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Dextran Derivative-Based pH-Sensitive Liposomes for Cancer Immunotherapy

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1. Introduction

Development of intracellular delivery systems is crucial for the establishment of advanced medicines such as gene therapy and cancer immunotherapy. For that reason, many attempts have been made to produce carriers that deliver their contents into cytosol. To date, various carrier systems have been used for cytoplasmic delivery of these bioactive molecules [1–5]. Among them, pH-sensitive liposomes are regarded as a promising system used as an intracellular delivery vehicle because they can protect contents entrapped in their interior from the outer environment until their uptake by a cell. Moreover, they can deliver contents into cytosol through destabilization and fusion with endosomes and lysosomes with acidic internal environments.

Conventionally, pH-sensitive liposomes have been prepared through the combination of nonbilayer-forming phospholipids and pH-sensitive amphiphiles [6,7]. These liposomes control lamellar-to-hexagonal II transition of the liposomal membrane in a pH-dependent fashion. This approach might meet with some difficulty in achieving somewhat conflicting properties, which are high stability and strong fusion ability, because the pH-sensitive fusogenic property of these liposomes originates from a strong tendency to take on a non-bilayer structure [6,7]. Therefore, the preparation of pH-sensitive liposomes has also been attempted based on other designs.

The surface modification of stable liposomes with pH-sensitive polymers is an efficient method for the production of pH-sensitive liposomes [8,9]. Therefore, pH-sensitive liposomes have been prepared using poly(carboxylic acid)s of various kinds obtained by polymerization of monomers having carboxyl groups such as poly(2-ethyl acrylic acid), poly(2-propyl acrylic acid), and methacrylic acid copolymers [8, 9]. We have prepared pH-sensitive polymers according to another strategy: incorporation of pH-sensitive moieties into polymers that have pH-insensitive but valuable properties for biological use. For instance, we have incorporated succinyl groups to side groups of poly(glycidol), which has a backbone structure resembling that of fusogenic polymer poly(ethylene glycol), by reacting succinic anhydride with poly(glycidol) [10]. Indeed, we showed that surface modification with succinylated poly(glycidol) provided pH-sensitive fusogenic property to stable egg yolk phosphatidyl choline (EYPC) liposomes. We observed that, after internalization into a cell via endocytosis, the succinylated poly(glycidol)-modified liposomes delivered contents into cytosol by fusion with endosome [11]. Similarly, by reaction with various carboxylic acid anhydrides such as 3-methyl glutarylic anhydride, we prepared poly(glycidol) derivatives having pH-sensitive moieties with varying hydrophobicities. Their abilities for pH-sensitization of liposomes increase concomitantly with increasing hydrophobicity of the pH-sensitive

moieties [12]. Furthermore, these pH-sensitive moieties were introduced into poly(glycidol)s with hyperbranched structures. The pH-sensitive poly(glycidol) derivatives with hyperbranched structures exhibited even higher ability for pH-sensitization of liposomes than those with a linear structure [13, 14].

In a previous study, we investigated the feasibility of these poly(glycidol) derivative-based pH-sensitive liposomes as antigen carriers which activate antigen-specific cellular immunity because these liposomes can deliver antigens into cytosol of dendritic cells (DCs), which play an important role in the antigen presentation and activation of antigen-specific immune responses [15–17]. After uptake of antigens *via* endocytosis, DCs are known to degrade them in lysosome and present them on major histocompatibility complex (MHC) class II molecules, leading to induction of humoral immunity. However, when antigens are delivered into cytosol of DCs, after degradation in proteasomes, antigens are expected to be presented by MHC class I molecules, which engender the induction of antigen-specific cytotoxic T leukocytes (CTLs), which attack the target cells directly and which eliminate them effectively. In fact, we found that these pH-sensitive poly(glycidol) derivative-based liposomes loaded with antigenic protein delivered OVA into DCs' cytosol and induced antigen-specific cellular immunity [18].

From the viewpoint of clinical application, the use of pH-sensitive polymers with biologically safe and biodegradable properties is strongly desired for the preparation of pH-sensitive liposomes. Candidates for such polymers might be naturally occurring polysaccharides [19]. In fact, many kinds of polysaccharide-based drug delivery systems have been developed for clinical applications [20,21]. Therefore, in this study, we applied our strategy for preparation of pH-sensitive polymers to polysaccharides and attempted to develop a new type of pH-sensitive polymers based on polysaccharides. pH-Sensitive moieties were incorporated to polysaccharide dextrans by reaction with 3-methyl glutaric anhydride. The resultant pH-sensitive dextran derivatives (MGlu-attached dextran: MGlu-Dex) were used for preparation of pH-sensitive liposomes. The ability of the dextran derivatives for pH-sensitization of liposomes and performance of the dextran derivative-modified liposomes as antigen delivery carriers for induction of antigen-specific immunity (Figure 1) have been investigated.

2. Materials and Methods

2.1. Materials

EYPC and L-dioleoyl phosphatidylethanolamine (DOPE) were kindly donated by NOF Co. (Tokyo, Japan). Lissamine rhodamine B-sulfonyl phosphatidylethanolamine

(Rh-PE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). 3-Methylglutaric anhydride, OVA, monophosphoryl lipid A (MPLA) and fluorescein isothiocyanate (FITC), Dextran having molecular weight of 40,000 (Dex_{40k}), Dextran having molecular weight of 450,000~650,000 (Dex_{500k}) were purchased from Sigma (St. Louis, MO.). Dextran having molecular weight of 70,000 (Dex_{70k}), 1-aminodecane, pyranine and Triton X-100 were obtained from Tokyo Chemical Industries Ltd. (Tokyo, Japan). 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). *p*-Xylene-bis-pyridinium bromide (DPX) was from Molecular Probes (Oregon, USA). FITC-OVA was prepared by reacting OVA (10 mg) with FITC (11.8 mg) in 0.5 M $NaHCO_3$ (4 mL, pH 9.0) at 4 °C for three days and subsequent dialysis [19].

2.2. Synthesis of Dextran derivatives

3-Methyl-glutarylated dextran (MGlu-Dex) was prepared by reaction of dextran with 3-methylglutaric anhydride. For $MGlu_{56}$-Dex, dextran (509 mg, 9.4 mmol of OH groups) and LiCl (511 mg) were dissolved in *N*,*N*-dimethylformamide (10 mL) and 3.0 equiv. of 3-methylglutaric anhydride (3.58 g, 27.9 mmol) was added to the solution. The mixed solution was kept at 120 °C for 24 h with stirring under argon atmosphere. Then, the reaction mixture was evaporated and dialyzed against water for 3 days. The

product was recovered by freeze-drying. Other MGlu-Dex polymers were also synthesized by reaction of dextran with various amounts of 3-methylglutaric anhydride by the same procedure. ^{1}H NMR for MGlu-Dex（400 MHz, D_2O+NaOD）: δ 0.9 (s, -CO-CH_2-CH(C*H*$_3$)-CH_2-), 1,9 – 2.3 (br, -CO-C*H*$_2$-C*H*(CH_3)-C*H*$_2$-), 3.5 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 5.0 (br, glucose 1*H*).

As anchor moieties for fixation of MGlu-Dex onto liposome membranes, 1-aminodecane was combined with carboxyl groups of MGlu-Dex. Each polymer was dissolved in water around pH 7.4, and 1-aminodecane (0.1 equiv. to hydroxyl group of polymer) was reacted to carboxyl groups of the polymer using DMT-MM (0.1 equiv. to hydroxyl group of polymer) at room temperature for 5 h with stirring. The obtained polymers were purified by dialysis in water. The ratios of MGlu units to decyl amide units for MGlu-Dex polymers were estimated using ^{1}H NMR. ^{1}H NMR for MGlu-Dex-C_{10}（400 MHz, D_2O+NaOD）: δ 0.8 - 0.9 (br, -CO-NH-CH_2-(CH_2)$_8$-C*H*$_3$), 0.9 (s, -CO-CH_2-CH(C*H*$_3$)-CH_2-), 1.2 – 1.4 (br, -CO-NH-CH_2-(C*H*$_2$)$_8$-CH_3), 1.9 – 2.3 (br, -CO-C*H*$_2$-C*H*(CH_3)-C*H*$_2$-), 3.2 (br, -CO-NH-C*H*$_2$-(CH_2)$_8$-CH_3), 3.5 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 5.0 (br, glucose 1*H*).

2.3. Titration

To 10 mL of an aqueous solution of each polymer (Carboxylate concentration: 3.0

$\times 10^{-4}$ M) was added an appropriate amount of 0.1 M NaOH solution to make pH 10.0. The titration was carried out by the stepwise addition of 0.1 M HCl and pH of the resultant solution was measured using an automated titration instrument (AUT-701, DKK-TOA Corporation, Tokyo, Japan).

2.4. Precipitation pH

Precipitation pHs of polymers were determined by measuring the optical density of aqueous polymer solutions (0.25 mg/mL) at various pH. Polymers were dissolved in 1.0 mL of acetate buffer (30 mM sodium acetate, 120 mM NaCl) adjusted to various pH. After 5 min incubation at 25 °C, optical densities (OD) of the polymer solutions at 500 nm were measured by using a spectrophotometer (Jasco V-560). Precipitation pH was determined by optical density-pH profile as the pH at which OD drastically rose.

2.5. Pyrene fluorescence

A given amount of pyrene in acetone solution was added to an empty flask, and acetone was removed under vacuum. Polymer (0.25 mg/mL) dissolving in 30 mM sodium acetate and 120 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.

2.6. 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 RPMI1640 supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine, 100 mM nonessential amino acid, 50 μM 2-mercaptoethanol (2-ME) and antibiotics at 37 °C [22]. EL4, a C57BL/6 mice-derived T lymphoma, was obtained from Tohoku University (Sendai, Japan). E.G7-OVA, which is a chicken egg OVA gene-transfected clone of EL4 and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) [23].

2.7. Animals

Female C57BL/6 mice (H-2^b, 7 weeks old) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The experiments were carried out in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

2.8. Preparation of Liposomes

To a dry, thin membrane of EYPC (10 mg) was added 1.0 mL of OVA/PBS solution (pH 7.4, 4 mg/mL), and the mixture was vortexed at 4 °C. The liposome suspension was further hydrated by freezing and thawing, and was extruded through a

polycarbonate membrane with a pore size of 100 nm. The liposome suspension was applied to a sepharose 4B column to remove free OVA from the OVA-loaded liposomes. Polymer-modified liposomes were also prepared according to the above procedure using dry membrane of a lipid mixture with polymers (lipids/polymer = 7/3, w/w). For induction of immune responses, MPLA (4 g/mol lipids) was introduced into liposomal membrane.

2.9. Dynamic light scattering and zeta potential

Diameters and zeta potentials of the liposomes were measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd, Worcestershire, UK). Data was obtained as an average of more than three measurements on different samples.

2.10. Release of Pyranine from Liposome

Pyranine-loaded liposomes were prepared as described above except that mixtures of polymers and EYPC were dispersed in aqueous 35 mM pyranine, 50 mM DPX, and 25 mM phosphate solution (pH 7.4). Release of pyranine from liposome was measured as previously reported [12, 13, 24]. Liposomes encapsulating pyranine (lipid concentration: 2.0×10^{-5} M) were added to PBS of varying pHs at 37 °C and fluorescence intensity (512 nm) of the mixed suspension was followed with excitation at 416 nm using a spectrofluorometer (Jasco FP-6500). The percent release of pyranine

from liposomes was defined as

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%).

2.11. Intracellular behavior of liposomes

The FITC-OVA-loaded liposomes containing Rh-PE were prepared as described above except that a mixture of polymer and lipid containing Rh-PE (0.6 mol%) was dispersed in phosphate-buffered saline containing FITC-OVA (4 mg/mL). DC2.4 cells (3×10^5 cells) cultured 2 days in 35-mm glass-bottom dishes were washed with Hank's balanced salt solution (HBSS), 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 by a flow cytometric analysis.

2.12. Antibody titration

E.G7-OVA cells (1×10^6 cells) were subcutaneously inoculated into the left backs

of C57BL/6 mice under anesthesia. On days 7 and 14, 100 μg of OVA-loaded liposomes were subcutaneously injected into the right backs of the mice under anesthesia. On day 26, whole blood was collected by heart puncture under deep anesthesia with isoflurane. OVA (0.5 μg/50 μl) diluted with PBS was coated onto microplates (Maxisorp, Nunc) at 4 °C overnight. The plates were washed three times in PBS containing 0.05% Tween 20 (PBS-T) and blocked by 1% bovine serum albumin (BSA) solution at 37 °C for 2 h. After washing three times with PBS–T, serial two-fold dilutions of sera were performed and then the plates were incubated at 4 °C overnight. The plates were washed again and reacted with a 5000-fold dilution of horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (American Qualex), IgG1 and IgG2b antibody (Zymed Laboratories). Following incubation for 2 h at 37 °C, the detection of antigen-antibody complexes was performed by using coloring kit for peroxidase (sumilon). The plates were washed and 100 μl of substrate solution was added. The plates were allowed to stand for 10 min at room temperature, and 100 μl of 2 M sulfuric acid were added to stop the reaction. The optical density of each well was read at 490 nm on a microplate reader (Wallac1420, Perkin Elmer). ELISA titers were determined according to the previous report [25].

2.13. *In vitro* cytokine release measured by enzyme-immunosorbent assay (ELISA)

On days 7 and 14, 100 μg of OVA-loaded liposomes were subcutaneously injected

into the right backs of the mice under anesthesia. On day 19, mice were sacrificed and splenocytes were suspended in RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-ME, and 20 U/mL recombinant murine IL-2 (Peprotech, London, UK). Splenocytes (2×10^6 in 2 mL) were incubated with mitomycin C-treated E.G7-OVA cells at a ratio of 10:1 for 5 days. After incubation, the concentration of IFN-γ was measured using murine IFN-γ ELISA development kit (Peprotech, London, UK) as follows. Microplates (Maxisorp, Nunc) were coated with affinity-purified rabbit anti-mouse IFN-γ (100 μl, 1 μg/ml) in PBS, incubated overnight at room temperature, and washed 4 times with PBS-T. The plates were blocked with 1% BSA in PBS for 1 h at room temperature and washed 4 times with PBS-T. Supernatant of splenocyte culture (100 μl) and standard recombinant murine IFN-γ diluted in PBS containing 0.05% Tween 20 and 0.1% BSA (Diluent) was added to the wells, and the plates were incubated for 2 h at room temperature and then washed 4 times with PBS-T. A biotin-labelled rabbit anti-mouse IFN-γ (100 μl, 0.25 μg/ml) was added to the wells, after which the plates were incubated for 2 h at room temperature and then washed 4 times with PBS-T. HRP-conjugated avidin (100 μl, 1/2000 diluted with Diluent) was added to the wells, and the plates were incubated for 30 min at room temperature and then washed 4 times with PBS-T. Finally, colorimetric signals were generated using ABTS liquid

substrate solution (Sigma). After 30 min, the reaction was stopped with 1% SDS and the absorbance at 405 nm and 595 nm was measured using a a microplate reader (Wallac1420, Perkin Elmer). The concentration of IFN-γ in the samples was interpolated on the basis of standard curves.

2.14. CTL assay

E.G7-OVA cells (1×10^6 cells) were subcutaneously inoculated into the left backs of C57BL/6 mice under anesthesia. On day 7, 100 μg of OVA-loaded liposomes were subcutaneously injected into the right backs of the mice under anesthesia. On day 14, splenocytes were suspended in RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-ME, and 20 U/mL recombinant murine IL-2 (Peprotech, London, UK). Splenocytes were then stimulated with mitomycin C-treated E.G7-OVA cells at a ratio of 10:1 for 5 days. The stimulated splenocytes were used as effector cells for the cytotoxicity assay. The CTL activity was evaluated at various ratios of effector cells to target cells (E.G7-OVA or EL4 cell), which were defined as E/T ratios, using a lactate dehydrogenase (LDH) cytotoxicity detection assay (Takara Biomedicals, Tokyo, Japan).

2.15. Treatment of tumor-bearing mice with liposomes

E.G7-OVA cells (1×10^6 cells) were subcutaneously inoculated into the left backs

of C57BL/6 mice under anesthesia. On days 7, when the tumor volumes were about 500 mm^3, and day 14, 100 μg of OVA-loaded liposomes were subcutaneously injected into the right backs of the mice under anesthesia. Tumor sizes were monitored from the day of inoculation. Mice immunized with PBS were used as controls to confirm the development of cancer following the first inoculation with E.G7-OVA cells. Mice were sacrificed when tumor volumes become over 2,500 mm^3. All treated groups contained four mice.

3. Results and Discussion

3.1. Characterization of dextran derivatives

Several kinds of pH-sensitive dextran derivatives (MGlu-Dex) with different contents of MGlu residues and different backbone lengths of dextran were prepared by reacting dextrans with molecular weights of 40,000, 70,000, and 450,000–650,000, which are designated respectively as Dex_{40k}, Dex_{70k}, and Dex_{500k}, with various amounts of 3-methylglutaric anhydride. Decyl groups were further introduced to these polymers by reaction of decylamine with carboxyl groups of MGlu units for fixation of dextran derivatives onto liposome membrane (Scheme 1). The obtained dextran derivatives were characterized using ^1H NMR. Figure 2 depicts ^1H NMR spectra of Dex_{70k},

$MGlu_{76}$-Dex_{70k}, and $MGlu_{56}$-Dex_{70}-C_{10}. In comparison of spectra for Dex_{70k}, which was used as a starting material, (Fig. 2A) and for $MGlu_{76}$-Dex_{70k}, (Fig. 2B), which was the product, introduction of MGlu residues to dextran was confirmed from the existence of new peaks corresponding to MGlu residues (0.9 ppm, 2–2.3 ppm) in Fig. 2B. From the integration ratio of peaks of MGlu residues to those of dextran backbone (3.5–4.0 ppm), 76% of hydroxyl groups of dextran were estimated as combined with MGlu residues. Similarly, from the integration ratio between dextran backbone, MGlu residues, and decyl groups (0.9–1.5 ppm), decyl-amidated MGlu residues and MGlu residues were found to be combined to 7% and 56% of hydroxyl groups of Dex_{70k} respectively in the product obtained using the reaction of $MGlu_{76}$-Dex_{70k} and decylamine, which is designated as $MGlu_{56}$-Dex_{70k}-C_{10}. Compositions of dextran derivatives prepared in this study are presented in Table 1.

Acid–base titration of MGlu-Dex was done (Fig. 3). In general, MGlu-Dex changed their ionized states depending on pH in neutral and weakly acidic regions. However, the pH region in which protonation of the polymer was enhanced tends to shift toward neutral pH with increasing MGlu residue contents when comparing titration curves for $MGlu_{13}$-Dex_{70k}, $MGlu_{48}$-Dex_{70k}, and $MGlu_{76}$-Dex_{70k}. In contrast, $MGlu_{76}$-Dex_{70k}, $MGlu_{66}$-Dex_{40k} and $MGlu_{72}$-Dex_{500k}, which are with similar MGlu-residue

contents but with very different molecular weights, showed ionization state changes in similar pH regions, suggesting that the chain length of dextran backbone only slightly affects the protonation behavior of MGlu-Dex. These results indicate that the density of carboxyl groups on the polymer controls the ionization characteristics of the MGlu-Dex. The apparent pK_a for these MGlu-Dex were determined from their titration curves. They are listed in Table 2.

These dextran derivatives were soluble in water at neutral pH; their solutions were transparent. However, when pH was decreased, these solutions suddenly became turbid at a specific pH, where the polymer loses its water solubility and changes its character from hydrophilic to hydrophobic. This pH was defined as the *precipitation pH*. We estimated the pH-sensitive property of the dextran derivatives from their precipitation behavior induced in acidic environments. The precipitation pH values of the dextran derivatives were determined by measuring the optical densities of their solutions with decreasing pH, as listed in Table 2. $MGlu_{13}$-Dex_{70k} showed no precipitation throughout the experimental pH region, probably because this polymer has a hydrophilic nature even after all MGlu units are protonated in the polymer chain. However, other polymers exhibited precipitation in the acidic region. Their precipitation pH tends to increase concomitantly with increasing MGlu unit% of MGlu-Dex,

indicating that polymers with higher content of MGlu-Dex lose their water solubility and exhibit hydrophobic characters at higher pH. In a previous study, we observed that precipitation pH for carboxylated poly(glycidol) derivatives of various kinds increased concomitantly with increasing hydrophobicity [12,13,26]. In general, degrees of protonation for these dextran derivatives was around 0.92–0.98 at the precipitation pH, indicating that most carboxyl groups must be protonated to elicit precipitation of the polymers.

Hydrophobicity of the polymers was further investigated using a fluorescence probe: pyrene. The 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 [27]. Consequently, this ratio has been widely used to estimate the hydrophobic nature of polymers [28,29]. Figure 4 depicts the I_1/I_3 ratio of pyrene fluorescence in the buffer dissolving various polymers as a function of pH. In buffers dissolving $MGlu_{13}$-Dex_{70k} and $MGlu_{35}$-Dex_{70k}, the I_1/I_3 ratios of pyrene were around 1.8 at pH 4, suggesting that these polymers formed few domains with a hydrophobic nature, even after protonation of carboxyl groups of the polymer chain. In contrast, a significant decrease in the I_1/I_3 ratio was seen in the presence of $MGlu_{76}$-Dex_{70k} or $MGlu_{85}$-Dex_{70k} under weakly acidic conditions. These results suggest that

MGlu-Dex with higher MGlu unit% formed more hydrophobic domains, which is consistent with their precipitation pH (Table 2).

3.2. Preparation of dextran derivative-modified liposomes

We prepared liposomes by dispersing mixed membranes of EYPC and the anchor-attached dextran derivatives in the buffer and extruding the suspension through a polycarbonate membrane with a pore size of 100 nm. Then the liposomes were purified using gel permeation chromatography. Obtained liposomes were characterized from average diameters and zeta potentials (Table 3). These liposomes exhibited similar diameters of around 100–150 nm, which corresponds roughly to the pore size of the filter membrane used for extrusion of these liposomes. Compared with the zeta potentials of the unmodified liposomes, the liposomes modified with dextran derivatives showed highly negative zeta potentials of around -50 mV, which indicates that the these polymers provided a negatively charged surface to the liposomes.

We examined pH-responsive properties of the liposomes by measuring their content release behaviors using the water-soluble dye molecule pyranine as the liposomal content [24] (Fig. 5). Figure 5A shows the time courses of pyranine release from liposomes modified with based dextran (70k)-based derivatives and unmodified liposomes at pH 7.4 and 5.0. The unmodified liposomes tightly retained their contents at

both levels of pH. Similarly, the polymer-modified liposomes retained their contents tightly at neutral pH. However, at pH 5.0, a large fraction of the contents was immediately released, indicating that the liposomes were destabilized strongly at acidic pH. The percentage release of the contents for these polymer-modified liposomes after 10 min-incubation is portrayed in Fig. 5B as a function of pH. $MGlu_{26}$-Dex_{70k}-modified liposomes enhanced content release below pH 5.5, although enhancement was observed below pH 6.5 for $MGlu_{56}$-Dex_{70k}-modified and $MGlu_{77}$-Dex_{70k}-modified liposomes. Because p*K*a and hydrophobicity of the MGlu-Dex tends to increase concomitantly with increasing MGlu residues on the polymer chains (Table 2 and Figure 4), the MGlu-Dex with higher MGlu residue contents become protonated at higher pH and acquired a hydrophobic character, which enables strong interaction with the liposome membranes. This result indicates that MGlu residue content of the derivatives affects the pH region where the content release is triggered for the polymer-modified liposomes.

We also compared pH-dependent content release behaviors among the liposomes modified with the derivatives of different molecular weights of dextrans (Figs. 5C, 5D). Apparently, these liposomes exhibited similar enhancement of content release around pH 6. Complete release was achieved below pH 5, irrespective of the molecular weights of the dextran chains. These results indicate that the ability of the derivatives to provide

pH-responsive properties of the liposomes is determined by MGlu unit content on the dextran backbone, but is not influenced by the chain length of the polymer backbone.

3.3. Liposome-mediated OVA delivery to DCs

Next, we examined the capability of these dextran derivative-modified liposomes to deliver OVA, which was used as a model antigen, into DCs. We used liposomes labeled with Rh-PE and containing FITC-OVA, respectively, for detection of liposomes and OVA molecules inside cells. DC2.4 cells were incubated with Rh-PE-labeled and FITC-OVA-loaded liposomes. Then the cells were observed using confocal laser scanning microscopy (Fig. 6). As presented in Fig. 6A, the cells treated with the unmodified liposomes showed weak and punctate fluorescence of Rh-PE and FITC-OVA. In addition, the merge image suggests that the fluorescence of Rh-PE and that of FITC-OVA are located mostly in the same positions in the cells, indicating that both OVA molecules and the liposomes were still trapped in endosomes and/or lysosomes. The cells treated with liposomes modified with MGlu-Dex also exhibited punctate fluorescence of Rh-PE but diffuse fluorescence of FITC-OVA was visible in the cells (Figs. 6B–6F), suggesting that OVA molecules were transferred into cytosol while membrane moiety of the liposomes were trapped in endosomes and lysosomes. Considering that significant destabilization of the polymer-modified liposomes was

induced under weakly acidic condition (Fig. 5), these liposomes might be destabilized strongly in the weakly acidic environment of endosomes, which might cause fusion with and destabilization of endosomal membrane. In fact, MGlu-Dex-modified liposomes were shown to generate fusion activity at weakly acidic pH (Fig. S1). Therefore, it is likely that, after internalization into the cell through endocytosis, MGlu-Dex-modified liposomes might be destabilized quickly and strongly upon exposure to weakly acidic environment of endosome interior and release OVA there. At the same time, association of the destabilized liposomes with endosomal membrane might cause destabilization of endosome membrane and their fusion. As a result, OVA molecules might be transferred efficiently from the endosome interior to the cytosol [12,13].

Comparison among the liposomes having polymers of different MGlu residue contents (Figs. 6B–6D) reveals that these liposomes similarly exhibited diffuse fluorescence of FITC-OVA inside cells, although FITC-OVA fluorescence in the nucleus is apparently somewhat weaker than that in cytosol. Probably, permeation of OVA through nuclear membrane is suppressed because of the large mass of OVA [30]. However, these results indicate that these liposomes are capable of delivering OVA into cytosol of DC. We further compared abilities for intracellular delivery of FITC-OVA among liposomes modified with those of polymers of different chain lengths (Figs. 6C,

6E, 6F). These liposomes were again observed to deliver FITC-OVA into cytosol space of DCs, irrespective of the polymer chain length. In addition, it should be noted that the cells treated with MGlu-Dex liposomes displayed not only diffuse fluorescence but also punctate fluorescence (Figs. 6B-6F). This fact indicates that not all of OVA molecules were transferred to the cytosol but a fraction of OVA molecules were retained in the endosomes.

Additionally, cellular association of liposomes was evaluated by measuring the Rh-PE fluorescence intensity of the liposome-treated cells using flow cytometry (Fig. 7). The MGlu-Dex -modified liposomes showed a higher degree of cellular association than unmodified liposomes did. An earlier report described that MGlu-attached poly(glycidol)-modified liposomes were taken up by DC2.4 cells effectively through specific binding with scavenger receptors, which recognize anionic polymers or lipids [13]. Similarly, these MGlu-Dex covering the liposomes might be recognized by scavenger receptors, resulting in higher association of the liposomes. When cellular association was compared among liposomes having $MGlu_{26}$-Dex_{70k}-C_{10}, $MGlu_{56}$-Dex_{70k}-C_{10}, and $MGlu_{77}$-Dex_{70k}-C_{10}, and unmodified liposomes (Fig. 7A), the liposomes having $MGlu_{56}$-Dex_{70k}-C_{10} exhibited much more efficient association to DCs than those without polymers or with $MGlu_{26}$-Dex_{70k}-C_{10}. This result underscores the importance of

MGlu-residues for efficient cellular association of the liposomes. However, the liposomes with $MGlu_{77}$-Dex_{70k}-C_{10} exhibited lower cellular association than $MGlu_{56}$-Dex_{70k}-C_{10} did. Probably, too many carboxylates on the polymers induce the electrostatic repulsion to negatively charged cellular surface and might be less recognized by scavenger receptors. Cellular association of the polymer-modified liposomes was also influenced to some degree by the chain length of the dextran backbone. Those with molecular weight of 70k or higher provided efficient cell association properties to liposomes (Fig. 7B). MGlu residues attached onto the long polymer chains might be recognized more efficiently by the cellular receptors.

3.4. Liposome-mediated induction of antigen-specific immunity *in vivo*

We next examined the ability of the MGlu-Dex-modified liposomes for induction of antigen-specific immune responses *in vivo*. OVA-expressing E.G7-OVA cells were inoculated to mice. Then OVA-loaded liposomes of various types were administered twice subcutaneously to mice. 12 days later, whole blood was collected from immunized mice. OVA-specific antibody titer in serum was first evaluated by ELISA (Fig. 8A). Compared to immunization with OVA solution, immunization with liposome-entrapped OVA provided more effective production of OVA-specific IgG. In addition, the use of MGlu-Dex-modified liposomes induced higher IgG titers. Those

having $MGlu_{56}$-Dex_{70k} –C_{10} showed the highest IgG titer, which exhibited highly efficient cellular association among MGlu-Dex-modified liposomes (Fig. 7).

We also measured IgG subclasses IgG1 and IgG2b titers, which are known to be derived respectively from Th2 cells and Th1 cells, and calculated IgG2b titer/IgG1 titer ratios to estimate the Th1/Th2 balance [31,32]. As Fig. 8B shows, administration of $MGlu_{56}$-Dex_{70k}-modified liposomes loaded with OVA induced a high IgG2b/IgG1 ratio compared to the cases of immunization with free OVA and OVA encapsulated in unmodified or $MGlu_{26}$-Dex_{70k}-modified liposomes. This result suggests that $MGlu_{56}$-Dex_{70k}-modified liposomes might be advantageous for the induction of efficient cellular immunity because such liposomes might induce cellular immunity through intracellular delivery of antigen and might simultaneously activate cellular immunity through Th1 cells.

We also examined the activation of OVA-specific immunity by measuring the IFN-γ secretion of splenocytes of immunized mice upon antigen-stimulation. Splenocytes were collected from mice that had been immunized with OVA-loaded liposomes or OVA solution twice. The splenocytes were then cultured with OVA for 5 days. Then, amounts of produced IFN-γ in culture medium were measured using ELISA (Fig. 9). Compared with the splenocytes of the free OVA- or OVA-loaded unmodified

liposome-immunized mice, those of mice treated with OVA-loaded MGlu-Dex liposomes exhibited higher production of IFN-γ responding to OVA. Especially, treatment with OVA-loaded $MGlu_{56}$-Dex_{70k}-C_{10}-modiifed liposomes generated splenocytes with the highest production of IFN-γ. Combined with the results of Fig. 8, $MGlu_{56}$-Dex_{70k}-liposomes induced generation of antigen-specific Th1 cells effectively. Th1 cells secrete IFN-γ in the presence of antigen. Therefore, Th1 cell-rich splenocytes might produce IFN-γ in the OVA-containing medium.

We further examined CTL induction upon immunization with OVA of free or liposome-encapsulated forms. Mice bearing E.G7-OVA tumor were immunized with OVA-loaded liposomes. After 7 days from immunization, splenocytes were collected and stimulated with mitomycin C-treated E.G7-OVA cells. Then their toxicity toward E.G7-OVA was measured and compared with their toxicity toward EL4 cells to estimate the induction of OVA-specific CTLs. Figure 10 depicts percent lysis for E.G7-OVA cells or EL4 cells (target cells) induced by the stimulated splenocytes (effector cells) at varying effector cell/target cell (E/T) ratios. Although the splenocytes obtained with the OVA-loaded unmodified liposomes exhibited low cytotoxicity to E.G7-OVA cells, the splenocytes obtained with OVA-loaded MGlu-Dex liposomes induced a much stronger level of CTL responses. These splenocytes only slightly exhibited cytotoxicity

against EL4 cells. This result indicates that these MGlu-Dex liposomes can induce antigen-specific CTLs efficiently. Despite a difference in antigen-specific Th1 cell induction ability between $MGlu_{26}$-Dex_{70k}-liposomes and $MGlu_{56}$-Dex_{70k}-liposomes (Figs. 8 and 9), the splenocytes derived from these liposome-immunized mice exhibited an almost identical level of target-specific CTL activities. For CTL assay, splenocytes collected the immunized mice 7 days after immunization. Indeed, induction of immunity is time-dependent. It is therefore possible that a difference of OVA administration methods affected the time-dependence of immune activation.

3.5. Therapeutic effect of liposome-mediated immunization

We examined the therapeutic effects of immunization with MGlu-Dex liposomes. Mice were inoculated with E.G7-OVA cells. On days 7 and 14 from the tumor cell inoculation, OVA-loaded liposomes of various types were administered subcutaneously to mice. Then tumor growth and the survival of mice were monitored (Fig. 11). First, we examined tumor-suppressive effects of the liposomes having MGlu-Dex with different MGlu contents. As Fig. 11A shows, immunization with the OVA solution and OVA-loaded unmodified liposomes only slightly exhibited a tumor suppressive effect. However, when mice were immunized with OVA-loaded $MGlu_{26}$-Dex_{70k}-C_{10}-modified liposomes and $MGlu_{56}$-Dex_{70k}-C_{10}-modified liposomes, tumor volume decreased

remarkably after 5–6 days from their administration, indicating that the OVA-specific immunity was effectively induced in mice. Mice survival was extended about 20 days by immunization with the OVA-loaded liposomes, compared to survival of mice treated with PBS (Fig. 11C). Comparison of tumor-suppressive effects between these MGlu-Dex-liposomes revealed that $MGlu_{56}$-Dex_{70k}-liposomes showed somewhat higher antitumor effect than that of $MGlu_{26}$-Dex_{70k}-liposomes. This result might have derived from the induction of stronger Th1 responses by the $MGlu_{56}$-Dex_{70k}-liposomes (Figs. 8 and 9).

We also compared the efficacy for tumor-suppression between the OVA-loaded liposomes having MGlu-Dex with different chain lengths (Figs. 11B and 11D). Despite a large difference in the polymer chain lengths, both $MGlu_{58}$-Dex_{40k}-C_{10}-modified liposomes and $MGlu_{55}$-Dex_{500k}-C_{10}-modified liposomes exhibited marked tumor regression. As described above, chain lengths of MGlu-Dex covering liposomes only slightly affect their abilities for cellular association (Fig. 7) and cytoplasmic delivery of antigen (Fig. 6). Results show that chain lengths of MGlu-Dex only slightly influence the tumor-suppressive effects of MGlu-Dex-modified liposomes.

In a previous study, we developed pH-sensitive liposomes for the induction of antitumor immunity using synthetic polymers, poly(glycidol)s [18]. In fact,

administration of these liposomes induced OVA-specific immune response efficiently and improved the survival of E.G7-OVA tumor-bearing mice [18]. Compared to these synthetic polymer-modified liposomes, dextran-based construction of pH-sensitive liposomes might be more preferred from a viewpoint of biodegradability and biological safety. Furthermore, MGlu-Dex liposomes exhibited comparable performance to poly(glycidol)-based pH-sensitive liposomes for the induction of tumor-suppressive immunity.

4. Conclusion

For this study, we developed pH-sensitive polymers by derivatizing naturally occurring polysaccharide dextran with MGlu residues. These MGlu-Dex changed their charged state in neutral and weakly acidic pH region and concomitantly changed their characteristics from hydrophilic to hydrophobic. Using surface modification with these MGlu-Dex with stable EYPC liposomes yielded pH-sensitive liposomes, which exhibited pH-sensitive significant destabilization in the weakly acidic pH region. Moreover, the MGlu-Dex-modified liposomes were taken up by DCs and delivered their contents efficiently into the cytosol. These MGlu-Dex-modified liposomes were found to be useful as an antigen delivery system for the induction of antigen-specific

immunity, which showed marked therapeutic effects on tumor-bearing mice. Therefore, we expect that these pH-sensitive polysaccharide derivative-modified liposomes can lead to production of safe and potent antigen delivery systems will be able to contribute to the establishment of efficient cancer immunotherapy.

Acknowledgments

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Figure captions

Figure 1. Design of MGlu-Dex-modified liposomes for induction of antigen-specific immunity. MGlu-Dex-modified liposome is taken up by DC via endocytosis and trapped in endosome. Its weakly acidic environment triggers destabilization of the liposome, which induces release of antigen molecules in endosome and their transfer to cytosol via fusion with endosome. Antigen molecules in cytosol cause antigen-specific cytotoxic T lymphocytes (CTL) via presentation by MHC class I, resulting in induction of cellular immunity. Antigen molecules in endosome induce antigen-specific Th1 and Th2 cells via presentation by MHC class II. Th1 cells activate antigen-specific cellular immunity, whereas Th2 cells lead to induction of antigen-specific humoral immunity.

Figure 2. ^{1}H NMR spectra of (A) Dex_{70K}, (B) $MGlu_{76}$-Dex_{70k}, and (C) $MGlu_{56}$-Dex_{70k}-C_{10} in D_2O/NaOD.

Figure 3. Acid-base titration curves for $MGlu_{13}$-Dex_{70k} (closed squares), $MGlu_{48}$-Dex_{70k} (closed diamonds), $MGlu_{76}$-Dex_{70k} (closed triangles), $MGlu_{66}$-Dex_{40k} (open triangles) and $MGlu_{72}$-Dex_{500k} (open circles).

Figure 4. pH-Dependence of I_1/I_3 of pyrene fluorescence in the absence (open triangles) or presence of $MGlu_{13}$-Dex_{70k} (closed triangles), $MGlu_{34}$-Dex_{70k} (closed circles), $MGlu_{76}$-Dex_{70k} (closed diamonds), and $MGlu_{85}$-Dex_{70k} (closed squares) dissolving in 30

mM sodium acetate and 120 mM NaCl solution of varying pHs. Concentration of polymers and pyrene were 0.25 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 5. pH-Sensitive contents release behaviors of dextran derivative-modified liposomes. Time courses (A) and pH-dependence (B) for pyranine release from liposomes modified with $MGlu_{26}$-Dex_{70k}-C_{10} (triangles), $MGlu_{56}$-Dex_{70k}-C_{10} (circles), $MGlu_{77}$-Dex_{70k}-C_{10} (diamonds), and unmodified (squares) liposomes. Open and closed symbols in (A) express at pH 7.4 and pH 5.0, respectively. Percent release after 30 min-incubation were shown in (B). Time courses (C) and pH-dependence (D) for pyranine release from liposomes modified with $MGlu_{58}$-Dex_{40k}-C_{10} (diamonds), $MGlu_{56}$-Dex_{70k}-C_{10} (circles), $MGlu_{55}$-Dex_{500k}-C_{10} (triangles), and unmodified (squares) liposomes. Open and closed symbols in (C) express at pH 7.4 and pH 5.0, respectively. Percent release after 30 min-incubation were shown in (D). Lipid concentrations were 2.0×10^{-5} M. Each point is the mean ± SD (n = 3).

Figure 6. Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with Rhodamine-PE-labeled and FITC-OVA-loaded EYPC liposomes modified with or without MGlu-Dex-C_{10} having various amounts of MGlu groups or molecular weights for 4 h at 37 °C in serum-free medium. Bar represents 10 μm.

Figure 7. Fluorescence intensity for DC2.4 cells treated with Rhodamine-PE-labeled EYPC liposomes modified with or without MGlu-Dex-C_{10} having various amounts of MGlu groups (A) or molecular weights (B). DC2.4 cells were incubated with liposomes (lipid concentration: 0.5 mM) for 4 h at 37 °C in serum free medium. Cellular autofluorescence was corrected.

Figure 8. Serum OVA-specific IgG titers (A) and IgG2b/IgG1 ratio (B) for E.G7-OVA tumor-bearing C57BL/6 mice immunized with OVA solution, unmodified and MGlu-Dex-C_{10}-modified liposomes loaded with OVA. OVA-specific IgG titers were measured by ELISA at 12 days after the second administrations.

Figure 9. IFN-γ production of splenocytes of OVA-immunized mice. Splenocytes were isolated from C57BL/6 mice immunized with immunized with free OVA or OVA encapsulated in various kinds of liposomes and the corrected cells (2×10^6/mL in 2 mL) were incubated with OVA (25 μg/ml) for 5 days. Concentration of IFN-γ in the medium was measured by ELISA.

Figure 10. OVA-specific CTL induction in spleen of tumor-bearing mice after 7 days from subcutaneous immunization with unmodified liposomes (squares), $MGlu_{26}$-Dex_{70k}-C_{10}-modified liposomes (triangles), and $MGlu_{56}$-Dex_{70k}-C_{10}-modified liposomes (diamonds). Cytotoxic activity was measured at various effecter cells/target cell (E/T)

ratios using the LDH assay. Aliquots of OVA (100 μg) was administered per mouse. E.G7-OVA cells (closed symbols) and EL4 cells (open symbols) were used as target cells. Each point represents the mean ±SD (n = 3).

Figure 11. Antitumor effect induced by subcutaneous administration of OVA-loaded liposomes. E.G7-OVA cells were subcutaneously inoculated into the left backs of C57BL/6 mice and tumor volume was monitored. Tumor volumes (A, B) and survival (%) (C,D) of tumor-bearing mice were followed from tumor cell inoculation. (A,C) OVA solution (inverted triangles), unmodified liposomes (squares), $MGlu_{26}$-Dex_{70k}-C_{10}-modified liposomes (triangles), and $MGlu_{56}$-Dex_{70k}-C_{10}-modified liposomes (circles) containing 100 μg of OVA were subcutaneously administered into the right backs of the mice twice on day 7 and day 14. (B, D) $MGlu_{56}$-Dex_{70k}-C_{10}-modified liposomes (circles) $MGlu_{58}$-Dex_{40k}-C_{10}-modified liposomes (squares), and $MGlu_{55}$-Dex_{500k}-C_{10}-modified liposomes (triangles) containing 100 μg of OVA were subcutaneously administered into the right backs of the mice twice on day 7 and day 14. Mice administered with PBS (diamonds) were used as controls to confirm the development of tumor. All treated groups contained four mice.

Scheme 1. Synthetic route of pH-sensitive dextran derivatives

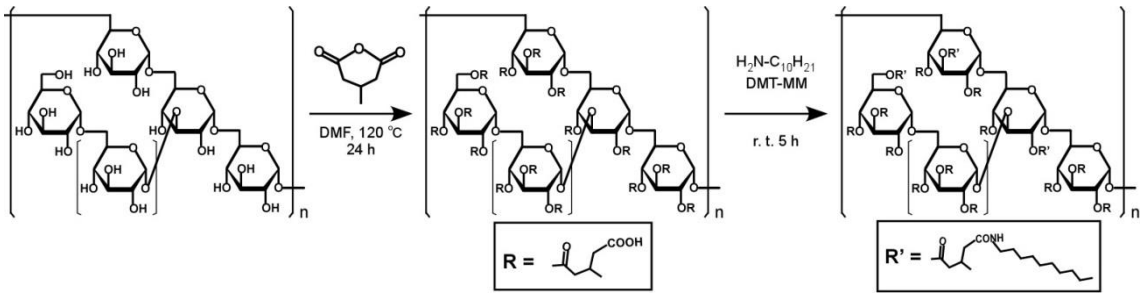


Table 1. Compositions of dextran derivatives

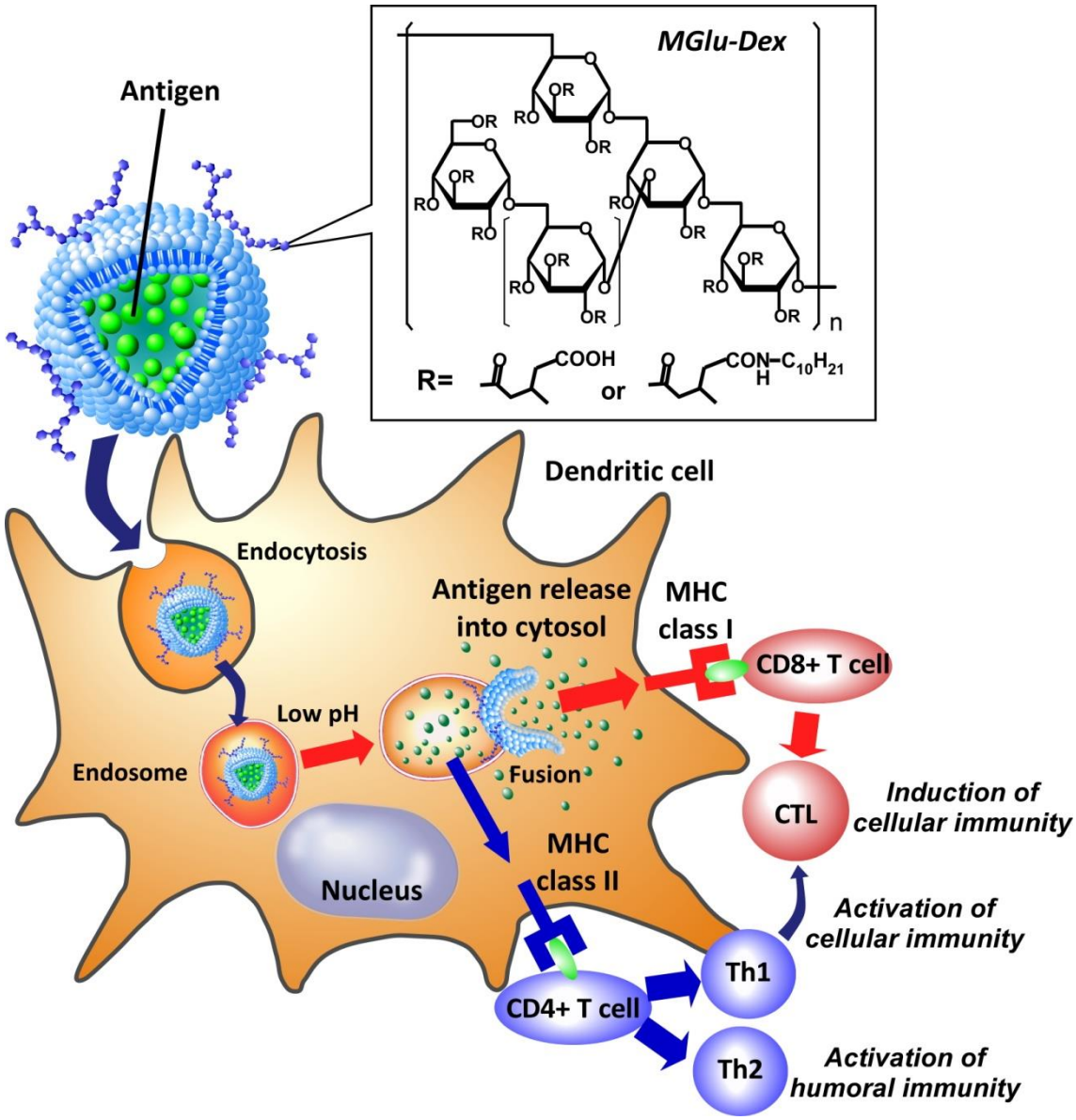
Polymer	-OH(mol%)	-MGlu(mol%)	-Anchor(mol%)
$MGlu_{13}$-Dex_{70k}	87	13	—
$MGlu_{34}$-Dex_{70k}	66	34	—
$MGlu_{48}$-Dex_{70k}	52	48	—
$MGlu_{76}$-Dex_{70k}	24	76	—
$MGlu_{85}$-Dex_{70k}	15	85	—
$MGlu_{66}$-Dex_{40k}	34	66	—
$MGlu_{72}$-Dex_{500k}	28	72	—
$MGlu_{26}$-Dex_{70k}-C_{10}	68	26	6
$MGlu_{56}$-Dex_{70k}-C_{10}	37	56	7
$MGlu_{77}$-Dex_{70k}-C_{10}	19	77	4
$MGlu_{55}$-Dex_{40k}-C_{10}	43	55	6
$MGlu_{58}$-Dex_{500k}-C_{10}	36	58	6

Table 2. p*K*a and precipitation pH for various dextran derivatives

Polymer	pK_a	precipitation pH	Degree of protonation at precipitation pH
$MGlu_{13}$-Dex_{70k}	5.30	N.D.	—
$MGlu_{48}$-Dex_{70k}	5.81	4.3	0.92
$MGlu_{76}$-Dex_{70k}	6.63	4.5	0.98
$MGlu_{66}$-Dex_{40k}	6.26	4.5	0.95
$MGlu_{72}$-Dex_{500k}	6.24	4.5	0.95

Table 3. Particle sizes and zeta potentials of liposomes

Polymer	Particle size (nm)	Zeta potential (mV)
Unmodified	96.5±1.0	-18.6±1.1
$MGlu_{26}$-Dex_{70k}-C_{10}	103.1±0.4	-49.5±1.1
$MGlu_{56}$-Dex_{70k}-C_{10}	109.3±4.3	-46.5±2.1
$MGlu_{77}$-Dex_{70k}-C_{10}	104.8±1.6	-40.7±0.7
$MGlu_{58}$-Dex_{40k}-C_{10}	142.7±0.3	-54.0±1.5
$MGlu_{55}$-Dex_{500k}-C_{10}	150.9±3.3	-54.1±1.3



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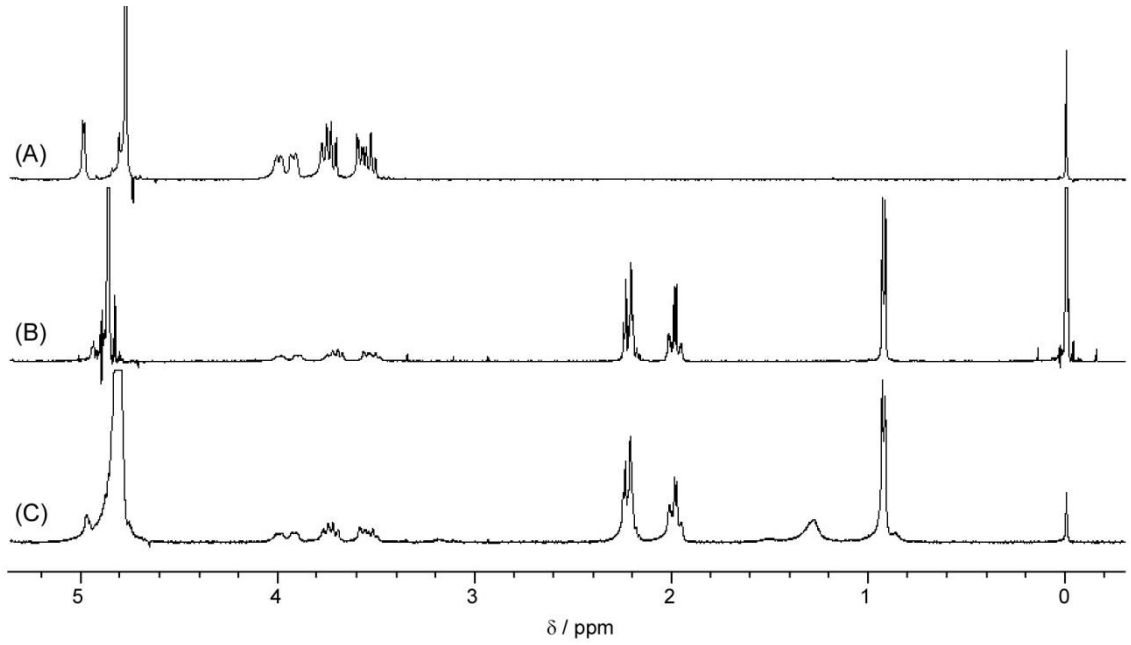

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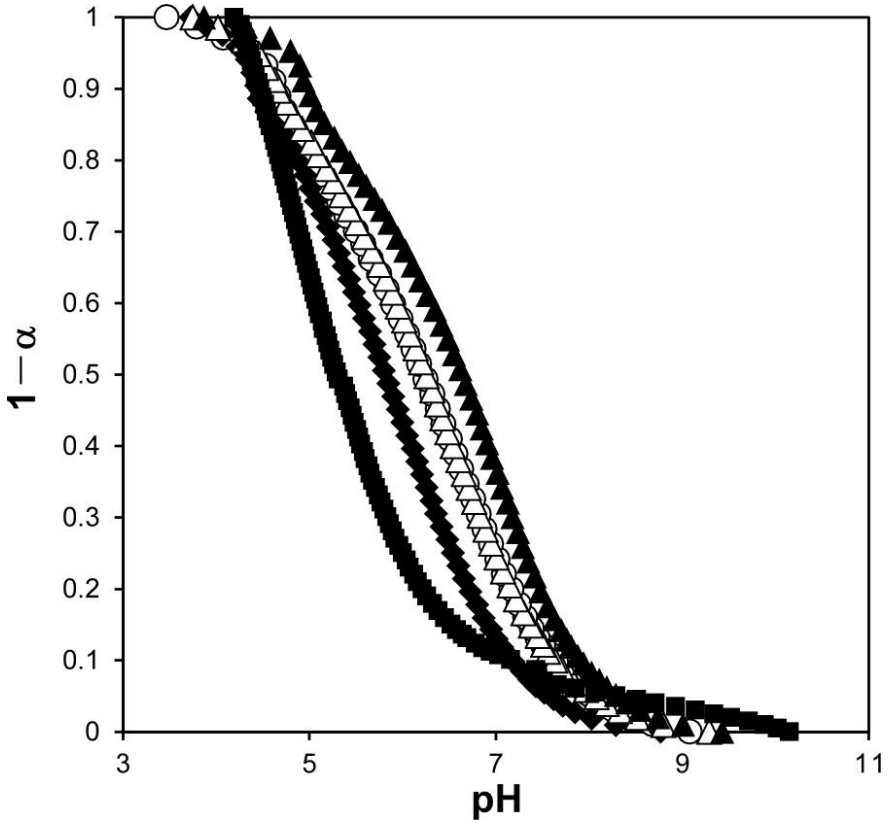

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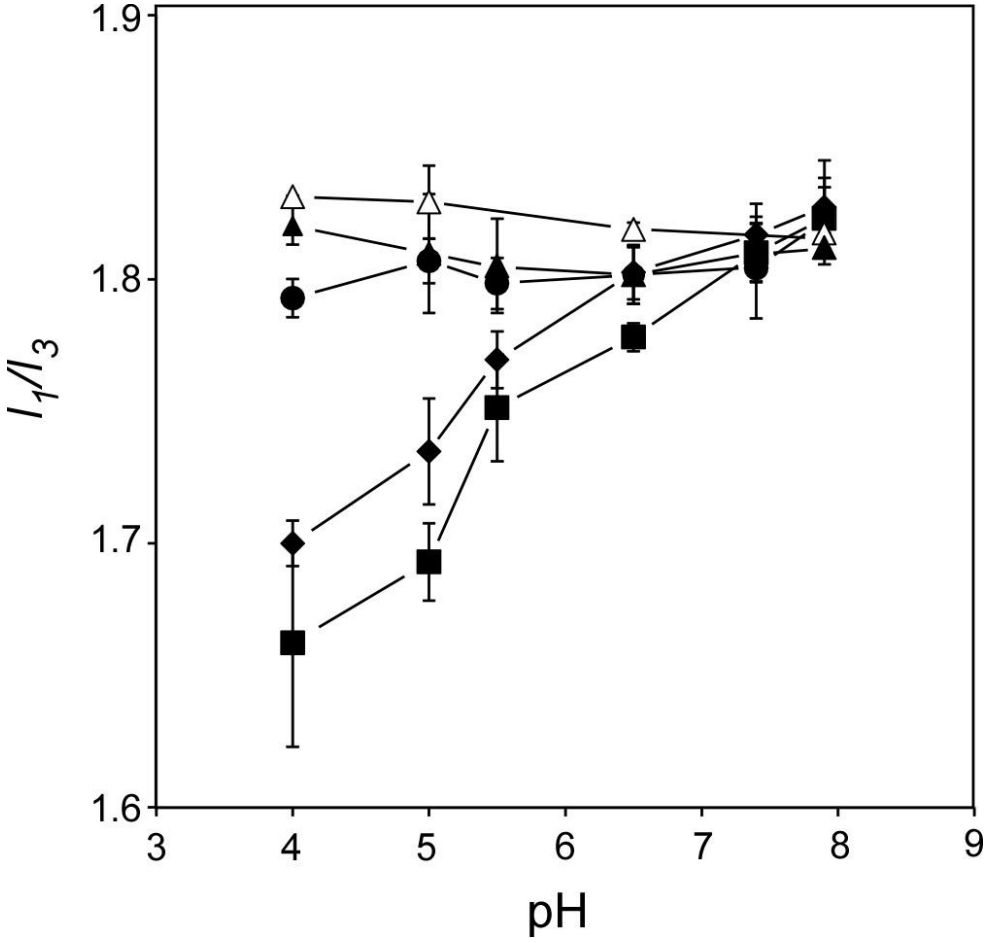

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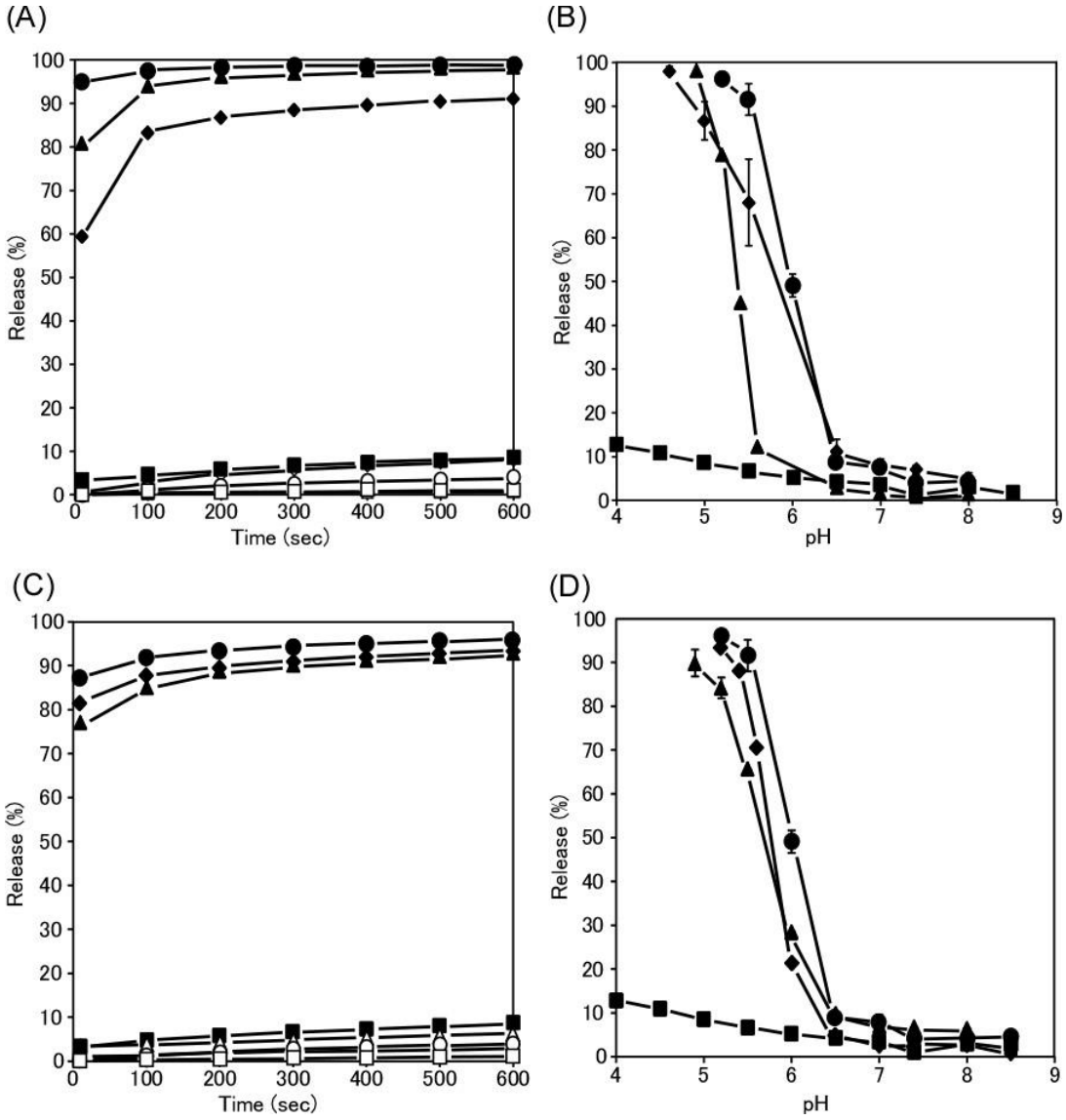

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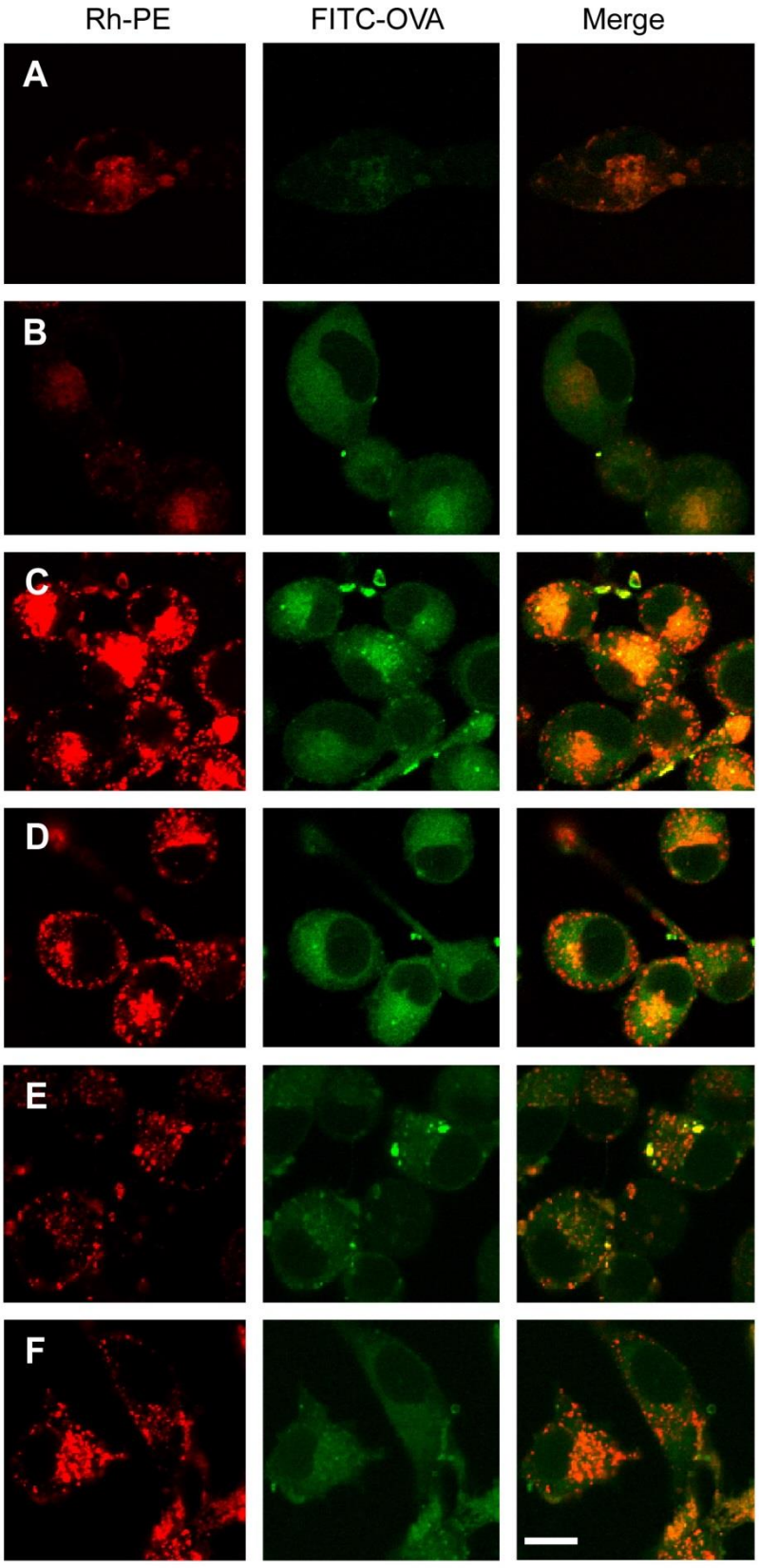

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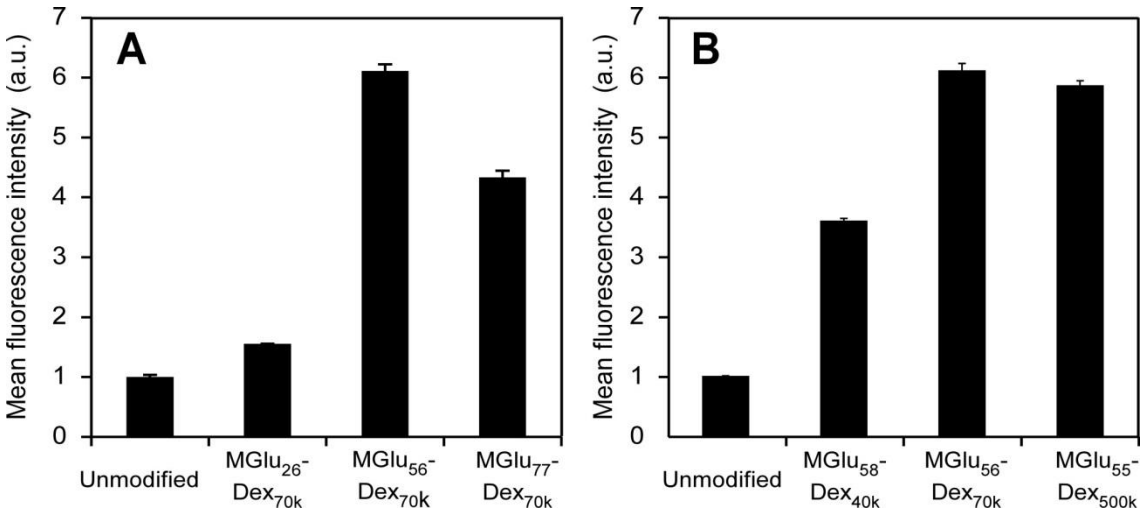

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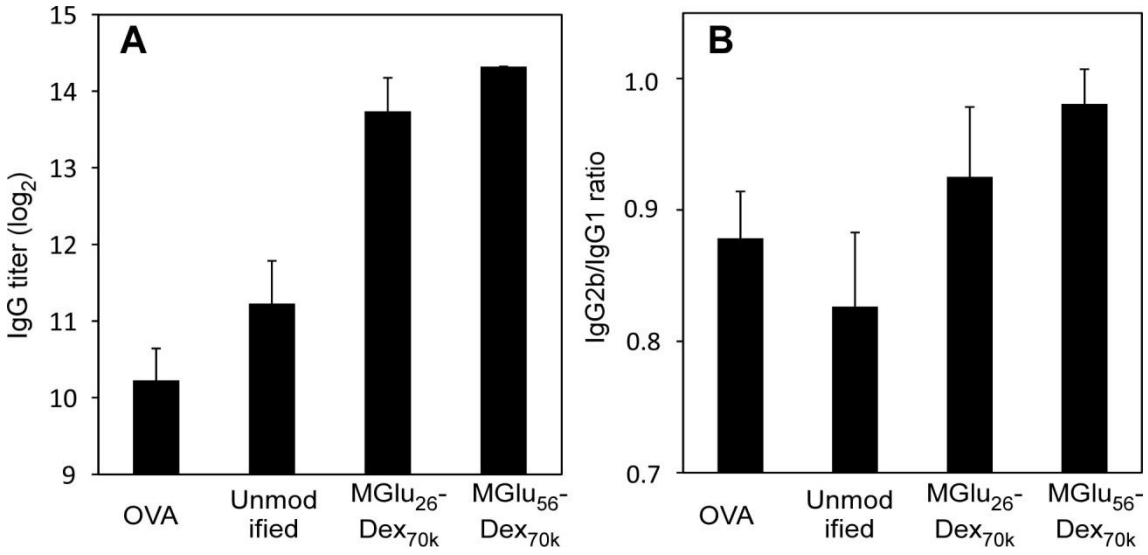

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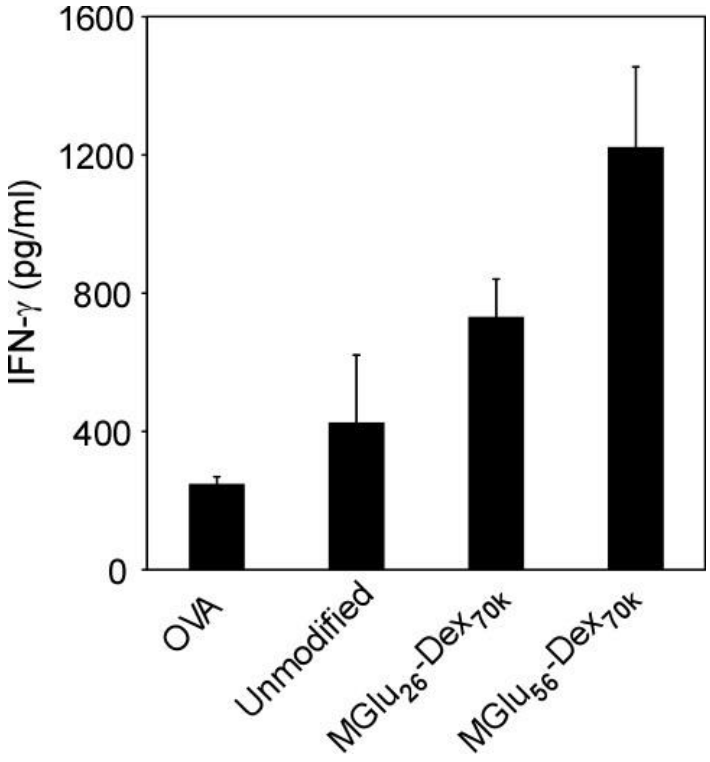

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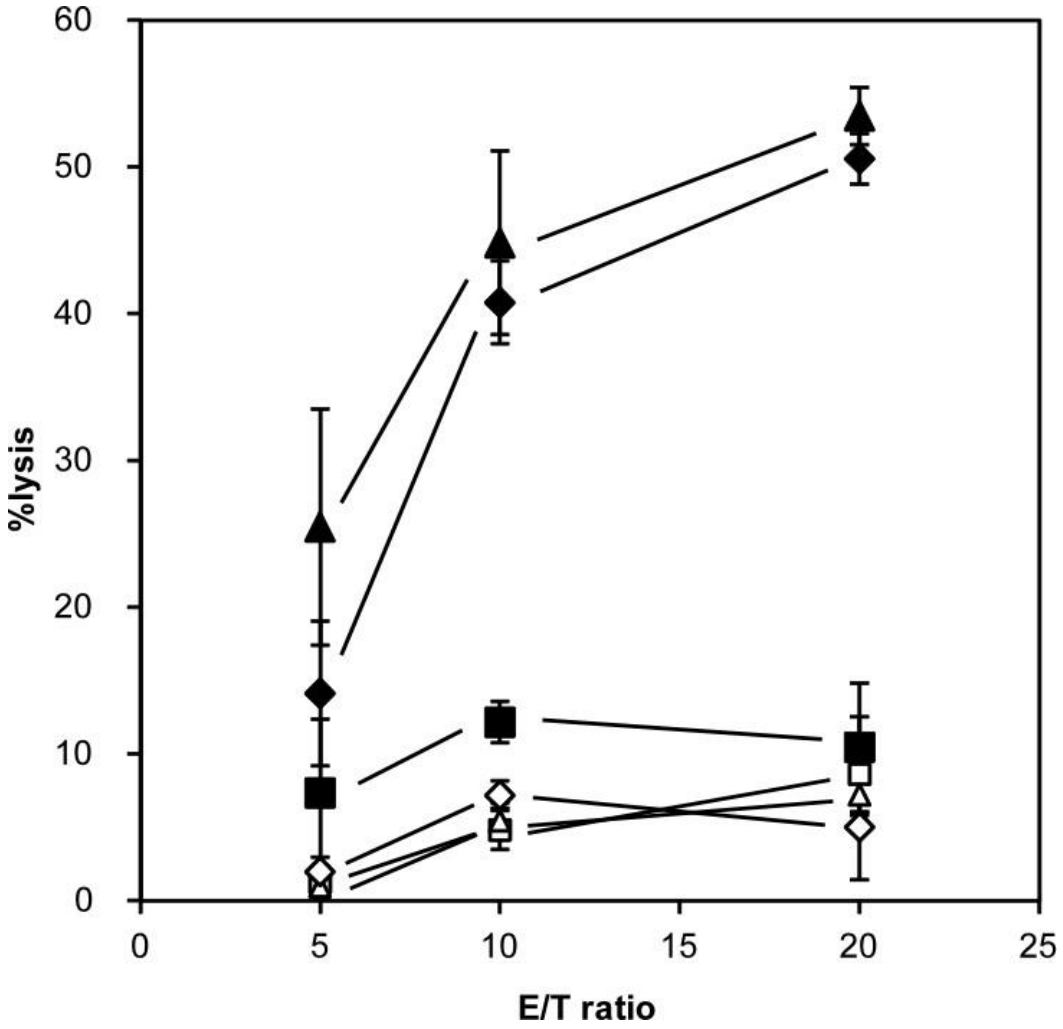

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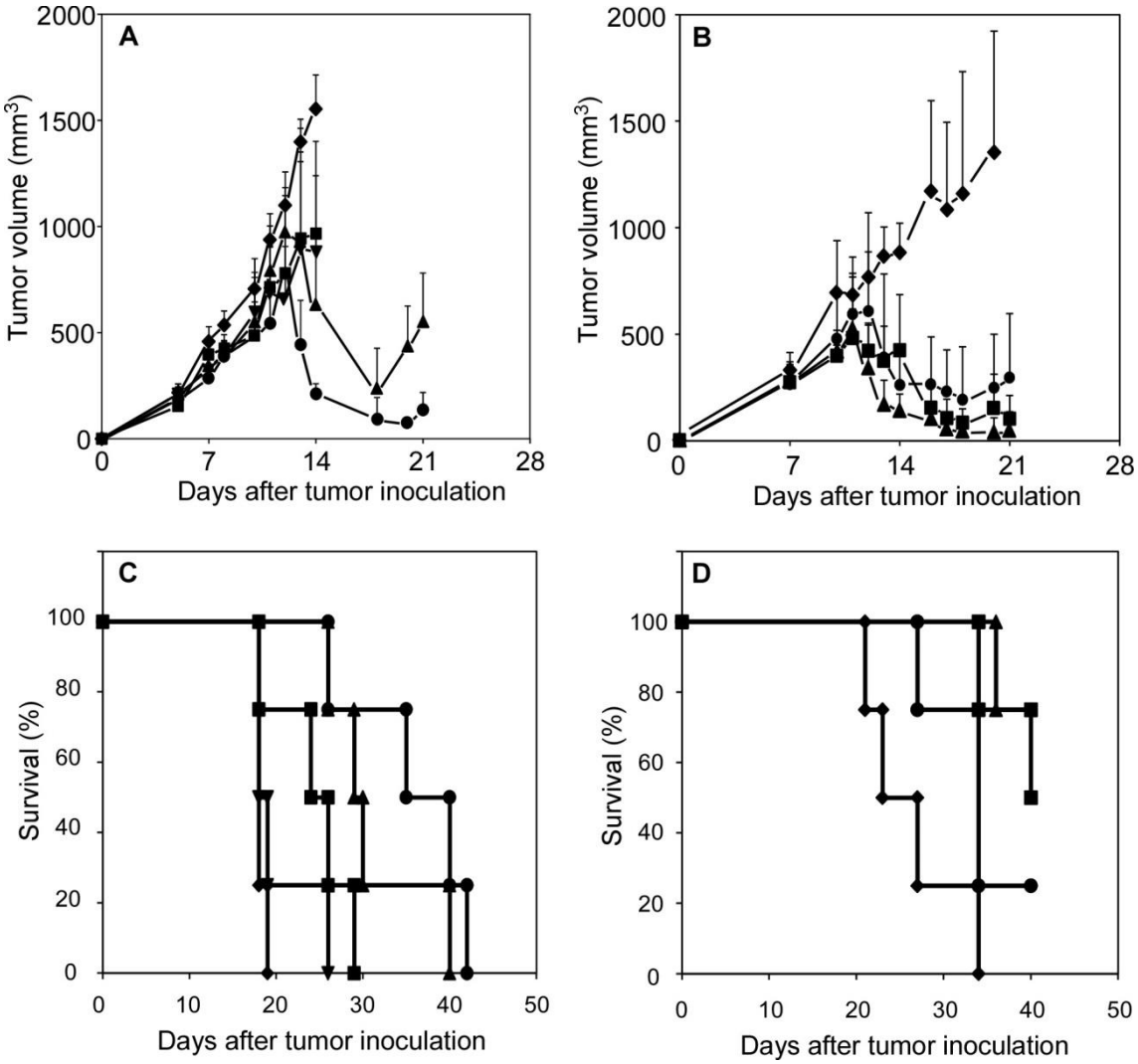

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