) was dispersed in phosphate-buffered saline (PBS) containing OVA
197 (4 mg/mL) by a brief sonication at room temperature, and the liposome suspension was
198 further hydrated by freezing and thawing and was extruded through a polycarbonate
199 membrane with a pore size of 100 nm at room temperature. The liposome suspension
200 was purified with Sepharose 4B column using PBS as an eluent at 4°C.

201 2.5. Characterization of liposomes

202 The concentrations of lipid and OVA in liposome suspension were measured using
203 phospholipid C test-Wako and Coomassie Protein Assay Reagent, respectively.
204 Diameters in PBS and zeta potentials in 0.1 mM phosphate aqueous solution of
205 dendron lipid assemblies or liposomes (0.1 mM of lipid concentration) were measured
206 using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Data were
207 obtained as an average of more than three measurements on different samples.

208 Pyranine-loaded liposomes were prepared as described above except that mixture of
209 EYPC and dendron lipid was dispersed in aqueous 35 mM pyranine, 50 mM DPX, and
210 25 mM phosphate solution. Liposomes encapsulating pyranine (lipid concentration: 2.0
211 $\times 10^{-5}$ M) were added to PBS of varying pH at 37ºC and fluorescence intensity at 512
212 nm of the mixed suspension was followed with excitation at 416 nm using a
213 spectrofluorometer (Jasco FP-6500 or FP-6200). The percent release of pyranine from
214 liposomes was defied as:

215 $$\text{Release}(\%) = (F_\text{t} - F_\text{i})/(F_\text{f} - F_\text{i}) \times 100$$

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

219 A given amounts of lipid-containing PBS was sealed in Ag pan. Differential
220 scanning calorimetry (DSC) measurements were performed with a DSC 120
221 microcalorimeter (Seiko Electronics). The heating rate was 1.0°C/min.

222 2.6. Confocal laser scanning microscopy

223 Rh-PE-labeled liposomes containing calcein were prepared as described above
224 except that a mixture of EYPC and dendron lipid containing Rh-PE (0.1 mol%) was
225 dispersed in 63 mM calcein solution. HeLa cells (1.0×10^5 cells) or DC2.4 cells

226 (murine dendritic cell line, 3.0×10^5 cells) cultured for 2 days in a glass-bottom dish
227 were washed twice with PBS and then incubated in a medium with 10% FBS (1.0 mL).
228 Rh-PE-labeled liposomes (1.0 mM lipid concentration, 1.0 mL) were added gently to
229 the cells and incubated for 1, 4 or 24 h at 37°C. After incubation, the cells were washed
230 with PBS three times and observed using confocal laser scanning microscopy LSM5
231 EXCITER (Carl Zeiss). For staining of nucleus, Hoechst33342 (Thermo) was used
232 according to the manufacture's instruction. For selective staining of early endosomes,
233 late endosomes or lysosomes, CellLight™ (Thermo) was used according to the
234 manufacture's instruction.

235 2.7. Cellular association of liposomes

236 Rh-PE-labeled liposomes containing FITC-OVA were prepared as described above
237 except that a mixture of EYPC and dendron lipid containing Rh-PE (0.1 mol%) was
238 dispersed in PBS containing FITC-OVA (4 mg/mL). DC2.4 cells (3.0×10^5 cells)
239 cultured for 2 days in a glass bottom dish were washed twice with PBS and then
240 incubated in the culture medium (1.0 mL). Liposomes (1 mM lipid concentration, 1.0
241 mL) were added gently to the cells and incubated for 4 h at 37°C. After incubation, the
242 cells were washed with PBS three times. Fluorescence intensity of these cells was
243 determined by a flow cytometric analysis (CytoFlex, Beckman Coulter, Inc.).

2.8. Cytokine production from dendritic cell line

DC2.4 cells (3×10^5 cells) cultured for 2 days in a six-well plate were washed with PBS twice and then incubated in the culture medium. OVA-loaded liposomes containing MPLA (4 g/mol lipids) (final lipid concentration: 0.5 mM) were added gently to the cells, followed by incubation for 4 h at 37°C. After incubation, the cells were washed with PBS three times and were additionally cultured for 20 h at 37°C. After incubation, supernatants were collected, and cytokine (IL-6 and TNF-α) production was measured using an enzyme-linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd.) according to the manufacturer's instruction.

2.9. Mice

Seven-week-old female C57BL/6 mice (H-2^b) were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Animal experiments using OVA were approved by the Institutional Animal Experimentation Committee in Osaka Prefecture University (Approval No. 24-2) and were performed in compliance with the institutional guidelines of animal care and use. Nine-week-old BALB/c mice or CBF1 mice (H-$2^{b/d}$) were bred in SPF facility of Kochi University. Animal experiments using antigenic peptides were approved by the Institutional Animal Experimentation Committee in Kochi University

(Approval No. H-00127) and were performed in compliance with the institutional guidelines of animal care and use.

2.10. Detection of antigen-specific antibodies in sera

On days 0 and 7, 50 μg of OVA-loaded liposomes containing MPLA (4 g/mol lipids) were subcutaneously injected into the back of the mice under anesthesia with isoflurane. After 7 days from final immunization, sera were collected and OVA-specific antibody in sera was detected by ELISA.

2.11. Induction of antitumor immunity

E.G7-OVA cells, OVA-expressing T-lymphoma, (1.0×10^6 cells/mouse) were subcutaneously inoculated into the left back of C57BL/6 mice under anesthesia with isoflurane. On days 5 and 12, 50 μg of OVA-loaded liposomes containing MPLA (4 g/mol lipids) were subcutaneously injected into the right back of the mice under anesthesia with isoflurane. Tumor sizes were monitored from the day of tumor inoculation. Mice immunized with PBS were used as a control to confirm the development of tumors following the first inoculation of E.G7-OVA cells. Mice were sacrificed when tumor volumes became over 2,000 mm^3.

2.12. *In vivo* induction of peptide-specific Th cells

An I-A^d-binding ovalbumin peptide PtOVII (PSVHAAHAEINEAGRPβA, 327-339) bearing peptidase-resistant flanking sequences (to be published elsewhere), or a tumor peptide from mouse WT1 tumor antigen sW336 (KLSHLQMHSRK, 336-346) were synthesized manually by Fmoc chemistry and purified by reverse phase HPLC to the purity of > 95%. Peptide-loaded liposomes were prepared as described above except that a mixture of EYPC, CHexDL-G1U and MPLA was dispersed in PBS containing peptides (1 mg/mL). A group of three mice, BALB/c for PtOVII and CBF1 (BALB/c x C57BL/6, H-$2^{b/d}$) for sW336 were immunized three times for 20 nmol PtOVII or four times for 50 nmol sW336 every week by footpad injection. For comparison, 100 μL of the 1:1 mixture of 50 nmol peptide and Freund's complete adjuvant (FA) was used for priming of sW336 followed by boost immunizations with the peptide in Freund's incomplete adjuvant containing pertussis whole cell vaccine[27]. Seven days after final immunization, splenocytes were harvested from the mice and were cultured for 4 days in the presence of corresponding antigenic peptide. Peptide-specific cell proliferation in splenocytes was detected using ^{3}H thymidine uptake.

2.13. Statistical analysis

Statistically significant differences between experimental groups were determined using Prism software (v8, GraphPad). Where one-way ANOVA followed by Tukey's

296 HSD post hoc test was used, variance between groups was found to be similar by
297 Brown-Forsythe test. Log-rank test was employed for analysis of survival of mice (Fig.
298 8b). The symbols *, **, *** and **** indicate *P* values less than 0.05, 0.01, 0.001 and
299 0.0001, respectively.
300

301 **3. Results and Discussion**

302 **3.1. Synthesis and characterization of carboxylated dendron-bearing lipids**

303 Carboxylated units were introduced to dendron lipids via reaction of primary
304 amines of dendron lipid and acid anhydrides (Scheme S1). Syntheses of dendron lipids
305 were confirmed by the presence of carboxylated unit-derived proton peaks at 1.03 ppm
306 for MGlu unit and 1.2–2.0 ppm for CHex unit in ^{1}H NMR charts and by mass
307 spectrometric analysis, as reported in the *Materials and Experiments* section.

308 Assembling structures of carboxylated dendron lipids were investigated by
309 measuring the π-A isotherm of each dendron lipid monolayer at various degrees of pH
310 (Fig. 3). Surface pressures of all dendron lipid monolayers increased along with the
311 decrease of the occupied area of lipids (Fig. 3A–D). To elucidate the behavior of
312 dendron lipids at liposomal membrane, the occupied area of dendron lipids at surface

pressure 25 mN/m, which corresponds to typical surface pressure of EYPC liposomal membrane[28], was found to be a function of subphase pH (Fig. 3E and 3F). CHexDL-G1S and CHexDL-G1U showed a higher occupied area than that of MGluDL-G1U (Fig. 3E), indicating that CHex units possess bulkier exclusion volume than that of MGlu units. Results show that CHexDL-G2S exhibited a markedly greater occupied area than CHexDL-G1S by the increase of dendron generation (Fig. 3F). In addition, the occupied area increased concomitantly with the decrease of subphase pH (Fig. 3E and 3F). After protonation of carboxy groups at low pH, these carboxylated units are expected to participate in the air–water interface, as shown in Fig. 3G. Furthermore, protonation of tertiary amines of dendron moiety induced electrostatic repulsion among dendron lipid molecules, thereby increasing the occupied area.

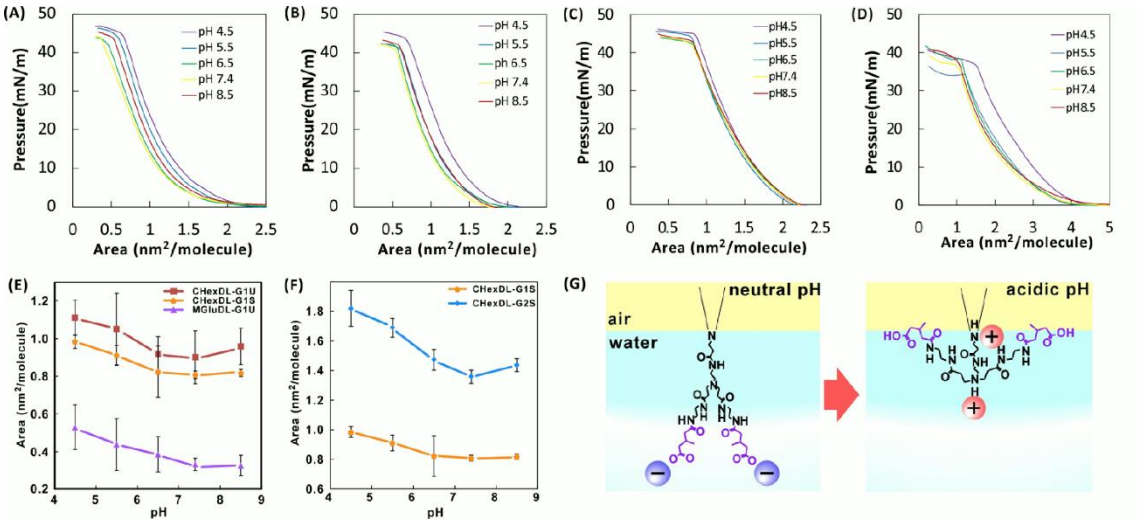


Figure 3. π-A isotherms of (A) MGluDL-G1U, (B) CHexDL-G1U, (C) CHexDL-G1S, and (D) CHexDL-G2S monolayers at various pH and 37 °C. (E and F) pH-Dependence of the molecular surface area for various dendron lipids at surface pressure of 25 mN/m at 37 °C. (G) Plausible molecular behaviors of dendron lipids at the air–water interface.

In our earlier report, dendron lipid without carboxylated units (DL-G1S) takes a vesicle structure at alkaline and neutral pH, whereas the vesicle structure was collapsed into a micelle-like structure responding to acidic pH[24]. Such a change in the assembling structure is understood according to the change in the apparent hydrophilic and hydrophobic balance of the lipid molecule after protonation of primary and tertiary amines of dendron moiety. For this study, primary amines of dendron lipid are converted to carboxylated units, which are expected to change the pH-sensitivity of dendron lipid considerably. Indeed, CHexDL-G2S suspension at pH 7.4 was transparent, but it became turbid at pH 5.5 and became transparent again at pH 3.0 (Fig. S1A). Figures S1B and S1C respectively depict the diameters and size distribution of dendron lipid suspensions at various pH. The MGluDL-G1U suspension exhibited an almost identical diameter change profile to that of the CHexDL-G2S suspension: small size at alkaline pH, aggregation at pH 6.0–4.0, and a marked decrease in diameter at

343 pH<4. In contrast, CHexDL-G1U and CHexDL-G1S showed a small size at alkaline pH
344 and aggregation in the low pH region. Both MGluDL-G1U and CHexDL-G2S might
345 form aggregates after protonation of the carboxy group, but these aggregates might be
346 re-dispersed after protonation of tertiary amines in dendron moiety at lower pH. Also,
347 CHexDL-G1S and CHexDL-G1U formed aggregates after protonation of carboxy
348 groups, but the strong hydrophobic interaction between cyclohexyl units might interfere
349 with the redispersion of the aggregates, even after protonation of tertiary amines at low
350 pH.
351
352 **3.2. pH-sensitive behaviors of dendron lipid-containing liposomes**
353 Next, carboxylated dendron lipid-containing liposomes were prepared for pH-
354 sensitizing of conventional liposome carriers. Pyranine and its quencher (DPX) were
355 encapsulated into these liposomes to evaluate the pH-responsive content release
356 behavior. Pyranine fluorescence after disruption of liposomes was almost identical for
357 all liposomes (Table S1), suggesting that the encapsulation amounts of pyranine was
358 same between samples. Liposomes without carboxylated dendron lipids (EYPC)
359 showed negligible content release at any pH, indicating that EYPC liposome has no pH-
360 sensitivity (Fig. S2). In contrast, carboxylated dendron lipid-containing liposomes

retained pyranine at alkaline pH and physiological pH, although these liposomes induced content release at a lower pH condition (Fig. S2). The content release behavior varied with carboxylated units, alkyl chain structure, dendron generation, and dendron lipid contents. To compare these differences, release% at 15 min was used as shown in Fig. 4. Figure 4A depicts the pH-dependence of pyranine release for 25 mol% dendron lipid-containing liposomes. All dendron lipid-containing liposomes induced content release with pH decreasing. At lower pH, protonated carboxylated units might destabilize liposomal membrane via hydrophobic interaction, as reported earlier in the literature[19]. Compared with MGluDL-G1U, CHexDL-G1U liposomes induced significantly higher content release at weakly acidic pH, indicating that bulkier CHex units disrupted liposomal membranes more effectively than MGlu units did. The content release% of CHexDL-G1S liposomes was less than CHexDL-G1U liposomes. Incorporation of octadecyl groups of CHexDL-G1S might increase membrane stability compared with unsaturated oleyl groups of CHexDL-G1U, resulting in decreased content release at the same pH condition. According to results of DSC analysis of CHexDL-G1S assemblies and CHexDL-G1S-containing liposomes and comparison of ΔH (Fig. S3), CHexDL-G1S took a gel phase at 37 °C. Approx. 12% of CHexDL-G1S formed the domain in the EYPC liposomal membrane. We were unable to obtain 25

379 mol% CHexDL-G2S-containing liposomes. Instead, 10 mol% CHexDL-G2S-containing

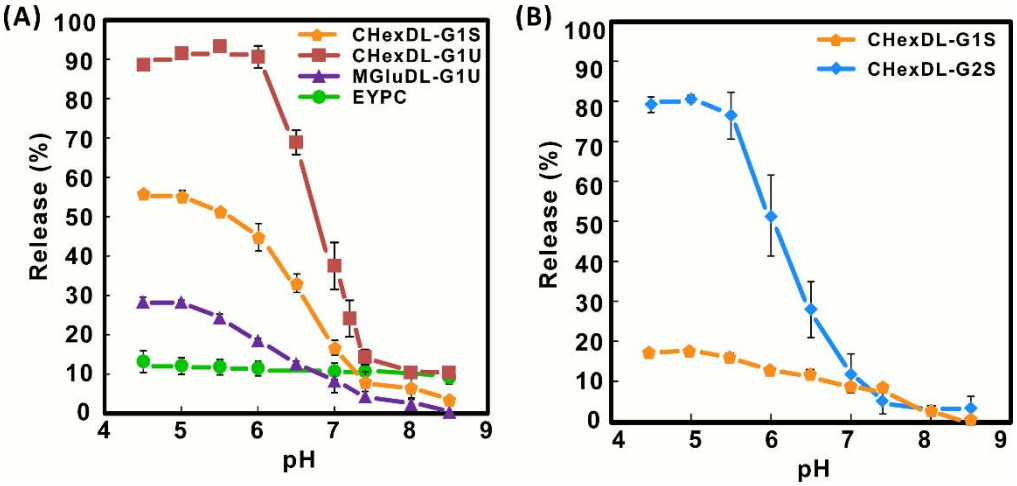
380 liposomes showed higher content release than 10 mol% CHexDL-G1S-containing

381 liposomes (Fig. 4B). CHexDL-G2S having 4 CHex units might destabilize the

382 liposomal membrane more effectively than CHexDL-G1S with 2 CHex units, whereas

383 high content (25 mol%) of CHexDL-G2S might destabilize lipid membrane, even in a

384 physiological condition.



385

386 **Figure 4.** pH-Dependence of pyranine release at 15 min from various dendron

387 lipid/EYPC liposomes in PBS at 37 °C. Lipid concentration was 2.0×10^{-5} M: (A)

388 Various G1 dendron lipid (25 mol%)-containing liposomes and (B) CHexDL-G1S and

389 CHexDL-G2S (10 mol%)-containing liposomes.

390

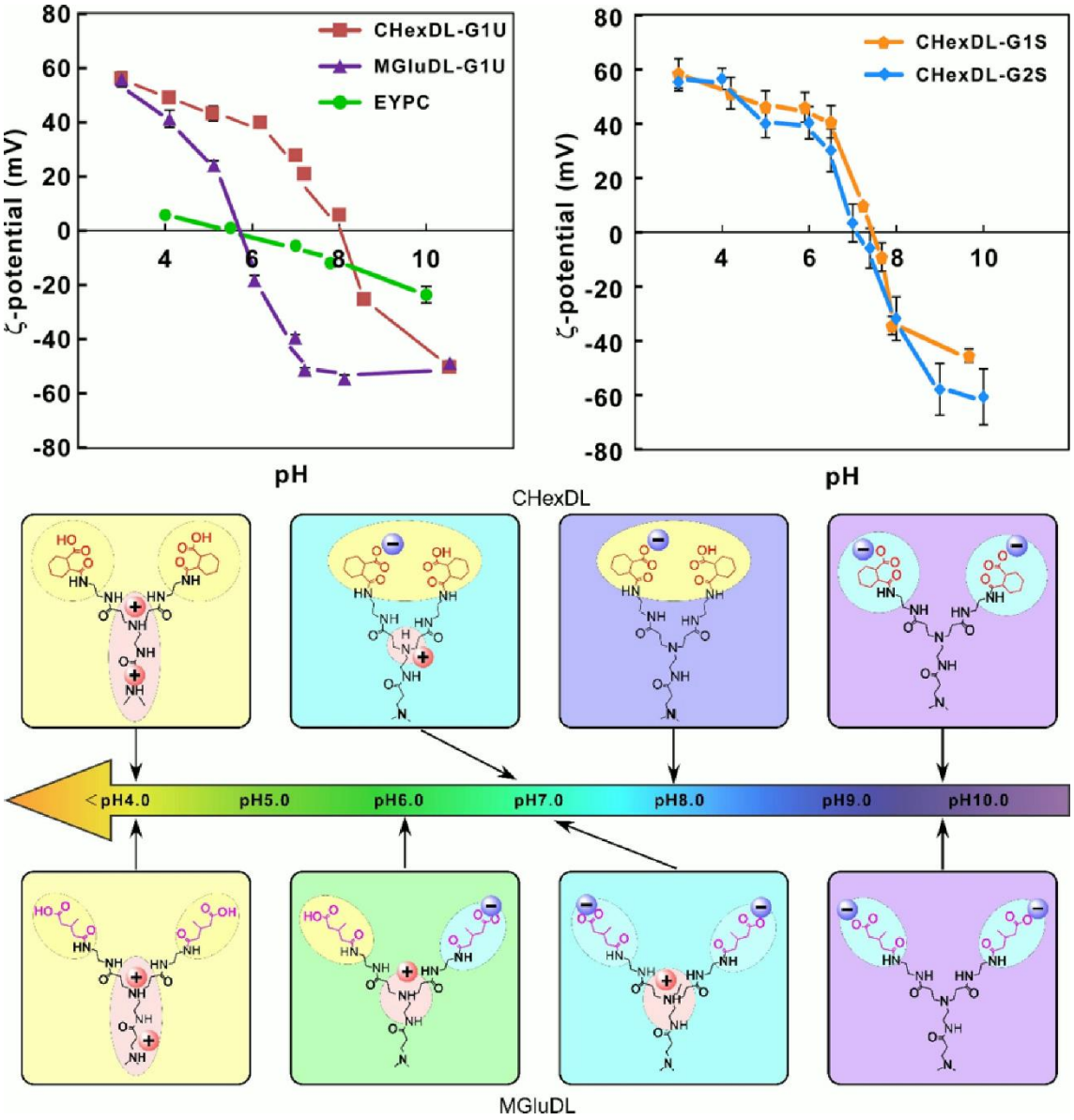
391 To elucidate the pH-sensitive content release behavior of carboxylated dendron

392 lipid-containing liposomes, diameters at various pH were investigated (Fig. S4). All

393 liposomes had around 100 nm size in the high pH region. However, MGluDL-G1U
394 liposomes and CHexDL-G2S liposomes exhibited a marked increase in size at the
395 weakly acidic pH region, whereas particle sizes of these liposomes became around 100
396 nm at even lower pH regions. These liposomes might be mutually associated at weakly
397 acidic pH via hydrophobic interaction between protonated MGlu units or CHex units,
398 although these aggregates are dissociated by electrostatic repulsion of protonated
399 tertiary amines at low acidic pH. By contrast, no change was observed for the diameter
400 of CHexDL-G1S and CHexDL-G1U liposomes at any pH (Fig. S4). These results
401 suggest that CHexDLs interacted with their own liposomal membranes rather than other
402 liposomes and induced content release from the liposomes.
403 Figure 5 shows the zeta potential of liposomes at various pH. Carboxylated dendron
404 lipid-containing liposomes possess negative zeta potential at the alkaline pH region.
405 With decreasing pH, the zeta potential of these liposomes changed from negative to
406 positive charge. Results show that MGluDL-G1U liposomes possessed positive charge
407 at less than pH 6, whereas CHexDL-G1U, CHexDL-G1S and CHexDL-G2S-containing
408 liposomes possessed positive zeta potential below pH 8.0, 7.5 and 7.0, respectively.
409 Acid–base titration was performed to elucidate the protonation behavior of dendron
410 lipids (Fig. S5). From the titration curve of DL-G0.5U, which has only tertiary amines

411 to be protonated in dendron moiety, protonation of tertiary amines in dendron moiety
412 occurred below pH 7.0; it was promoted below pH 4.5. For MGluDL-G1U, the degree
413 of protonation at pH 7.0 was almost 50%, indicating that carboxy groups in MGlu units
414 were first protonated during pH 10–7.0; then tertiary amines were protonated. For
415 CHexDL-G1U and CHexDL-G1S, the protonation of CHexDL-G1U during pH 10–7.0
416 was promoted compared with CHexDL-G1S probably because of fluidic characteristic
417 of CHexDL-G1U assemblies. The degree of protonation at pH 6.0 was almost 25%,
418 indicating that one carboxy group in the CHex unit was protonated at pH 10–6.0. Then
419 tertiary amines were protonated (Fig. S5). These protonation behaviors of CHexDLs
420 remarkably differ from the change in zeta potential of CHexDL-G1U and CHexDL-
421 G1S-containing liposomes, for which zeta potentials became almost neutral at pH 7.5
422 and 8.0, respectively. The protonation of carboxy groups in CHexDL might be
423 promoted because CHexDL molecules are buried in lipid membrane having
424 hydrophobic parts near dendron moiety. Thus, accurate evaluation of ionic state of
425 dendron lipid molecules seems difficult only from titration curves because the titration
426 was performed as lipid assemblies, not as single lipid molecule. Considering the zeta
427 potential change, plausible protonation behaviors of CHexDL and MGluDL in
428 liposomes are presented in Fig. 5. From comparison between zeta potential change and

content release behaviors, the content release was induced when the zeta potential changed to a positive charge. Therefore, the content release is expected to be induced by destabilization of liposomal membrane via hydrophobic interaction of the protonated carboxylated units and by temporary pore formation on the lipid membrane via electrostatic repulsion between tertiary amines in the dendron moiety (Fig. 1). In addition, membrane fluidity derived from unsaturated allyl chains (CHexDL-G1U compared with CHexDL-G1S) and the bulkiness of head group (CHexDL-G2S compared with CHexDL-G1S as shown in Fig. 3F) might also affect the extents of pyranine leakage from these liposomes.



438

439 **Figure 5.** ζ-potentials of various dendron lipid/EYPC liposomes at various pH.

440 Comparison of MGluDL-G1U and CHexDL-G1U dendron lipid (25 mol%)-containing

441 liposomes is shown with a comparison of CHexDL-G1S and CHexDL-G2S dendron

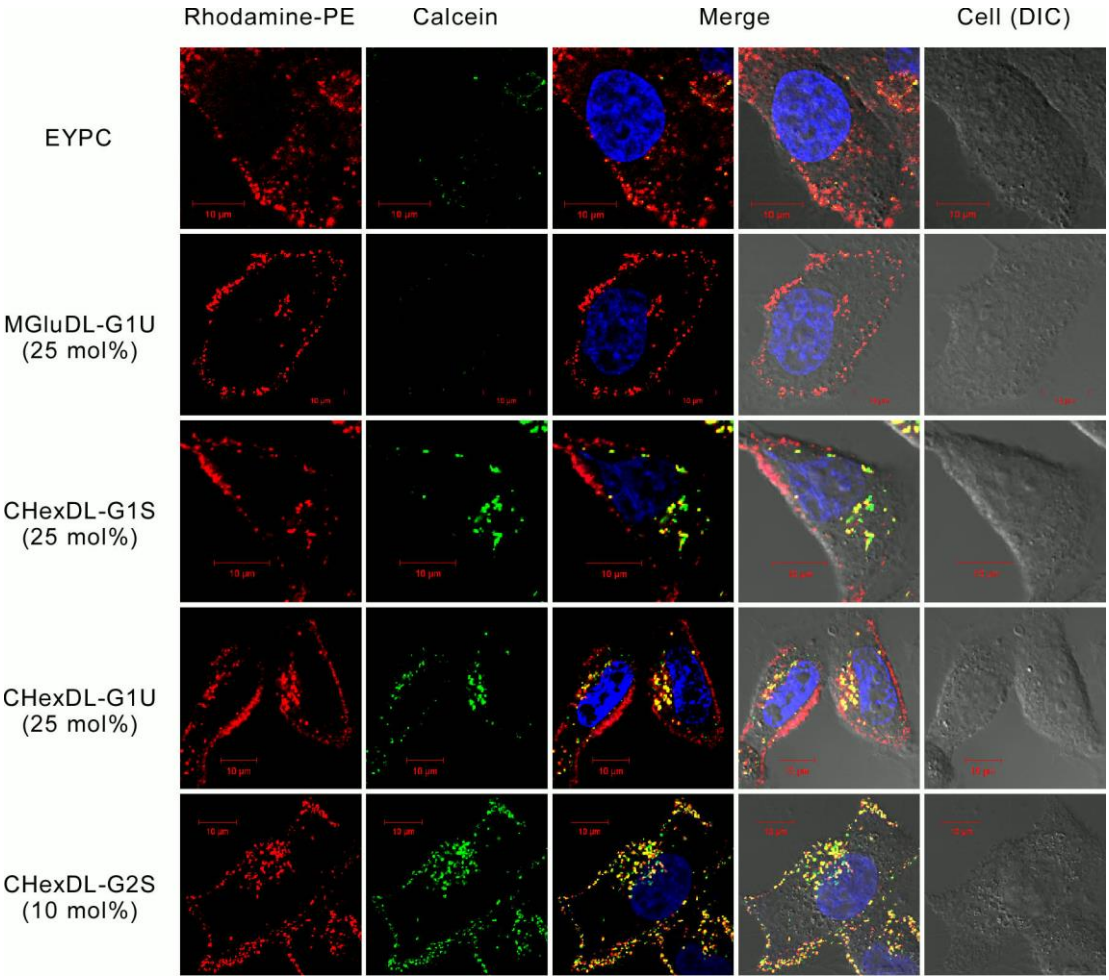
442 lipid (10 mol%)-containing liposomes, and proposed charged states of dendron lipids on

443 the liposome surface at various pH.

444

3.4. Intracellular distribution of dendron lipid-containing liposomes

Considering pH-responsive content release properties of carboxylated dendron lipid-containing liposomes, we used confocal laser scanning microscopy to investigate the intracellular distribution of liposomes. Rh-PE-labeled and almost same amount of calcein-loaded liposomes (Table S1) were incubated with HeLa cells for 4 h. Then lipid-derived fluorescence (Rh-PE) and released content-derived fluorescence (calcein fluorescence is quenched when encapsulated in the liposomes[29]) were observed (Fig. 6). For all liposomes, red fluorescence dots were observed from the cells, meaning that all liposomes were internalized to these cells via endocytosis. Slight fluorescence derived from calcein was detected from cells treated with EYPC liposomes and MGluDL-G1U-containing liposomes, suggesting that these liposomes did not induce calcein release under these experimental conditions. In contrast, calcein fluorescence dots were observed from cells treated with CHexDL-containing liposomes, indicating that calcein release was induced by these liposomes. It is particularly interesting that calcein release was observed near the nucleus in most cases.



460

461 **Figure 6.** Confocal laser scanning microscopic (CLSM) images of HeLa cells treated
462 with rhodamine-PE-labeled and calcein-loaded liposomes of various kinds for 4 h at 37
463 °C in the presence of serum. The cell nucleus was stained with Hoechst. Scale bar
464 represents 10 μm. Dendron lipid contents in the liposomes were 25 mol%, except for
465 CHexDL-G2S (10 mol%).

466 To ascertain the precise intracellular location of calcein release, early endosomes,
467 late endosomes and lysosomes were stained selectively (Fig. S6). For early endosome
468 staining, some calcein fluorescence by CHexDL-containing liposomes was colocalized
469 with early endosome-derived fluorescence. For late endosome/lysosome staining, most

calcein fluorescence was colocalized with these organelles. For Fig. S7, the observation time was changed to reveal the content-release dynamics. For CHexDL-containing liposomes, calcein fluorescence was overlapped with early endosome-derived fluorescence, even for 1 h incubation. Considering that CHexDL-containing liposomes showed immediate content release below pH 7.0 within 15 min (Fig. S2 and 4A), CHexDL-containing liposomes achieved content release for early endosomes immediately after internalization into the cells.

For EYPC and MGluDL-G1U liposomes, released calcein fluorescence dots were detectable after 24 h incubation and were colocalized with late endosome-derived or lysosome-derived fluorescence (Fig. S7). These results indicate that EYPC liposomes are degraded in late endosomes or lysosomes within 24 h and also indicate that MGluDL-G1U liposomes can induce content release at late endosomes or lysosomes by long-term incubation. Results show that intracellular content release behavior, timing, and sites can be changed by adjusting the pH-sensitivity of liposomes using different carboxylated units on dendron lipids.

3.5. Cellular association of dendron lipid-containing liposomes with immune cells

Precise control of the intracellular fate of cargo is expected to support a precision therapeutic approach. For induction of antigen-specific immunity, antigen-presenting cells such as dendritic cells, play a crucially important role[30]. Especially, the intracellular distribution of antigenic proteins or peptides strongly affects the induced immune responses[17, 18]. Antigen release into cytosol or at very weakly acidic

compartments such as early endosomes promotes MHC class I-mediated antigen presentation, resulting in the induction of cellular immune responses[17, 18]. In contrast, antigen released in lysosomes is carried onto MHC class II molecules to induce helper T cell (Th) responses, such as IgG antibody production or CTL activation. Considering the performance of carboxylated dendron lipid-containing liposomes, these liposomes were applied to antigen delivery to dendritic cells.

Figure 7A represents the cellular association of liposomes to the dendritic cell line. Modification of carboxylated dendron lipids into EYPC liposomes increased the cellular association 1.5–4 times. Figure S8 portrays the intracellular behavior of Rh-PE-labeled and calcein-loaded liposomes. Similarly to the case of HeLa cells, all liposomes were taken up by the cells, whereas remarkable calcein release in the cells was induced by CHexDL-containing liposomes. To evaluate intracellular antigen delivery, FITC-labeled OVA was encapsulated into the liposomes; also, cellular fluorescence was observed by CLSM (Fig. 7B). OVA encapsulation efficiency for each liposome was almost identical according to the analysis of protein and lipid concentrations for liposomes (Table S1). The FITC fluorescence is not quenched irrespective of encapsulation or release from the liposomes. For cells treated with FITC-OVA-loaded liposomes of all types, green fluorescence dots were observed and were colocalized with Rh-PE fluorescence. These results indicate that FITC-OVA molecules are located in endo/lysosomes, not in cytosol, even in cases of pH-responsive MGluDL-containing or CHexDL-containing liposomes, which is completely different from our earlier systems using MGlu or CHex unit-introduced polymer-based cytosolic delivery liposomes[21, 22, 31]. MGlu or CHex unit-introduced polymers interact with other membranes such as endosomes or lysosomes

via hydrophobic interaction after protonation of carboxy groups in MGlu or CHex units[21, 22, 31], whereas carboxylated DL-containing liposomes mainly interacted with their own liposomal membrane, as discussed above. These differences in intracellular delivery performance are expected to provide important information related to the molecular design of pH-sensitive materials for precise control of the intracellular fate of cargo.

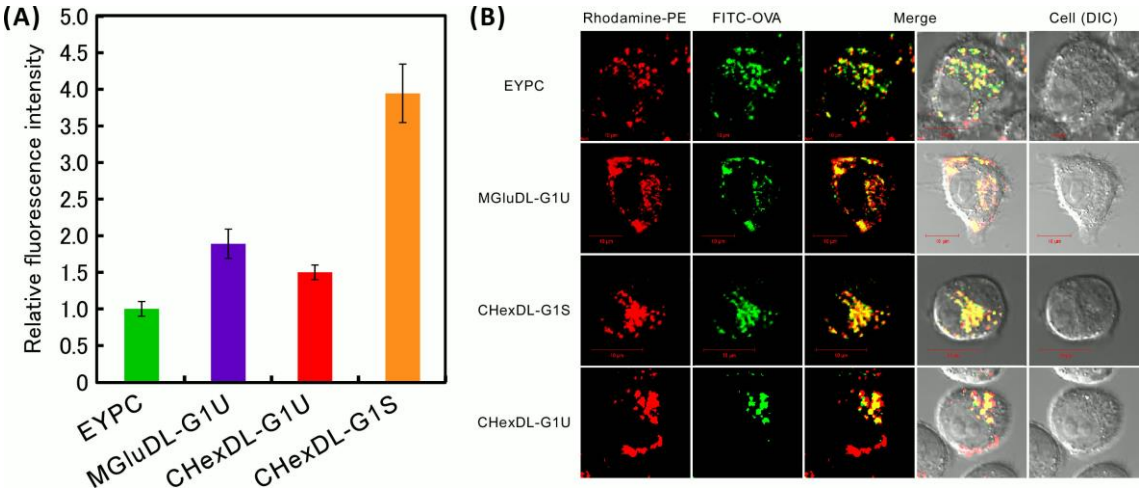


Figure 7. (A) Mean fluorescence intensity for DC2.4 cells treated with various dendron lipid-containing liposomes labeled with Rh-PE for 4 h at 37 °C in the presence of serum. (B) CLSM images of DC2.4 cells treated with Rh-PE-labeled and FITC-OVA-loaded liposomes of various kinds for 4 h at 37 °C in the presence of serum. The scale bar represents 10 μm.

3.6. Immunity-activating properties of dendron lipid-containing liposomes

529 Control of intracellular fate of antigen and maturation of dendritic cells are both
530 crucially important to induce antigen-specific immune responses. Therefore, MPLA
531 (TLR4 agonist) was incorporated into liposomes for providing adjuvant function. After
532 treatment of these liposomes, inflammatory cytokine production from dendritic cells
533 was promoted (Fig. S9A). CHexDL-containing liposomes induced slightly high
534 cytokine production, suggesting an additional adjuvant effect derived from CHex units,
535 as reported[21].

536 Because antigenic proteins were released in endo/lysosomes (Fig. 7A), the
537 induction of humoral immune responses in mice was evaluated. We twice
538 subcutaneously injected OVA-loaded liposomes into mice. After 7 days, OVA-specific
539 antibody in serum was detected using ELISA (Fig. S9B). No antibody was detected
540 from PBS-immunized mice, although OVA-specific IgG1 was produced in sera of mice
541 that had been immunized with OVA-loaded liposomes. CHexDL-G1U liposomes
542 induced slightly higher antibody production than other formulations, which might
543 reflect its additional adjuvant effect (Fig. S9A).

544

545 **3.7. Induction of anitumor effects by dendron lipid-containing liposomes**

546 To assess therapeutic effects of liposomes on immunotherapy, the prophylactic
547 effect of carboxylated dendron lipid-containing liposomes was evaluated (Fig. S10).
548 After immunization of PBS or OVA-loaded CHexDL-G1U liposomes, mice were
549 inoculated OVA-expressing tumor cells (E.G7-OVA cells). In the case of PBS-treated
550 mice, tumors started to grow 4 days after tumor inoculation. By contrast, no tumor
551 growth was observed from mice immunized with OVA-loaded CHexDL-G1U
552 liposomes. This finding suggests that OVA-loaded CHexDL-G1U liposomes can induce
553 not only humoral immunity (Fig. S9B) but also cellular immunity, which directly
554 attacks antigen-expressing tumor cells. Indeed, OVA-specific IFN-γ secretion from
555 splenocytes, which indicates the induction of Th1 cells and/or CTLs, was promoted by
556 pH-sensitive dendron lipid-containing liposomes (Fig. S11A). In addition, pH-sensitive
557 dendron lipid-containing liposomes induced higher OVA-specific CTLs in spleen than
558 EYPC liposomes did (Fig. S11B). Furthermore, many CD8-positive cells (CTLs) were
559 observed in the tumor tissues from the mice immunized with pH-sensitive dendron
560 lipid-containing liposomes (Fig. S11C). These results apparently indicate that pH-
561 sensitive dendron-bearing lipid-containing liposomes could induce strong OVA-specific
562 cellular immune responses via cross-presentation.

563 Encouraged by these results, therapeutic effects of OVA-loaded liposomes on
564 tumor-bearing mice were examined (Fig. 8). E.G7-OVA cells were inoculated to the
565 mice. Then, OVA-loaded liposomes, OVA solution or PBS was injected subcutaneously
566 at 5 and 12 days after tumor inoculation. Figure 8A shows that OVA solution exhibited
567 no tumor suppressive effect. The OVA-loaded EYPC liposomes induced some degree
568 of tumor suppression. Whereas, MGluDL-G1U- or CHexDL-G1U-containing liposomes
569 showed regression of tumor volume until day 20. Figure 8B shows that CHexDL-G1U-
570 containing liposomes induced a remarkably strong antitumor effect compared to that of
571 CHexDL-G1S-containing liposomes. Whereas tumor reoccurrence took place in some
572 mice after Day 20 (Fig. 8A) or 30 (Fig. 8B) even in CHexDL-G1U-containing
573 liposome-treated mice. Tumor reoccurrence might be induced after diminishing of CTL
574 activity but its occurring rate and timing varied in each mouse. Furthermore, mice
575 survival results are presented in Fig. 8C. All carboxylated dendron lipid-containing
576 liposomes extended mice survival significantly compared with PBS, OVA solution, and
577 EYPC liposomes. Especially, CHexDL-G1U-containing liposomes significantly
578 prolonged mice survival compared to other liposomes: 40% of mice were completely
579 cured. Although liposome uptake performance of MGluDL-G1U- and CHexDL-G1U-
580 containing liposomes was identical (Fig. 7A), CHexDL-G1U-containing liposomes

showed the highest content-release behavior (Fig. 4A), content release in early endosomes (Fig. S6–S8 and 7B), and additional adjuvant effect (Fig. S9A). Such highly intracellular delivery performance and adjuvant function can be expected to induce cross-presentation of OVA via vacuolar pathway to induce OVA-specific cellular immunity and CTL infiltration into tumor tissues (Fig. S11), thereby producing strong antitumor effects on tumor-bearing mice.

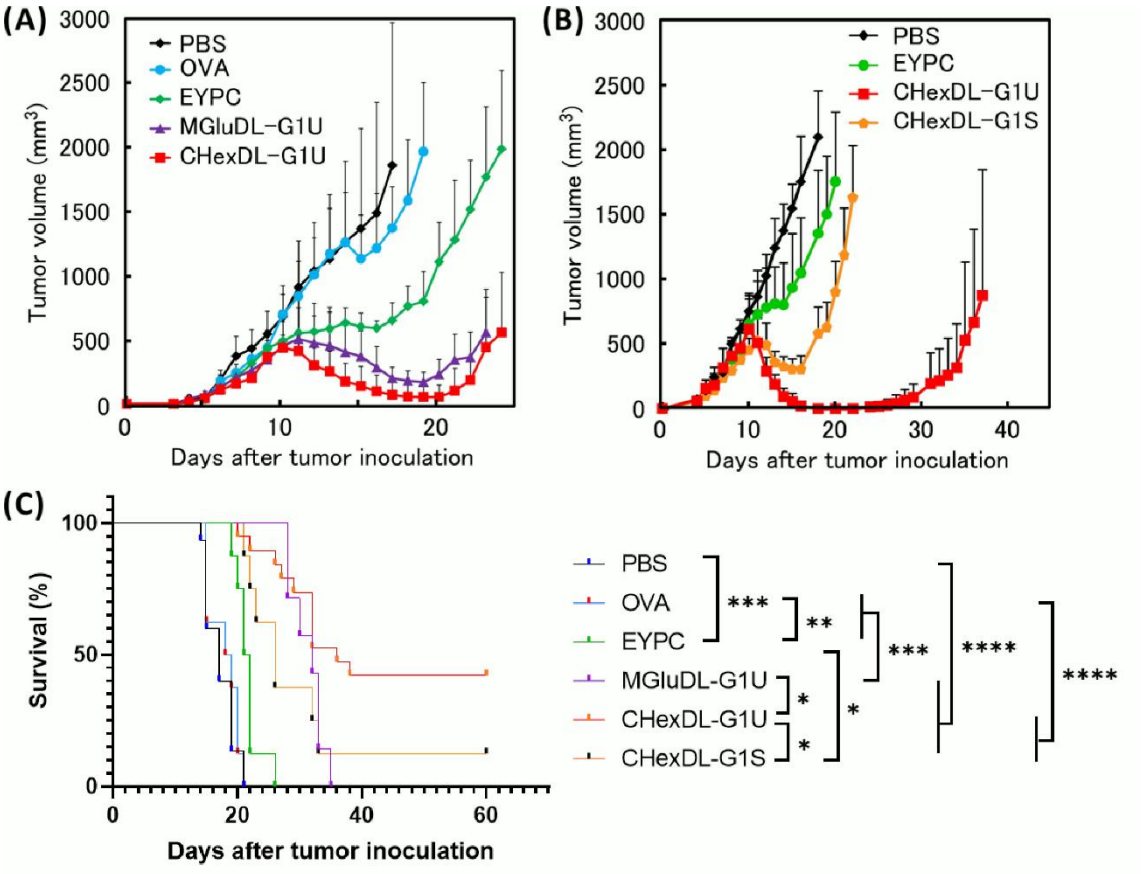


Figure 8. Antitumor effects induced by subcutaneous administration of OVA-loaded liposomes with or without various dendron lipids (25 mol%). E.G7-OVA cells were

590 inoculated subcutaneously into the back of C57BL/6 mice. Then tumor volume (A and
591 B) and survival of mice (C) were monitored. Mice were immunized with various
592 liposomes loaded with OVA (50 μg) or OVA solution twice at day 5 and day 12. PBS-
593 treated mice were used as a control. *P <0.05, ** P <0.01, *** P <0.001, and ****P
594 <0.0001.
595
596 **3.8. Induction of natural tumor antigen-specific Th cells by dendron lipid-**
597 **containing liposomes**
598 Carboxylated dendron lipid-containing liposomes were applied further to antigenic
599 peptide delivery. Compared with whole protein, peptide can be synthesized chemically
600 that excludes the problems of biological contaminants in producing drugs. In addition,
601 peptide sequence can be designed freely and are cost-efficient. First, OVA class II
602 epitope-containing peptide (PtOVII) was encapsulated into CHexDL-G1U-containing
603 liposomes. After immunization to mice, peptide-specific proliferation of helper T
604 lymphocytes in splenocytes was evaluated (Fig. 9A). Compared with PBS, peptide
605 alone, peptide-loaded CHexDL-G1U-containing liposomes induced significant cell
606 proliferation in splenocytes from low peptide concentration in *in vitro* culture, which
607 indicates that CHexDL-G1U-containing liposomes can promote induction of OVA-

608 specific Th subsets. Although OVA II peptides might be released at early endosomes as
609 a result of the pH-sensitivity of CHexDL-G1U-containing liposomes (Fig. S6 and S7),
610 OVA II peptide cannot bind to MHC class I molecules in early endosomes. After
611 transfer into late endosomes/lysosomes, OVA II peptide might bind to MHC II
612 molecules and be presented to CD4+ T cells.

613 Finally, WT-1 tumor antigenic peptide with wild type sequence was used to
614 evaluate the potential of CHexDL-G1U-containing liposomes to induce aggressive T
615 cell responses sufficient for clinical use. Tumor antigens are regarded as self thus are
616 under immune tolerance. Therefore, the induction of natural tumor antigen-specific
617 immune responses is quite challenging. WT1-derived helper T cell epitope peptide
618 (sW336) was incorporated into CHexDL-G1U-containing liposomes. After
619 immunization, peptide-specific T cell proliferation was observed (Fig. 9B).
620 Immunization of sW336 peptide emulsified in Freund's complete adjuvant as clinically-
621 used formulation, however, induced minimal proliferation. Surprisingly, CHexDL-
622 G1U-containing liposomes induced significantly high proliferation in an antigen-
623 specific manner. These results suggest that CHexDL-G1U-containing liposome is
624 applicable not only for immunization of highly antigenic foreign peptide (OVA
625 peptide), but also for natural tumor antigens of clinical use.

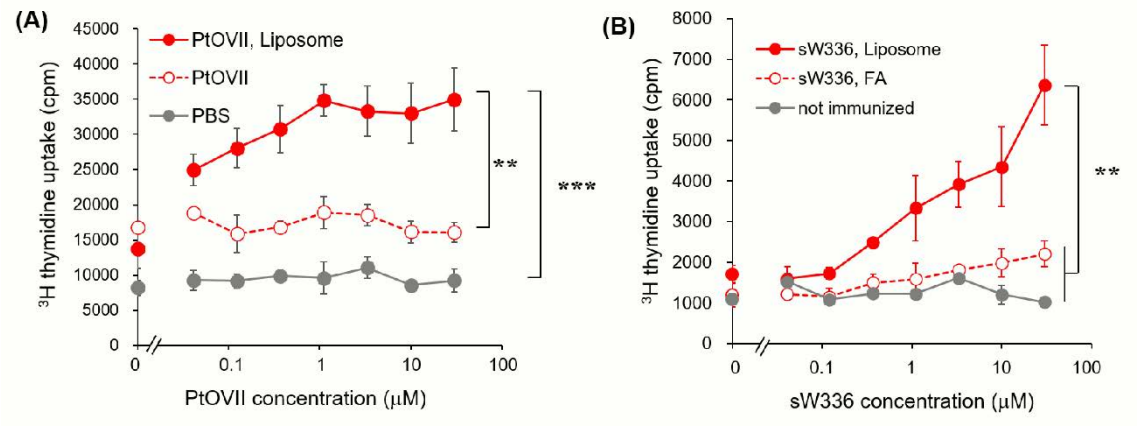


Figure 9. Proliferation of peptide-specific Th cells in splenocytes from mice immunized with (A) PtOVII peptide- or (B) WT1-derived peptide-loaded CHexDL-G1U-containing liposomes. (A) BALB/c mice were immunized in footpads with PtOVII (30 nmol)-loaded CHexDL-G1U-containing liposomes three times at one week intervals. (B) CBF1 mice were immunized in footpads with WT1-derived peptide (50 nmol)-loaded CHexDL-G1U-containing liposomes or peptide/Freund's complete adjuvant (FA) emulsion four times at one-week intervals. Seven days after the last immunization, splenocytes were harvested from the mice. Proliferation of peptide-specific Th cells was detected by ^{3}H thymidine uptake. Statistical analyses were done using analysis of variance (ANOVA) with Tukey's test. $^{**}P < 0.01$; $^{***}P < 0.001$.

4. Conclusion

For this study, carboxylated unit-introduced polyamidoamine dendron lipids were synthesized for precise control of intracellular distribution of the cargo. Carboxylated dendron lipid-containing liposomes induced pH-responsive content release in the very weakly acidic pH region and released their contents at early endosomes or late endosomes/lysosomes. Carboxylated dendron lipid-containing liposomes with early endosome release property achieved induction of antigen-specific antibody production, induction of antigen-specific CTLs, prophylactic effect of tumor cells, regression of solid tumors, and induction of WT1 peptide-specific Th responses. Results demonstrate that carboxylated dendron lipid-containing liposomes are promising as antigen delivery carriers for induction of antigen-specific cancer immunity and for establishment of peptide vaccine-based cancer immunotherapy.

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