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Rational Design of Liposome-Based
Immunity-Inducing System
for Cancer Immunotherapy

Yuta Yoshizaki

February 2017

Doctoral Thesis at Osaka Prefecture University
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Chapter 1: General Introduction

1.1. What is cancer immunotherapy?

Today, the main cause of death is cancer in Japan, and cancer kills approximately 300,000 people every year [1]. Many efforts have been continued to overcome cancer for more than 100 years, at the present time, progress in science and technology brings several effective cancer treatments. Major cancer treatment methods include surgical therapy that directly removes tumor burden, chemotherapy using cytotoxic drugs, radiation therapy using electron beams and particle beams [2]. Although these cancer treatments achieve a given success, these are heavily invasive for cancer patient. Recently, cancer immunotherapy attracts much attention as minimally invasive treatment modality that eliminates cancer cells from our body by the immune system [3]. It is generally known that cancer cells express cancer-specific antigen because cancer cells generate by accumulation of gene mutations in normal cells [4-5]. On the other hand, immunity is the biological defense system that protects our body from non-self-substances and pathogenic organisms. In addition, an important characteristic of immunity is very high specificity such as antigen-antibody reaction [5]. Therefore, cancer immunotherapy is paid attention as next-generation treatment modality that eliminates only cancer cells without damaging normal cells and maintains a quality of life of cancer patients.

The concept of cancer immunotherapy emerged more than 100 years ago. In 1890s, American surgeon, William B. Coley firstly demonstrated that immune response was able to be applied to cancer treatment. Coley observed that cancer (sarcoma) patients that were infected with erysipelas (acute streptococcal infection of skin) experienced spontaneous tumor regression and improved their survival time. Coley attempted local injection of streptococcal culture solution to sarcoma patient and succeeded in treatment of tumor. Then, Coley developed the mixture of heat-killed bacteria that was called as “Coley’s toxin” or “Coley’s vaccine” [6]. This treatment was high-risk because this
treatment deliberately infected patients with bacteria. Moreover, radiation therapy attracted attention in the same era, and “Coley’s toxin” became disused.

After 70 years from “Coley’s toxin”, advance in molecular biology and immunology has brought biological response modifier (BRM) to cancer immunotherapy. BRM uses polysaccharides extracted from bacteria or mushroom, monoclonal antibody and cytokine [7, 8]. Since the effect of these BRM was limited, many doctors doubted cancer immunotherapy.

In 2014, Japanese pharmaceutical company started to sell anti-PD-1 (programmed cell death-1) antibody, nivolmab. Immune cell such as T cell expresses PD-1 on its cell surface, and PD-1 works as brake of immune responses [9]. Cancer immunology reveals that cancer cells can escape from immune system using PD-1 pathway [9]. Nivolmab inhibits PD-1 pathway and shows better therapeutic effects than chemotherapy [10]. This result surprised the world, and cancer immunotherapy attracted much attention. However, it is the problem that nivolmab does not work if tumor-specific CD8+ T cell does not exist in patient’s tumor tissue [11]. Therefore, the development of immunity-inducing system such as cancer vaccine to induce CD8+ T cell becomes a crucial target now.

Cancer vaccine classifies peptide vaccine and cellular vaccine. Peptide vaccine uses peptides derived from cancer-specific antigen and aims to induce antigen-specific immune responses [12]. Cellular vaccine uses patient-derived immune cells such as antigen presenting cells (dendritic cell (DC), macrophage) which are cultured and stimulated by antigen ex vivo [13]. Then, administration of cultured immune cells on patient induces immune responses against cancer. However, cellular vaccine has the disadvantage that large-scale technical equipments are needed to culture large amounts of immune cells. Therefore, research on peptide vaccine attracts much attention, and the way of delivering antigenic peptide to DC is important for establishment of practical peptide vaccine [14]. As a target cell for vaccine delivery, DC is important to enhance the efficacy of vaccine because DC has the strongest activity of controlling immunity [15].
1. 2. Mechanism of induction of immunity by dendritic cells

In 1971, R. M. Steinman discovered DC, and DC controls two types of immune responses [16]. One is cellular immunity, and another is humoral immunity. In cellular immunity, DC presents the antigen where exists in the cytosol (endogenous antigen) to CD8+ T cells with major histocompatibility complex (MHC) class I to induce cytotoxic T cells (CTLs) [17]. On the other hand, in humoral immunity, the exogenous antigens are taken up by the endocytosis, then DC presents the antigen where exists in endosome with MHC class II to CD4+ T cells to induce helper T cells (Th) (Fig. 1-1) [18]. DC determines the induction of cellular immunity or humoral immunity depending on where the antigen exists in the cytosol or endosome [19]. CTLs recognize and eliminate abnormal cells such as cancer cells and virus-infected cells [20], and humoral immunity produces antibodies against bacteria [21]. Therefore, it is essential for cancer immunotherapy to develop the antigen carrier that delivers antigen to cytosol and induce cellular immunity.

Fig. 1-1. DC controls immune responses depending on the location of antigen.
In 1974, Allison reported for the first time antigen carriers based on liposome [22]. Liposomes are closed vesicle composed of phospholipid bilayers, and they have the feature of introducing various functions by introducing water-soluble molecules in the inner water phase and lipophilic molecules in the lipid membrane. For this reason, various researchers have studied various liposome-based antigen carriers. To date, cytosolic delivery of antigen using natural virus-mimicked liposome-based carriers have been studied focusing on membrane fusion phenomenon of virus [23]. For example, inactivated Sendai virus-derived fusogenic proteins or influenza virus-derived hemagglutinin-introduced liposome delivered antigen into the cytosol by fusion with cell membrane and endosomal membrane, and then these liposomes induced cellular immunity [24]. However, these viral protein-introduced liposomes have possibility to cause unexpected immune responses. Therefore, it is desirable to develop artificial membrane fusion molecules that do not contain viral components [25]. For example, liposome containing unsaturated phosphatidylethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) as membrane fusion molecules delivered antigen into the cytosol, then the liposome induced cellular immunity [26]. Thus, inclusion of fusogenic lipid to liposomal membrane can give membrane fusion activity to liposome.

Surface modification by fusogenic polymer can also give membrane fusion activity to liposome. D. A. Tirrel firstly reported synthetic fusogenic polymer using poly(acrylic acid) derivative as a pH-sensitive polymer [27]. At physiological pH 7.4, the polymer did not interact with liposomal membrane because the polymer has anionic charge moiety due to carboxylic acid of side chain. On the other hand, the polymer disrupted liposomal membrane with forming micelle when carboxylic acids were protonated in acidic condition, pH 5.5-6.5 [28]. K. Kono developed more biocompatible fusogenic polymer, succinylated poly(glycidol) (Suc-PG) having succinic acid ester structure in side chain and having poly(glycidol) as main chain with ether oxygen [29]. Suc-PG showed membrane fusion activity at pH < 6, while Suc-PG did not interact with membrane at pH 7.4 [30]. To improve membrane fusion activity, 3-methyl glutarylated poly(glycidol) (MGLu-PG) was developed which has
more hydrophobic side chain than Suc-PG. MGlu-PG showed stronger pH-sensitivity than Suc-PG at pH 6 [30]. E. Yuba reported that MGlu-PG-modified liposome delivered model antigen, ovalbumin (OVA) into the cytosol of DC. MGlu-PG-modified liposome induced cellular immunity in mice, and therapeutic effect is confirmed with tumor-bearing mice [31]. Furthermore, to investigate the influence of polymer architecture on activity of antigen delivery, pH-sensitive polymer having hyperbranched backbone (MGlu-HPG) was compared with MGlu-PG having linear backbone. MGlu-HPG-modified liposome showed higher cellular uptake and induced stronger cellular immunity than those of MGlu-PG-modified liposome [32].

1.4. Adjuvant

Considering the mechanism of antigen presentation by DC, it is inappropriate only to present antigen with MHC molecules (the first signal). If DC transmits only the first signal to T cell, DC induces immune tolerance [33]. In order to induce the desirable immune response, it is essential for DC to transmit the second signal by the co-stimulatory molecule and the third signal to T cells by the cytokine [34]. It is also known that cytokines produced by DC during antigen presentation determine the balance of cellular immunity or humoral immunity [34]. Activated DC highly expresses MHC molecules and co-stimulatory molecules, and the cell produces large amounts of cytokines. Therefore, antigen carrier requires not only to deliver antigen into cytosol but also to activate DC. The effect of activating DC or immune responses is called as adjuvant effect.

Inclusion of adjuvant molecules into liposome provides DC activation function to antigen carrier. Cationic lipids and Toll like receptor (TLR) ligands are known as adjuvant molecules [35, 36]. DC expresses TLRs that play crucial roles in recognizing pathogen specific molecular pattern such as bacteria and viruses, and the signal from TLRs activates DC strongly. Some TLR ligands are highly safe and cost-effective adjuvant because they are synthetic molecules [37]. In general, vaccine formulation includes adjuvant to activate DC. Therefore, to prepare rational immunity-inducing system needs to introduce adjuvant for inducing cellular immunity.
1.5 Objectives and outline in this thesis

This thesis aims to develop rational immunity-inducing system that improves therapeutic effects of cancer immunotherapy. Multiplex inclusion of functional molecules into liposome develops the high-performance immunity-inducing system that delivers antigen into the cytosol of DC and activates them simultaneously. Here, in order to enhance its membrane fusion activity, poly(carboxylic acid) derivatives were synthesized as pH-sensitive polymers, which have poly(glycidol) or polysaccharide as a main chain. In order to activate DC, various adjuvant molecules, cationic lipid, nucleic acid, and bioactive polysaccharide were introduced to liposome. These liposomes were investigated in terms of intracellular antigen delivery and induction of immune responses. Then, this thesis aims to reveal the rational design of immunity-inducing system to realize effective cancer immunotherapy.

This thesis consists of the following eight chapters.

Chapter 1 describes the introduction, the research background, objectives, and the outline of this thesis.

Chapter 2 describes the usefulness of peptide-encapsulated pH-sensitive polymer-modified liposome as a peptide carrier for cancer immunotherapy. OVA-derived peptide and monophosphoryl lipid A (MPL) as an adjuvant were introduced to MGlu-HPG-modified-liposome. We discuss validity of using pH-sensitive polymer-modified liposome as an antigen carrier for peptide vaccine.

Chapter 3 describes the preparation of cationic lipid-introduced MGlu-HPG modified-liposome. As a cationic lipid, 3,5-didodecylbenzoxamididine was used, and the liposomes were evaluated as an antigen carrier. The synthetic cationic lipid has a cost advantage as adjuvant molecules in comparison with the living organism-derived molecules such as MPL. This chapter demonstrates that cationic lipid inclusion into pH-sensitive polymer-modified liposome improves intracellular antigen delivery, and is effective approach for the preparation of high-performance antigen carrier.

Chapter 4 describes attempts to enhance adjuvant function of cationic lipid-incorporated pH-sensitive polymer-modified liposome. CpG-DNA is selected as an adjuvant molecule, which is the
ligand of TLR9 expressing in endosomes of DC. In this chapter, CpG-DNA complexation methods were investigated in terms of immunity-inducing activity. We discuss that adjuvant complexation methods to antigen carrier affects the intracellular distribution of adjuvant molecules and immunity-inducing function.

Chapter 5 describes the preparation of immunity-inducing system using pH-sensitive polymer modified-liposome and cationic liposome-IFN-γ gene complex (lipoplex). OVA-loaded pH-sensitive polymer-modified liposome and lipoplex of plasmid DNA (pDNA) encoding IFN-γ were used in this study. Two cases of immunity inducing system were constructed and compared as "Combination" when using them in combination and "Hybrid" when they were complexed with electrostatic interaction. Results demonstrated that immunized with the combination of liposome-lipoplex induced early infiltration of CTL in tumor and showed stronger therapeutic effect.

Chapter 6 aims to prepare safer fusogenic polymer and to synthesize pH-sensitive dextran derivatives. Biodegradable polysaccharide, dextran was reacted with 3-methylglutaric anhydrides to synthesize MGlu-Dex as safer fusogenic polymer. Results demonstrated that MGlu-Dex-modified liposome delivered antigen into the cytosol of DC and administration of the liposome to mice induced cellular immunity. Since dextran has been applied to clinical as a plasma expander, MGlu-Dex is promising polymer material with highly functionality and safety.

Chapter 7 describes the design of multifunctional polysaccharides, which combine cytosolic antigen delivery and adjuvant function. Curdlan and mannan were used as bioactive polysaccharides because they are known to activate DC via their respective interactions with Dectin-1 and Dectin-2 expressing on DC. To obtain polymers that have both cytosolic antigen delivery and DC activation function, curdlan and mannan were chemically modified with 3-methylglutaric acid ester as a pH-sensitive functional group. Their bioactive polysaccharides derivative-modified liposomes are discussed in terms of usefulness for cancer immunotherapy.

Chapter 8 summarizes the results obtained in Chapters 2 through 7.
1.6. References


2) American Cancer Society,


Chapter 2: Improvement of peptide-based tumor immunotherapy using pH-sensitive fusogenic polymer-modified liposomes

2.1. Introduction

Recent developments in tumor immunology have attracted much attention to cancer immunotherapy, a treatment to activate the patient’s own immunity against tumors or remove immunosuppression in tumor microenvironments. The induction of cancer-specific cytotoxic T lymphocytes (CTLs) is crucially important to achieve efficient therapeutic effects because CTLs can kill tumor cells directly [1–3]. Actually, adoptive transfer of tumor-infiltrating T-lymphocytes causes marked therapeutic effects in cancer patients [4]. Earlier studies show that CTLs recognize the complex of cancer antigen and major histocompatibility complex (MHC) class I molecules expressing on cancer cells [5,6]. Furthermore, a tumor-specific mutant antigen designated as neoantigen plays an important role for the recognition of cancer cells by CTLs in actual cancer [7–9]. Therefore, identification of neoantigen-derived cancer antigenic peptides and the induction of these peptide antigen-specific CTLs are important to establish personalized cancer treatments using peptide-based cancer vaccines.

Since the discovery of melanoma antigen MAGE, various tumor antigenic peptides have been reported, such as WT1, glypican-3, MART-1, and TRP-1 [1, 10–12]. Moreover, numerous clinical trials using these antigenic peptides have been attempted [3, 13–15]. However, their therapeutic efficacy in clinical trials is insufficient even though tumor antigenic peptides have specificity to a tumor and the ability to induce antigen-specific immune responses in vitro [2]. Therefore, although tumor antigenic peptide-based immunotherapy is eagerly sought, it is still under development.

Generally, peptide-based cancer vaccine is conducted using antigenic peptides emulsified in incomplete Freund’s adjuvant (IFA) [1]. After subcutaneous administration of peptide/IFA, peptides are released gradually from IFA emulsion and are bound to MHC molecules by displacement of endogenous peptides on antigen-presenting cells (APCs) such as dendritic cells (DCs),...
macrophages, and B cells. These peptides can also be taken up by APCs. Peptides are bound to MHC class I molecules via cross presentation or MHC class II molecules in endosome/lysosome. APCs present antigenic peptides to CD8$^+$ T cells and CD4$^+$ T cells, which differentiate, respectively, to antigen-specific CTLs and helper T cells [5,6]. Subsequently, CTLs migrate to tumor sites and induce tumor-killing effects. Helper T cells support the CTL’s tumor killing effect and B cell activation. The antigen processing pathway of peptides in antigen-presenting cells and immune-inducing process are known to be affected by the peptide length. Especially, immunization with antigenic short peptides emulsified in IFA induced trapping of CTLs at original vaccine sites [16,17]. Short antigenic peptides are presented continuously to CD8$^+$ T cells through MHC molecules, not only on DCs but also on B cells in peptide/IFA injected sites because peptide/IFA emulsion remains at the injected site. Continuously released short peptide molecules bind to MHC molecules on APCs. Such a peptide presentation by APCs induces the accumulation of CTLs at the peptide-injected site with high peptide concentration, which suppresses migration of CTLs into the tumor site (Fig. 2-1). CTLs accumulated at the peptide vaccine-injected site lose their activity and undergo apoptosis, consequently producing poor antitumor effects [17].

This fact suggests the importance of effective antigen presentation by DCs for the induction of CTLs which can migrate into tumor sites and which can eliminate tumor cells effectively. Therefore, to achieve efficient immunotherapy with short tumor antigenic peptides, it is crucially important to deliver them into DCs specifically and efficiently without excessive presentation for APCs of other kinds. Then, the short peptides taken up by DCs at vaccination sites might be presented on DCs and induce antigen-specific CTLs that can migrate into tumors (Fig. 2-1). Therefore, such DC-specific peptide delivery systems are expected to improve the efficacy of short peptide vaccine for cancer immunotherapy.

To date, carrier systems of various types have been examined for their feasibility for delivery of short antigenic peptides for the induction of antigen-specific immunity. For example, amphiphilic polyethyleneimine-based micelles were used for delivery of Trp2 peptide, which is an antigenic
After subcutaneous administration, micelles accumulate preferentially to lymph nodes, probably because of their small size (around 30 nm) and induced Trp2-specific CTLs in vivo [18]. Additionally, poly(DL-lactide-co-glycolide) (PLGA)-based polymeric nanoparticles loaded with short antigenic peptides of MART-1, gp100 or ovalbumin (OVA) have been shown to induce stronger antigen-specific CTLs and antitumor effects than IFA/peptide emulsion [19]. However, these systems might present shortcomings in toxicity derived from cationic polymers, peptide-loading capability and controllability of peptide delivery in the body or inside of APCs. Indeed, liposomes are promising systems for short peptide delivery from the perspectives of biocompatibility, high peptide-loading capability, and functionalization, which enhance the accuracy of peptide delivery to DCs after administration.

We previously developed liposomes modified with pH-sensitive fusogenic polymer MGlu-HPG (Fig. 2-1) and demonstrated that the MGlu-HPG liposomes have excellent capability for induction of antigen-specific immunity using OVA as antigenic protein [20–23]. In fact, MGlu-HPG-modified liposomes delivered antigenic protein OVA into cytosol of DCs via membrane fusion responding to weakly acidic pH of the endosomes/lysosomes and induced MHC class I-restricted antigen presentation [20–23]. Administration of these liposomes to OVA-expressing tumor-bearing mice induced OVA-specific cellular immunity and induced efficient suppression of tumors of mice [23]. In addition, these liposomes were taken up by DCs efficiently through the recognition by scavenger receptors of DCs because liposome surfaces are covered with many carboxylates derived from MGlu-HPG [20–23]. Based on these results, in chapter 2, we attempted to apply the pH-sensitive polymer-modified liposomes to short peptide vaccine delivery to DCs because we expected that the liposomes might solve the problem of the short peptide vaccine, trapping of CTLs at the vaccination sites, by delivering the short peptides into cytosol of DCs and inducing antigen-specific CTLs effectively (Fig. 2-1).
**2. 2. Materials and methods**

**2. 2. 1. Materials**

Egg yolk phosphatidylcholine (EYPC) and L-dioleoyl phosphatidylethanolamine (DOPE) were kindly donated by NOF Corp. (Tokyo, Japan). Rh-PE was purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA). OVA and MPLA were purchased from Sigma (St. Louis, MO, USA). 3-Methyl-glutarylated hyperbranched poly(glycidol) with polymerization degree of 60 (MGlu-HPG, Fig. 2-1) was used as a pH-sensitive polymer. It was prepared as explained in an earlier report [21].
OVA-I peptide (SIINFEKL) [29] and derivation of OVA-II peptide (PSIQAVHAHAINEAPEVA), a modified I-A\textsuperscript{d}-binding OVA epitope peptide described elsewhere [30], were used as model short antigenic peptides that bind respectively to MHC class I and II molecules on DCs. These peptides were synthesized using Fmoc chemistry as previously reported [26]. The peptides were purified by reverse phase HPLC to a purity of >95%. Their molecular weights were confirmed using MALDI-TOF mass spectrometry (Voyager DE–RP; Applied Biosystems, Carlsbad, CA., USA). The peptide concentration of was determined using a Micro BCA assay (Pierce, Carlsbad, CA., USA). FITC-labeled peptides were purchased from KareBay TM Biochem, Inc. (Monmouth Junction, NJ., USA). Bordetella pertussis whole cell vaccine (Wc), which is a suspension of inactivated bacteria in PBS, was produced from B. pertussis Tohama phase I bacteria and provided by Osaka University (Bikenkai, Japan).

### 2.2.2. Preparation of liposomes

For liposome preparation, 1.0 mL of peptides/PBS solution (pH 7.4, 1 mg/mL) was added to a dry, thin membrane of EYPC (5.0 mg), DOPE (4.7 mg), and MPLA (50 μg). The mixture was vortexed at 4 °C. The liposome suspension was hydrated further by freezing and thawing. It was extruded through a polycarbonate membrane with 100 nm pore size. The liposome suspension was centrifuged at 55,000 rpm for 2 h at 4 °C twice to remove free peptides from the peptide-loaded liposomes. MGlu-HPG-modified liposomes were also prepared according to the procedure described above using dry membrane of a lipid mixture with MGlu-HPG (lipids/polymer = 7/3, w/w). OVA whole protein-loaded liposomes were also prepared according to the procedure described above using OVA/PBS solution (pH 7.4, 4 mg/mL). The concentrations of lipids, peptides and proteins were determined using Wako phospholipids C (Wako Pure Chemical Inds. Ltd., Osaka, Japan), Micro BCA assay and Coomassie (Bradford) protein assay kit (Pierce Biotechnology Inc., Carlsbad, CA., USA), respectively.
2.2.3. Cell culture

DC2.4 cells, which were an immature murine DC line, were provided by K. L. Rock (Harvard Medical School, Worecester, MA., USA) and were grown in RPMI-1640 (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% FBS (MP Biomedicals, Santa Ana, CA., USA), 2 mM L-glutamine (Wako, Osaka, Japan), 100 mM MEM nonessential amino acid (Nacalai Tesque Inc.), 50 μM 2-mercaptoethanol (2-ME; Gibco, Carlsbad, CA., USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C [31]. EL4, a C57BL/6 mice-derived T lymphoma, was obtained from Tohoku University (Sendai, Japan). E.G7-OVA, a chicken egg OVA gene-transfected clone of EL4 that presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA, USA) [29].

2.2.4. Animals

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

2.2.5. Intracellular behavior of liposome

The FITC-labeled OVA peptide-loaded liposomes containing Rh-PE were prepared as described above except that a mixture of polymer and lipid containing Rh-PE (0.1 mol%) was dispersed in PBS containing FITC-OVA peptides (1 mg/mL). DC2.4 cells (3 × 10⁵ cells) cultured for 2 days in 35-mm glass-bottom dishes were washed with Hank’s balanced salt solution (HBSS). Then they were incubated in serum-free RPMI medium (1 mL). The FITC-OVA peptide-loaded liposomes (50 μg/mL peptide concentration, 1 mL) were added gently to the cells and were incubated for 4 h at 37 °C. After incubation, the cells were washed three times with HBSS. CLSM analysis of these cells was performed using LSM 5 EXCITER (Carl Zeiss Co., Oberkochen, Germany).
2. 2. 6. Cytokine production from cells treated with liposomes

The DC2.4 cells (4 × 10⁴ cells) cultured overnight in 96-well plates were washed with HBSS. Then they were incubated in serum-free RPMI medium (100 μL). OVA-I solution or OVA-I-loaded MGLu-HPG liposomes with/without Wc (4 × 10⁴ cells) (100 μg/mL peptide concentration, 100 μL) were added gently to the cells, followed by incubation for 4 h at 37 °C. After incubation, supernatants of cultured cells were collected for measurements of TNF-α using an enzyme-linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd., Rock Hill, NJ., USA) according to the manufacturer’s instructions.

2. 2. 7. CTL assay

Mice were administered PBS, 50 μg of OVA-loaded MGLu-HPG liposomes, OVA-I-loaded MGLu-HPG liposomes, OVA-II-loaded MGLu-HPG liposomes, free OVA, OVA-I, and OVA-II solution subcutaneously. Seven days later, splenocytes were recovered from each mouse and were suspended in RPMI1640 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 Inc.). 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 after 4 h-incubation was evaluated at a ratio of effector cells to target cells (E.G7-OVA or EL4 cell), which was defined as the E/T ratio, of 5 using a lactate dehydrogenase (LDH) cytotoxicity detection assay (Takara Bio Inc., Shiga, Japan).

2. 2. 8. Induction of antitumor immunity by liposomes

OVA-loaded or OVA-peptide-loaded liposomes and free OVA or OVA peptide solution (50 μg of protein or peptide) were administered subcutaneously into the right back of C57BL/6 mice 14 and 7 days before tumor cell inoculation under anesthesia with isoflurane. Seven days after the second immunization (Day 0), E.G7-OVA cells (1 × 10⁶ cells) were inoculated subcutaneously into the left back of anesthetized mice. The tumor size was monitored using the following formula: (major axis ×
minor axis $^3 \times 0.5$ and survival of the mice was also measured. Mice were killed when tumor volumes become greater than 2500 mm$^3$. Each treated group included four mice.

2. 2. 9. Therapeutic effects induced by liposomes on tumor-bearing mice

E.G7-OVA cells ($5 \times 10^5$ cells) were inoculated subcutaneously into the left back of C57BL/6 mice under anesthesia with isoflurane. On Days 6 and/or 13, 50 μg of OVA-loaded or OVA-peptide-loaded liposomes and free OVA or OVA peptide solution were injected subcutaneously into the right back 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 killed when tumor volumes become greater than 2500 mm$^3$. Each treated group included four mice.

2. 2. 10. Statistical analysis

Tukey–Kramer method (Figures 2-2, 4-6, and 2-S1–4) was used for statistical evaluation of the results. Log-rank tests were applied for the statistical analysis of survival data (Figures 2-5c, 6c, 6e, and 2-S4b).

2. 3. Results and Discussion

2. 3. 1. Preparation of peptide-loaded liposomes and selection of adjuvant

For the induction of efficient immune responses, both antigen delivery into DCs and the activation of DCs should be achieved simultaneously. In an earlier study, monophosphoryl lipid A (MPLA), which stimulates Toll-like receptor 4 (TLR4) [24], has been introduced to MGlu-HPG-modified liposomes to activate DCs [23]. MGlu-HPG-modified liposomes without adjuvant induced antitumor immunity. Their therapeutic effect was enhanced further by inclusion of MPLA [23, 25]. In this study, *B. pertussis* whole cell vaccine (Wc), which is known to induce Th1 immune responses [26, 27], was compared with MPLA as another adjuvant. The effect of Wc and MPLA inclusion to
MGLu-HPG liposomes on the induction of antigen-specific antitumor immunity was examined (Fig. 2-2a). PBS or OVA-loaded MGLu-HPG-modified liposomes and $1 \times 10^7$ cells of Wc were administered subcutaneously to E.G7-OVA tumor-bearing mice. For mice administered PBS, the E.G7-OVA tumor volume increased rapidly after Day 5 (Fig. 2-2a). However, mice administered the MGLu-HPG-modified liposomes containing MPLA or a combination with liposomes and Wc exhibited decreased E.G7-OVA tumor volume after Day 8 (Fig. 2-2a), which indicates that OVA-specific immunity was induced by administration of the OVA-loaded MGLu-HPG-modified liposomes. That result is consistent with the results of our earlier study [23]. The combination of Wc did not affect the antitumor effects induced by MGLu-HPG-modified liposomes containing MPLA. In both cases, E.G7-OVA tumor burden disappeared at Day 14, which indicates that antitumor immunity induced by MGLu-HPG-modified liposomes was sufficient to regress the established tumor.

**Fig. 2-2.** (a) Antitumor effect induced by immunization with MGLu-HPG-modified liposomes with or without Wc on tumor-bearing mice. The E.G7-OVA cells ($1 \times 10^6$ cells) were inoculated subcutaneously into the left back of C57BL/6 mice and MGLu-HPG-modified liposomes with Wc (closed squares) or without Wc (open squares) containing 50 μg of OVA were administered subcutaneously into the backs of the mice on Day 5. Mice immunized with PBS (closed diamonds) are shown as controls. Each treated group included four mice; * $p < 0.01$. (b) TNF-α production from DC2.4 cells treated with 10 μg of OVA-I or OVA-I-loaded MGLu-HPG-modified liposomes with (closed bars) or without Wc (open bars) for 4 h in the absence of serum; ** $p < 0.05$. 

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Next, the activation of DC2.4 cells by treatment with free peptide or peptide-loaded pH-sensitive polymer-modified liposomes was examined. OVA-I peptide (SIINFEKL)-loaded MGlu-HPG liposomes were prepared. Their diameters were about 100 nm according to dynamic light scattering (DLS) analysis (Fig. 2-S1a). A spherical structure for liposomes was also found using transmission electron microscopic (TEM) analysis (Fig. 2-S1b). Loading amounts of peptides in the liposomes were about 30 μg/μmol lipid (Fig. 2-S1c). The loading efficiency of peptide to liposome was 42.5% ± 1.5% for unmodified liposome and 44.5% ± 4.5% for MGlu-HPG liposome. MGlu-HPG liposomes retained encapsulated peptide at pH 7.4, but released at acidic pH (Fig. S1d). DC2.4 cells were treated with free OVA-I or OVA-I-loaded MGlu-HPG liposomes containing MPLA with or without Wc for 4 h. Furthermore, the production of TNF-α by DC2.4 cells was measured. Fig. 2b shows that production of TNF-α was enhanced by OVA-I-loaded MGlu-HPG liposomes, irrespective of the presence of Wc compared with free OVA-I solution. This result indicates that MPLA-introduced liposome treatment strongly activated DC2.4 cells, which might derive from the activation via MPLA and efficient intracellular delivery of OVA-I to DC2.4 cells (Fig. 2-3a). Therefore, we used MPLA-introduced MGlu-HPG-modified liposomes in the following experiments considering their ease of inclusion as a lipid membrane component.
Fig. 2-S1. (a) Diameters of peptide-loaded liposomes with or without MGlh-HPG determined by Dynamic light scattering (DLS). (b) TEM image for OVA-I-loaded liposomes with or without MGlh-HPG. (c) Peptide or protein amounts per lipid in various liposomes. ** $p < 0.01$. (d) Peptide release from liposomes. Liposomes were incubated at pH 7.4 or 5.0 for 30 min and then liposome suspension were centrifuged and peptide amounts in supernatant were determined by Micro BCA assay.

2.3.2. Intracellular distribution of peptide-loaded liposomes

Next, intracellular peptide delivery performance of MGlh-HPG-modified liposomes was investigated. DC2.4 cells were treated with FITC-labeled peptide and peptide-loaded liposomes labeled with lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE). Then they were observed using confocal laser scanning microscopy (CLSM) (Fig. 2-3). For free OVA-I peptides, most green fluorescence was observed at the cell periphery, which indicates that FITC-labeled peptides were bound mainly to the cellular membrane (Fig. 2-3a). For cells treated with unmodified liposomes, punctate red fluorescence was observed within cells, although green fluorescence was not observed under experimental conditions. This result suggests that unmodified liposomes are not taken up efficiently by DCs. Therefore, these liposomes were ineffective for delivery of OVA-peptides to the cytosol. For MGlh-HPG liposomes, strong red or green fluorescence was observed within the cells. In addition, some green fluorescence was found at different locations from red fluorescence. This result suggests that MGlh-HPG-modified liposomes were taken up efficiently by DCs through recognition by scavenger receptors on DCs, as reported previously [20–23]. Their
contents (OVA-peptides) were delivered into the cytosol of DCs through membrane fusion responding to acidic pH of endosomes [20–23]. Efficient delivery of short peptides into the interior of DCs by MGlue-HPG-modified liposomes is important to avoid antigen presentation by other APCs at the injected site after administration to mice. In addition, some OVA-I peptides were delivered to cytosol, which induces MHC class I-mediated presentation and the induction of cellular immune response. OVA-II peptides (PSISQAVHAAHAINEAPβA) were also delivered efficiently to the interior of DCs by MGlue-HPG-modified liposomes as well as OVA-I peptides (Fig. 2-3b). To evaluate the endosomal escape efficiency, the colocalization ratio of green fluorescence with red fluorescence was calculated from CLSM images (Fig. 2-S2). As Fig. 2-S2 shows, about 50% of green fluorescence was merged with red fluorescence, indicating that about 50% of FITC-peptides exist in endosome/lysosome, and 50% exist in cytosol. For OVA-II peptides, release in endosomes or lysosomes is necessary for MHC class II presentation [5,6]. Therefore, efficient antigen presentation via MHC class II pathway might be expected by MGlue-HPG liposomes from peptides released in endosome/lysosome.

<table>
<thead>
<tr>
<th>(a) Rh-PE</th>
<th>FITC-peptide</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGlue-HPG liposome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmodified liposome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide only</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2-3.** Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with FITC-OVA-I peptides (a); FITC-OVA-II peptides (b); and peptide-loaded EYPC/DOPE/Rh-PE liposomes modified with or without MGlue-HPG. Cells were treated with peptide or liposomes with peptide concentration of 25 μg/mL for 4 h in the absence of serum. Scale bars represent 10 μm.
2.3.3. Induction of cellular immune responses in vivo

The induction of CTLs is crucially important for establishing tumor regression. Therefore, the induction of CTL in spleen was measured for mice immunized with liposomes. Fig. 2-4a and Fig. 2-S3a depict the percent lysis for E.G7-OVA cells or EL4 cells induced by the stimulated splenocytes at E/T ratio of 5. Splenocytes obtained from mice treated with OVA-I-loaded MGlu-HPG liposomes induced CTL response as well as OVA-loaded MGlu-HPG liposomes. In contrast, splenocytes obtained from mice treated with OVA-II-loaded MGlu-HPG liposomes induced no detectable CTL responses under experimental conditions. This result is reasonable because OVA-II peptide induces MHC class II-mediated presentation, which induces helper T cells, not CTLs. These splenocytes exhibited no cytotoxicity against EL4 cells. Therefore, CTLs induced by OVA-I-loaded MGlu-HPG liposomes were specific to OVA expression on the cells. Moreover, the administration of free OVA or free OVA-peptides showed quite weak CTL response in the spleen (Fig. 2-4b and 2-S3b). These results suggest that OVA-I-loaded MGlu-HPG liposomes can induce antigen-specific CTLs efficiently because of their efficient cytoplasmic delivery of OVA peptide to DCs (Fig. 2-3a).
Therefore, OVA-I-loaded liposomes are expected to induce tumor-specific therapeutic effects against E.G7-OVA tumor-bearing mice.

Fig. 2-4. CTL response in spleen seven days after subcutaneous immunization with: (a) 50 μg of OVA-loaded, OVA-I-loaded, and OVA-II-loaded MGl-HPG-modified liposomes; and (b) 50 μg of free OVA, OVA-I, or OVA-II. Cytotoxicity against E.G7-OVA cells was measured using an LDH assay at an effector cells/target cell (E/T) ratio of (a) 5 or (b) 1. Each bar represents means ± SD (n = 3). * p < 0.05, ** p < 0.01. No significant difference was found between any groups in (b).

Fig. 2-S3. CTL response in spleen 7 days after subcutaneous immunization with (a) 50 μg of OVA-, OVA-I-, OVA-II-loaded MGl-HPG-modified liposomes and (b) 50 μg of free OVA, OVA-I or OVA-II. Cytotoxicity against EL4 cells was measured by a LDH assay at effector cells/target cell (E/T) ratio of (a) 5 or (b) 1. Each bar represents means ± SD (n = 3). No significant difference was found between any groups according to Tukey-Kramer method.

2.3.4. Induction of antitumor responses

The induction of antitumor immunity was investigated using MGl-HPG-modified liposomes containing OVA peptides (Fig. 2-5). OVA peptide-loaded liposomes were administered subcutaneously into mice and then, E.G7-OVA cells were inoculated to the mice. OVA protein-
loaded MGlu-HPG liposomes were also administered as a positive control to confirm the tumor rejection by OVA-specific CTLs [20–23]. The tumor size and mice survival were monitored. As presented in Fig. 5a, mice immunized with free OVA-I or OVA-II peptide solution exhibited a rapid increase in tumor volume after five days from the inoculation of tumor cells. However, mice immunized with these peptides-loaded MGlu-HPG liposomes showed a marked delay in the day on which the rapid tumor volume increase took place, indicating that the encapsulation of OVA-peptides by MGlu-HPG liposomes improved antitumor immune responses. For OVA-I-loaded MGlu-HPG liposomes, OVA-specific CTLs induced by these liposomes might kill the tumor cells efficiently (Fig. 2-4a). Compared to the OVA-I-loaded MGlu-HPG liposomes, OVA-II-loaded MGlu-HPG liposomes also showed tumor growth suppression, although these liposomes did not induce OVA-specific CTLs under the experimental conditions (Fig. 2-4a). Although details of the mechanism remain unclear at present, presumably, efficient delivery of OVA-II peptide to DCs by MGlu-HPG liposomes might induce MHC class II-mediated antigen presentation and the production of Th1 cytokine such as IFN-γ from OVA-specific Th1 cells. Th1 cytokine might activate NK cells or other innate immune cells to attack the tumor cells, resulting in tumor cell rejection. Survival rates of tumor-inoculated mice with immunization are presented in Figure 5b. Encapsulation of OVA-peptides by MGlu-HPG liposomes caused a significant improvement of the survival rates compared with administration of OVA-peptide solution \( (p = 0.00673 \text{ for OVA-I, } p = 0.00912 \text{ for OVA-II, Table 2-S1}) \). Actually, 25% or 50% mice survived, respectively, for longer than 200 days by the immunization with the liposome-encapsulated OVA-I or OVA-II peptide. Therefore, the use of MGlu-HPG liposomes enables the improvement of antigenic short peptides for the induction of antitumor immunity. Although the reason why a subset of mice treated with peptide-loaded MGlu-HPG liposomes survived remains unclear at present, individual differences of mice might affect the difference of mouse survival.
Fig. 2-5. Induction of OVA-specific immunity by immunization with MGlu-HPG-modified liposomes containing OVA proteins or OVA peptides on mice. C57BL/6 mice were subcutaneously immunized with 50 μg of OVA-I solution (open circles), OVA-I-loaded MGlu-HPG-modified liposomes (closed circles), OVA-II solution (open triangles), OVA-II-loaded MGlu-HPG-modified liposomes (closed triangles), and OVA-loaded MGlu-HPG-modified liposomes (closed squares) 14 and seven days before tumor cell inoculation. Seven days after second immunization, E.G7-OVA cells (1 × 10^6 cells) were inoculated subcutaneously into the left back of each mouse. The tumor volume was monitored. A timeline of experiments (a); change in tumor volume (b); and survival of mice (c) are shown. Each point represents means ± SD (n = 4). Mice were killed when tumor volumes became greater than 2500 mm^3. *p < 0.05 compared with free peptide-treated groups. Results of Log-rank test are shown in Table 2-S1.

**Table 2-S1.** Survival analysis by log-rank test for Figs 2-5c.

<table>
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<th>Comparison</th>
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</tr>
</thead>
<tbody>
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<td>OVA-I solution vs. OVA-I/MGlu-HPG liposome</td>
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</tr>
<tr>
<td>OVA-I solution vs. OVA-II solution</td>
<td>0.0169 *</td>
</tr>
<tr>
<td>OVA-I solution vs. OVA-II/MGlu-HPG liposome</td>
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</tr>
<tr>
<td>OVA-I/MGlu-HPG liposome vs. OVA-II solution</td>
<td>0.00673 **</td>
</tr>
<tr>
<td>OVA-I/MGlu-HPG liposome vs. OVA-II/MGlu-HPG liposome</td>
<td>0.00912 **</td>
</tr>
<tr>
<td>OVA-II/MGlu-HPG liposome vs. OVA/MGlu-HPG liposome</td>
<td>0.693</td>
</tr>
<tr>
<td>OVA-I/MGlu-HPG liposome vs. OVA/MGlu-HPG liposome</td>
<td>0.0401 *</td>
</tr>
<tr>
<td>OVA-II solution vs. OVA-II/MGlu-HPG liposome</td>
<td>0.00912 **</td>
</tr>
<tr>
<td>OVA-II solution vs. OVA/MGlu-HPG liposome</td>
<td>0.00912 **</td>
</tr>
<tr>
<td>OVA-II/MGlu-HPG liposome vs. OVA-MGlu-HPG liposome</td>
<td>0.127</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01.
Finally, we investigated the improvement of antigenic short peptides by encapsulation with MGlu-HPG liposomes for the treatment of tumor-bearing mice (Fig. 2-6). E.G7-OVA cells were inoculated subcutaneously to mice. After six days, free or liposome-encapsulated OVA-I or OVA was injected subcutaneously into the mice. Tumor sizes were monitored from the day of inoculation. Figure 6b shows that tumor volumes of mice treated with PBS increased rapidly. However, the mice treated with OVA-loaded MGlu-HPG-modified liposomes, which were used as a positive control, showed a decrease in tumor volume after Day 12, indicative of tumor suppression induced by OVA-specific CTL [23]. Administration of free OVA-I or OVA-I-loaded unmodified liposomes showed no tumor suppression, indicating that neither free OVA-I nor OVA-I-loaded unmodified liposomes are effective for induction of antitumor immunity. In contrast, OVA-I-loaded MGlu-HPG-modified liposomes exhibited significant suppression of tumor growth after Day 12, as was the case of OVA-loaded MGlu-HPG-modified liposomes based on $p$-value. Compared to the OVA-loaded liposomes, the OVA-I-loaded liposomes exhibited a lower degree of tumor suppression, probably because OVA protein has higher antigenicity than short peptide OVA-I. Effects of boost immunization were also examined. These liposomes were administered to mice twice (Days 6 and 13) (Fig. 2-S4). Then, their therapeutic efficacy was compared to that of their single administration. As presented in Figures 2-6b and 2-S4b, and in Table 2-S2, the survival period increased significantly ($p = 0.0285$) by additional administration of the OVA-I-loaded MGlu-HPG liposomes.
Fig. 2-6. Antitumor effects induced by immunization with MGl-u-HPG-modified liposomes containing OVA proteins or OVA peptides on tumor-bearing mice. (a) Timeline of experiments. (b,c) The E.G7-OVA cells (5 × 10^5 cells) were inoculated subcutaneously into the left back of C57BL/6 mice and MGl-u-HPG-modified liposomes containing 50 μg of OVA proteins (squares), MGl-u-HPG-modified liposomes containing 50 μg of OVA-I peptides (circles), unmodified liposomes containing 50 μg of OVA-I peptides (diamonds) and OVA-I solution (triangles) were administered subcutaneously into the right back of the mice once on Day 6 (open symbols) or twice on Days 6 and 13 (closed symbols). Data for mice immunized with PBS (dotted line) are presented as control data. * p < 0.05 compared with PBS-treated groups. † p < 0.05 compared with unmodified liposomes-treated groups. Results of a Log-rank test are shown in Table S2. (d, e) The E.G7-OVA cells (5 × 10^5 cells) were inoculated subcutaneously into the left back of C57BL/6 mice and MGl-u-HPG-modified liposomes containing 50 μg of OVA proteins (closed squares), MGl-u-HPG-modified liposomes containing 50 μg of OVA-II peptides (closed triangles) and OVA-II solution (open triangles) were administered subcutaneously into the right back of the mice on Day 6. Mice immunized with PBS (closed diamonds) are shown as controls. All treated groups included four mice. Changes in tumor volumes (b,d); and mice survival (c,e) are shown. Mice were killed when tumor volumes became greater than 2500 mm^3. ‡ p < 0.05 compared with OVA-II solution-treated groups. ‡ p < 0.05 compared with OVA-II-loaded MGl-u-HPG-modified liposome-treated groups. Results of Log-rank tests are shown in Table 2-S3.
Fig. 2-S4. Antitumor effect induced by immunization with MGlu-HPG-modified liposomes containing OVA proteins or OVA-I peptides on tumor-bearing mice. The E.G7-OVA cells (5 × 10⁵ cells) were subcutaneously inoculated into the left backs of C57BL/6 mice and MGlu-HPG-modified liposomes containing 50 μg of OVA proteins (squares), MGlu-HPG-modified liposomes containing 50 μg of OVA-I peptides (circles), were subcutaneously administered into the right backs of the mice twice on days 6 and 13. Mice immunized with PBS (dotted line) were shown as controls. Change in tumor volume (a) and survival of mice (b) were shown. Mice were sacrificed when tumor volumes became over 2,500 mm³. * p < 0.05 compared with PBS-treated group. Results of Log-rank test was shown in Table S2.

Table 2-S2. Survival analysis by log-rank test for figs 2-6c and 2-S4b.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>PBS vs. OVA-I/MGlu-HPG liposome (twice)</td>
<td>0.00912 *</td>
</tr>
<tr>
<td>PBS vs. OVA-I/unmodified liposome (twice)</td>
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</tr>
<tr>
<td>PBS vs. OVA/MGlu-HPG liposome (once)</td>
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</tr>
<tr>
<td>PBS vs. OVA/MGlu-HPG liposome (twice)</td>
<td>0.0285 *</td>
</tr>
<tr>
<td>PBS vs. OVA-I solution (twice)</td>
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</tr>
<tr>
<td>OVA-I/MGlu-HPG liposome (once) vs. OVA-I/MGlu-HPG liposome (twice)</td>
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<tr>
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</tr>
<tr>
<td>OVA-I/MGlu-HPG liposome (twice) vs. OVA-I solution (twice)</td>
<td>0.0415 *</td>
</tr>
<tr>
<td>OVA-I/unmodified liposome (twice) vs. OVA/MGlu-HPG liposome (once)</td>
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<tr>
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<td>OVA-I/unmodified liposome (twice) vs. OVA-I solution (twice)</td>
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<td>OVA/MGlu-HPG liposome (once) vs. OVA-I solution (twice)</td>
<td>0.0404 *</td>
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<tr>
<td>OVA/MGlu-HPG liposome (twice) vs. OVA-I solution (twice)</td>
<td>0.0292 *</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01.
Immunization with OVA-II-loaded MGlu-HPG liposomes before tumor cell inoculation showed tumor growth suppression (Fig. 2-5). Therefore, we also examined therapeutic effects of OVA-II peptide-loaded MGlu-HPG-modified liposomes (Fig. 2-6d, e, Table 2-S3). However, the OVA-II-loaded liposomes exhibited tumor suppressive effects only slightly. Indeed, OVA-II peptide was designed to be presented on MHC class II, which engenders the induction of OVA-specific helper T cells, not CTL.

Table 2-S3. Survival analysis by log-rank test for Fig 2-6e.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS vs. OVA/MGlu-HPG liposome</td>
<td>0.01 *</td>
</tr>
<tr>
<td>PBS vs. OVA-II/MGlu-HPG liposome</td>
<td>0.774</td>
</tr>
<tr>
<td>PBS vs. OVA-II solution</td>
<td>0.0404 *</td>
</tr>
<tr>
<td>OVA/MGlu-HPG liposome vs. OVA-II/MGlu-HPG liposome</td>
<td>0.0171 *</td>
</tr>
<tr>
<td>OVA/MGlu-HPG liposome vs. OVA-II solution</td>
<td>0.00815 **</td>
</tr>
<tr>
<td>OVA-II/MGlu-HPG liposome vs. OVA-II</td>
<td>0.317</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01.

Therefore, it is likely that the MGlu-HPG liposomes achieved efficient association to DCs through interaction with scavenger receptors and efficient delivery of OVA-I peptide into DC cytosol using their pH-sensitive membrane fusion ability [20–23]. Subsequent presentation of OVA-I peptide on MHC class I might cause efficient induction of target-specific CTL. As a result, significant tumor suppression was induced. Indeed, OVA-I peptide encapsulated with MGlu-HPG liposomes exhibited much stronger ability for induction of tumor-specific immunity and therapeutic effects than the free form of the same peptide. The immunity-inducing effect by OVA-I peptide encapsulated with MGlu-HPG liposomes remained lower than that of OVA protein-loaded MGlu-HPG liposomes, probably because OVA protein has many epitopes and can induce various CTLs and Th cells [22,23]. In the case of actual cancer treatment, limited amounts of tumor whole proteins can be recovered from patients. In contrast, tumor peptides can be synthesized to an unlimited degree, which is the most effective point of using of tumor peptides instead of whole tumor proteins. Future efforts will be undertaken to improve the immunity-inducing ability of MGlu-HPG liposomes by introduction of
adjuvant functions such as cationic lipid, CpG-DNA, cytokine gene. In addition, higher antigenic long peptides have been developed for the induction of both CTL and Th cells [28]. The use of such long peptides is also expected to enhance the therapeutic effects of peptide-based vaccines.

2. 4. Conclusion

This study investigated the improvement of antigenic short peptide OVA-I through their encapsulation with MGlu-HPG-modified liposomes, which have both a high affinity to DC and pH-sensitive membrane fusion ability. Indeed, we observed that encapsulation of the peptide in the liposomes strongly improved the immune-induction ability of the peptide. Immunization with the liposome-encapsulated peptide achieved growth suppression of established tumors and complete rejection of tumors in a subset of mice. Results demonstrate that the use of appropriate carriers such as MGlu-HPG liposomes, which deliver short peptide vaccines into DC cytosol efficiently, can greatly potentiate their efficacy. Therefore, MGlu-HPG liposomes offer great potential to improve the efficacy of peptide vaccine for antigen-specific CTL, which is crucially important for the establishment of efficient cancer immunotherapy.

2. 5. References


Chapter 3: Potentiation of pH-sensitive polymer-modified liposomes with cationic lipid inclusion as antigen delivery carriers for cancer immunotherapy

3.1. Introduction

Cancer immunotherapy, which activates cancer-specific immune responses for the treatment, has received much attention [1-5]. Efficient delivery of cancer-specific antigens into antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages is crucial important to activate such immune responses [1,4,5]. Actually, through the presentation of antigen, DCs can activate antigen-specific immunities of two types: humoral immunity and cellular immunity [6,7]. When exogenous antigenic proteins are taken up by DCs via endocytosis, these molecules are degraded to peptide fragments in lysosome. In addition, the antigen-derived peptides are presented on major histocompatibility complex (MHC) class II molecules to the CD4+ T cells. Then, CD4+ T cells differentiate to subsets of helper T cells such as Th1 cells and Th2 cells, which activate cytotoxic T lymphocytes (CTLs) or B cells, respectively, via secretion of various cytokines. In contrast, endogenous antigenic proteins existing in cytosol of DCs are degraded by proteasomes. Their derived peptide fragments are presented on MHC class I molecules to the CD8+ T cells, which then differentiate to antigen-specific CTLs. The induction of the tumor-specific CTLs is generally regarded as important to achieve efficient cancer immunotherapy because these CTLs attack the target cells directly and eliminate them effectively. Therefore, using antigen delivery systems that generate antigen-specific CTLs and Th1 cells is important for the induction of effective cellular immunity with high tumor-specificity.

To date, many antigen delivery systems have been produced using polymer-based or membrane-based nanoparticles for induction of cellular immunity [8-15]. Some membrane-based nanoparticles such as liposomes with membrane-active properties are regarded as especially good candidates for this purpose because such systems can introduce antigenic proteins into cytosol of DCs by
destabilizing or fusing with cellular or endosomal membranes and thereby inducing the antigen-derived peptide presentation on MHC class I molecules. To date, liposomes modified with fusion active or membrane-lytic molecules such as viral proteins and their derived peptides and pH-sensitive polymers have been studied for antigen delivery into DCs for induction of cellular immunity [11-15]. From a safety perspective, to avoid unexpected biological effects including immune responses derived from viral molecules, synthetic molecules might be more desirable than virus-derived molecules for the construction of liposomes as an antigen delivery vehicle. Although synthetic molecules of various types have been used to provide membrane disruptive or fusion abilities to liposomes [16-22], synthetic polymers might be chosen this purpose because of the high efficiency attributable to their large molecular size and the wide freedom they provide for the design of molecular architectures. Especially, pH-sensitive fusogenic polymers, which generate a strong fusion capability under weakly acidic conditions, are beneficial because these polymers generate fusion activity only under weakly acidic environments, such as that inside an endosome. Therefore, when taken up by APCs via endocytosis, liposomes modified by these polymers can introduce entrapped antigens into their cytosol by fusion with endosomal membrane.

In a previous study, we used egg yolk phosphatidylcholine (EYPC) liposomes modified with 3-methylglutarylated hyperbranched poly(glycidol) (MGlu-HPG), which exhibit highly fusogenic and membrane disruptive abilities under weakly acidic conditions, for the delivery of antigenic protein ovalbumin (OVA) into cytosol of DCs. Results showed that the MGlu-HPG-modified liposomes delivered OVA efficiently into cytosol of DCs [15,21,22] and that they induced antigen presentation via MHC class I molecules, which causes induction of antigen-specific cellular immunity. In addition, subcutaneous administration of the MGlu-HPG-modified, OVA-loaded liposomes into mice bearing OVA-expressing tumors induced the shrinkage of tumors through the OVA-specific immunity.

For T cell activation, DCs must express co-stimulatory molecules such as CD80 and CD86 molecules on their surface, which stimulates T cells via interaction with CD28, together with the
antigen peptide presentation on MHC molecules [23-25]. The expression of these co-stimulatory molecules on DCs is enhanced when DCs are activated [26, 27]. Therefore, antigen delivery systems should have capabilities not only to deliver antigen into DCs but also to activate DCs, which is known as an adjuvant function, for the induction of effective antigen-specific immunity. Molecules and materials of many types have adjuvant functions: monophosphoryl lipid A (MPLA), CpG-DNA, β-glucan, and so on [27-32]. Among them, cationic lipids might be potent adjuvants because liposomes containing cationic lipids, such as 1, 2-dioleoyl-3-(trimethyl ammonium) propane (DOTAP), have been shown to activate APCs and to induce antigen-specific immune responses effectively [32-34].

Toward development of potent antigen delivery systems that achieve induction of antigen-specific immunity, we designed MGlu-HPG-modified liposomes containing cationic lipids as a membrane component, which introduce antigen molecules into cytosol of DCs and activate them simultaneously (Fig. 3-1). For this study, we used 3, 5-didodecylxybenzamidine (TRX) as a cationic lipid because TRX is stably incorporated in phospholipid-based membranes and because its pDNA complexes induced up-regulation of MHC class I molecules when taken up by DCs [35]. Here, we examine the capabilities of MGlu-HPG-modified, TRX-containing liposomes to deliver antigenic proteins into DCs, to activate DCs, and to induce antigen-specific immune response. In addition, we investigate the importance of synergistic effect of MGlu-HPG-induced antigen delivery into DCs and TRX-induced DC activation for the induction of antigen-specific immunity.
3.2. Materials and methods

3.2.1. Materials

EYPC was kindly donated by NOF Co. (Tokyo, Japan). 3,5–Didodecyloxybenzamidine hydrochloride (TRX) were kindly donated by Terumo Corp., Ltd. (Kanagawa, Japan). Lissamine rhodamine B–sulfonyl phosphatidylethanolamine (Rh–PE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). OVA, bovine serum albumin (BSA), 3-O-desacyl-4’-monophosphoryl lipid A (MPLA), and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO.). Pyranine, sodium azide and Triton X–100 were obtained from Tokyo Chemical Industries Ltd. (Tokyo, Japan). p–Xylene–bis–pyridinium bromide (DPX) was from Molecular Probes (Oregon, USA). Tween20 was obtained from Nacalai Tesque, Inc., (Kyoto, Japan). FITC–OVA was prepared by reacting OVA (10 mg) with FITC (11.8 mg) in 0.5 M NaHCO3 (4 mL, pH 9.0) at 4 °C for three days and subsequent dialysis [21]. 3–Methylglutarylated hyperbranched poly(glycidol) with polymerization degree of 60 (MGluc–HPG) was prepared as previously reported [21]. The ratios of hydroxyl units, MGluc units and decyl amide units for MGluc–HPG was 9/80/11, as estimated using 1H NMR [21].
3. 2. 2. Cell culture

DC2.4 cells, which were an immature murine DC line, were provided from Dr. K. L. Rock (Harvard Medical School, USA) and were grown in RPMI–1640 (Nacalai Tesque) supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L–glutamine (Wako), 100 mM MEM nonessential amino acid (Nacalai Tesque), 50 μM 2–mercaptoethanol (2–ME, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C [36]. 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) [37]. CD8–OVA1.3 cells, a T–T hybridoma against OVA257–264/H–2Kb complex, were kindly provided by Dr. C.V. Harding [38], and were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% FBS, 50 μM 2–ME, 100 U/mL penicillin and 100 μg/mL streptomycin. OT4H.1D5 cells, a T–T hybridoma against OVA265–277/I–Aβ complex, were kindly provided by Dr. J.A. Kapp [39], and were cultured in RPMI–1640 medium supplemented with 10% FBS, 50 μM 2–ME, 100 U/mL penicillin and 100 μg/mL streptomycin.

3. 2. 3. Animals

Female C57BL/6 mice (H–2b, 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.

3. 2. 4. Preparation of liposomes

To a dry, thin membrane of EYPC and various mol% of TRX (total lipids; 1.25 × 10⁻⁵ mol) was added 500 μL of 35 mM pyranine, 50 mM DPX, and 25 mM phosphate solution (pH 7.4) and the mixture was sonicated for 2 min using a bath–type sonicator. 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 centrifuged with the speed of 55,000 rpm for 2 h at 4 °C twice to remove free pyranine from the pyranine–loaded liposomes. MGlut–HPG–modified
liposomes were also prepared according to the above procedure using dry membrane of a lipid mixture with MGl u–HPG (lipids/polymer = 7/3, w/w).

3.2.5. Dynamic light scattering and zeta potential

OVA–loaded liposomes were prepared as described above except that mixtures of polymers and lipids were dispersed in OVA–containing phosphate buffered saline (PBS) solution (pH 7.4, 4 mg/mL). Diameters and zeta potentials of the liposomes (0.1 mM lipids) in 0.1 mM phosphate aqueous solution 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.

3.2.6. Release of pyranine from liposome

Release of pyranine from liposome was measured as previously reported [20,40]. Liposomes encapsulating pyranine (lipid concentration: 2.0 × 10^{-5} M) were added to PBS of varying pHs at 37 °C and fluorescence intensity at 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

\[
\text{Release} \% = \left( \frac{F_t - F_i}{F_f - F_i} \right) \times 100
\]

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 Triton X–100 (final concentration: 0.1%).

3.2.7. Intracellular behavior of liposomes

The FITC–OVA–loaded liposomes containing Rh–PE were prepared as described above except that a mixture of polymer and lipids containing Rh–PE (0.1 mol%) was dispersed in PBS 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 (1 mL). The FITC–OVA–loaded liposomes (0.5 mM of lipid concentration, 1 mL) 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 DC2.4 cells treated with these liposomes without FITC–OVA was also determined by a flow cytometric analysis (EPICS XL, Beckman Coulter, Inc).

3.2.8 Analysis of liposome-treated DC phenotype

The DC2.4 cells (3 × 10⁵ cells) cultured for 2 days in a 6-well plate were washed with HBSS, and then incubated in serum–free RPMI–1640 medium (500 μL). The OVA–loaded liposomes (0.5 mM of lipid concentration, 500 μL) were added gently to the cells and incubated for 4 h at 37 °C. After the incubation, supernatants of cultured cells were collected for measurements of cytokines (IFN–γ and IL–10) production in the following section. The cells were washed with HBSS three times and cultured for 24 h. DC2.4 cells treated with 10 μg/mL LPS (Nacalai Tesque, Inc., Kyoto, Japan) and 100 units/mL recombinant mouse IFN–γ (PeproTech EC LTD., London, England) for 24 h were used as positive controls for phenotypical DC maturation. At 24 h, supernatants of cultured cells were collected for measurements of cytokines (TNF–α, IL–6) production in the following section. Cell phenotype was confirmed by a flow cytometric analysis. Briefly, 10⁶ cells in 100 μL of staining buffer (PBS containing 0.1% BSA and 0.01% sodium azide) were incubated for 30 min on ice with the anti–Fc γRII/III monoclonal antibody (eBioscience, 2.4G2) to block nonspecific binding of the subsequently used antibody reagents. The cells were re–suspended in 100 μL of staining buffer and incubated for 30 min on ice, using the manufacturer’s recommended amounts of biotinylated antibodies: anti–mouse H–2Kb/Db (BD Pharmingen, 28–8–6) and FITC–labeled anti–CD80 (abcam, 16–10A1). The cells were then re–suspended in 100 μL of staining buffer containing 10 μL of R–Phycoerythrin (PE)–conjugated streptavidin (Sigma), and nonspecific binding was measured using PE–conjugated streptavidin alone. After incubation for 30 min on ice, 10,000 events of the stained
cells were analyzed for surface phenotype, using a flow cytometer (EPICS XL, Beckman Coulter, Inc). Between all incubation steps, cells were washed three times with staining buffer.

3. 2. 9. Cytokine from DC2.4 cells treated with liposomes

Cytokine (IFN–γ, IL–10, TNF–α and IL–6) production in supernatants of DC2.4 cells collected in the above section was measured using an enzyme-linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd.) according to the manufacture’s instruction.

3. 2. 10. Antigen presentation assay

The DC2.4 cells (3 × 10^5 cells) cultured for 2 days in a 6–well plate were washed with HBSS, and then the cells were treated with OVA–loaded liposomes. After 4 h incubation at 37 °C, the cells were washed three times with HBSS. Subsequently, the cells (2 × 10^4 cells) were co–cultured with CD8–OVA1.3 cells (2 × 10^4 cells) or OT4H.1D5 cells (2 × 10^4 cells) for 24 h at 37 °C in 96–well round bottom dish. The response of stimulated CD8–OVA1.3 or OT4H.1D5 cells was assessed by determining the amount of IL–2 released into an aliquot of culture medium (100 μL) using a murine IL–2 ELISA Kit (PeproTech EC Ltd.) according to the manufacture’s instruction.

3. 2. 11. Antibody production

On days 0 and 7, 50 μg of OVA–loaded liposomes were subcutaneously injected into the right backs of the mice under anesthesia with isoflurane. On day 14, 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% 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 IgG2a 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). Antibody titers were represented as the reciprocal of endpoint dilution exhibiting an optical density more than 2.5 times that of background [41].

3. 2. 12. In vitro cytokine release measured by ELISA

50 µg of OVA-loaded liposomes or PBS were subcutaneously injected into the right backs of the mice under anesthesia twice at a week intervals. After a week from second immunization mice were sacrificed and splenocytes were suspended in RPMI–1640 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 × 10⁶ in 2 mL) were cultured with or without 50 µg/mL of OVA for 5 days. After incubation, the concentration of IFN–γ was measured using murine IFN–γ ELISA development kit (Peprotech, London, UK) according to the manufacture’s instruction.

3. 2. 13. Treatment of tumor-bearing mice with liposomes

E.G7–OVA cells (1 × 10⁶ cells) were subcutaneously inoculated into the left backs of C57BL/6 mice under anesthesia with isoflurane. On days 5 and day 12, 50 µg of OVA-loaded liposomes with or without MPLA (4 g/mol lipid) were subcutaneously injected into the right backs of the mice under anesthesia with isoflurane. 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 mm³. All treated groups contained four mice.
3. 3. Results and Discussion

3. 3. 1. Preparation of cationic lipid-incorporated liposomes modified with pH-sensitive polymers

Table 3-1 presents diameters and zeta potentials of liposomes used for this study. Most liposomes exhibited mean diameters around 100nm, which correspond to the pore size of the filter membrane used for extrusion of these liposomes. The zeta potentials of MGlù-HPG-modified liposome without TRX were, respectively, $-15.7$ mV and $1.3$ mV at pH 7.4 and pH 5.5, suggesting that the surface properties of the liposomes were affected by the charged state of the polymer chains with carboxyl groups attached onto the liposome surface. The MGlù-HPG-modified liposomes containing cationic lipid TRX exhibited even more negative zeta potentials despite the positive charge of liposomal membrane at pH 7.4. Probably, positively charged surface of the liposome membrane enhanced attachment of the polymer chains, resulting in more efficient coverage of the liposome membranes with the polymer chains compared to the polymer-modified liposomes without TRX. In contrast, these polymer-modified liposomes with TRX exhibited positively zeta potentials at pH 5.5. Because carboxyl groups of the polymer chains became protonated at that pH, the polymer chains without charge might take on a compact conformation, resulting in exposure of the positively charged surface of the liposome membranes [21]. In fact, the zeta potentials of the polymer-modified liposomes at pH 5.5 increased concomitantly with increasing TRX contents, indicating that their zeta potentials were reflected by the cationic lipid content of liposome membranes (Table 3-1).

<table>
<thead>
<tr>
<th>TRX content / mol%</th>
<th>Mean diameter / nm</th>
<th>(\zeta)-potential / mV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>0</td>
<td>115 ± 2.4</td>
<td>-15.7 ± 2.5</td>
</tr>
<tr>
<td>10</td>
<td>111 ± 9.4</td>
<td>-28.4 ± 2.9</td>
</tr>
<tr>
<td>20</td>
<td>185 ± 11</td>
<td>-50.7 ± 1.5</td>
</tr>
<tr>
<td>30</td>
<td>120 ± 5.2</td>
<td>-46.3 ± 1.4</td>
</tr>
</tbody>
</table>

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3. 3. 2. Effects of cationic lipid inclusion on pH-sensitivity of liposomes

Previously, we reported that MGlu-HPG-modified EYPC liposomes are stable and that they retain water soluble molecules such as pyranine at neutral pH, but release their contents at mildly acidic pH where carboxyl groups on the polymer chains are protonated [21]. The polymer chains on a protonated state can interact strongly with the liposome membrane and can induce content release at weakly acidic pH. Therefore, we examined the effect of cationic lipid inclusion on the pH dependent destabilization of the polymer-modified liposomes (Fig. 3-2). Fig. 3-2A presents examples of pyranine release profiles from MGlu-HPG-modified liposomes with various TRX contents at pH 6.0. Apparently, the polymer-modified liposomes exhibited more intensive content release than the polymer-modified liposomes without TRX. Considering that the inclusion of TRX in the liposome membrane did not affect the stability of the membrane under experimental conditions, inclusion of the cationic lipid might enhance interaction of the liposome membranes with polymers at that pH.

Fig. 3-2B depicts the percentage release of polymer-modified liposomes with varying TRX contents as a function of pH. Irrespective of TRX contents, these liposomes only slightly release pyranine at neutral pH. However, as the environmental pH decreased below around 6.5-6.0, the content release from the polymer-modified liposomes increased considerably. Moreover, compared to the polymer-modified liposomes without TRX, the polymer-modified liposomes with TRX triggered the content release even at more weakly acidic pH. This result again suggests that the polymer-lipid membrane interaction was enhanced by the inclusion of TRX.

In addition, this TRX-dependent enhancement of content released from the polymer-modified liposomes was observed only in weakly acidic pH regions (Fig. 3-2A). At neutral pH, where carboxyl groups of the polymer chains are largely dissociated, the polymer chains with highly hydrophilic property were unable to destabilize the liposome membranes irrespective of the TRX inclusion. At low pH, where carboxyl groups of the polymer chains are mostly protonated, the polymer chains with highly hydrophobic property were able to destabilize the liposome membranes irrespective of the TRX inclusion. In contrast, at weakly acidic pH, where carboxyl groups of the polymer chains are
partially protonated, the polymer chains with insufficient hydrophobicity might not interact with the
TRX-free membrane through hydrophobic interaction but interact strongly with TRX-incorporated
membrane synergistically through hydrophobic interaction and electrostatic interaction.

3.3.3. Effects of cationic lipid inclusion on interaction of the liposomes with dendritic cells

We examined the effects of cationic lipid inclusion on association of MGlue-HPG-modified liposomes with DCs. DC2.4 cells, a murine DC line, were incubated with the liposomes labeled with fluorescent lipid Rh-PE. Then the fluorescence intensity of cells was measured using a flow cytometer (Fig. 3-3). Compared with MGlue-HPG-modified liposomes without TRX, TRX-incorporated liposomes exhibited much higher fluorescence intensity. Especially, liposomes with 20 mol% of cationic lipids showed 27 times higher association with the cells than that of liposomes without cationic lipids. Poly(carboxylic acid)-modified liposomes are known to be taken up by macrophages and DCs via scavenger receptors [42,43]. In our previous studies, we showed that EYPC liposomes modified with carboxylated poly(glycidol)s or carboxylated dextrans were taken up by DCs through interaction with scavenger receptor [21,22,35]. As shown in Table 3-1, inclusion of TRX generated
a highly negative charged surface of the MGLu-HPG-modified liposomes, probably because of enhanced association of the negatively charged polymer chains onto the positively charged liposome membranes. Therefore, such liposome surfaces with densely accumulated MGLu-HPG chains might induce efficient recognition of liposomes via scavenger receptors on DCs, resulting in highly efficient uptake of the TRX-incorporated liposomes by DCs.

**Fig. 3-3.** Histograms (A) and mean fluorescence intensity (B) of DC2.4 cells treated with MGLu-HPG-modified liposomes with various TRX content mol% containing 0.1 mol% of Rh-PE. Cells were incubated with various liposomes at 0.5 mM lipid concentration for 4 h in the absence of serum. © 2014 Elsevier Ltd.

We described previously that MGLu-HPG-modified liposomes delivered entrapped contents such as OVA into cytosol of DC [15,21]. Therefore, we examined the influence of cationic lipid inclusion on their intracellular delivery ability. We prepared liposomes containing Rh-PE and FITC-OVA for the respective detection of liposomes and OVA molecules inside of the cells. DC2.4 cells were incubated with Rh-PE-labeled and FITC-OVAloaded liposomes. Then the cells were observed using confocal laser scanning microscopy (Fig. 3-4). DC2.4 cells treated with MGLu-HPG liposomes without TRX exhibited diffuse fluorescence of FITC-OVA as well as punctate fluorescence FITC-OVA (Fig. 3-4A). In addition, the punctate fluorescence of FITC-OVA and Rh-PE fluorescence were observed mostly at the same sites of the cells. Considering that these liposomes are taken up by DCs through endocytosis, diffuse fluorescence and punctate fluorescence of FITC-OVA derive respectively from those locating in cytosol and those locating in the endosome or lysosome [15]. Nevertheless, it seems that the punctate fluorescence of FITC-OVA tends to increase concomitantly
with increasing TRX content in the liposome membrane, although diffuse fluorescence of FITC-OVA is also observed in these liposome-treated cells (Fig. 3-4B-D). This result suggests that, after incubation with the liposomes, the cells contained OVA molecules both in cytosol and in endosomal or lysosomal compartments but use of the liposomes with higher TRX contents tends to increase the fraction of OVA molecules trapped in the endosomes and/or lysosomes. The MGlut-HPG chains getting a hydrophobic character at acidic pH are likely to interact strongly with the liposome membrane and the endosome membrane, resulting in their significant destabilization and the transfer of OVA from endosome interior into cytosol. However, as the TRX content of the liposome membrane increases, MGlut-HPG chains might be associated tightly on the surface of positively charged liposome membranes through electrostatic interaction. Therefore, the polymer chains might tend to interact preferentially with the liposome membranes rather than the endosomal membrane, resulting in elevation of the OVA fraction remaining in endosomes. These results demonstrate that intracellular distribution of OVA molecules might be controlled by the cationic lipid content of the liposome membranes.

![Fig. 3-4. Histograms (A) and mean fluorescence intensity (B) of DC2.4 cells treated with MGlut-HPG-modified liposomes with various TRX content mol% containing 0.1 mol% of Rh-PE. Cells were incubated with various liposomes at 0.5 mM lipid concentration for 4 h in the absence of serum. © 2014 Elsevier Ltd.](image-url)
3. 3. 4. Activation of dendritic cells by cationic lipid-incorporated liposomes

For the induction of antigen-specific immunity, antigen-derived epitope peptides must be presented by APCs such as DCs via MHC class I molecules and MHC class II molecules, which respectively induce activation of CD8+ cytotoxic T cells (CTLs) and CD4+ helper T cells. In addition, stimulation signals via co-stimulatory molecules such as CD80 and CD86 on APCs are crucial for the efficient activation of antigen-specific T cells [22-25]. Therefore, we analyzed activation of DC2.4 cells in terms of co-expression of MHC class I molecules and CD80 molecules on DC2.4 cells treated with the OVA-loaded liposomes using flow cytometry with MHC class I-specific or CD80-specific antibodies. As presented in Fig. 3-5A, 58% of DC2.4 cells exhibited co-expression of MHC class I and CD80 molecules before the liposome treatment. However, after the liposome treatment, the co-expressing cells increased to 73-76%, irrespective of the TRX content of the liposomes. Considering that the treatment with strong adjuvants LPS and IFN-γ induced 87% of the co-expressing DC2.4 cells, these polymer-modified liposomes might be regarded as having a high ability to activate DCs irrespective of cationic lipid contents.

The liposome-induced activation of DC2.4 cells was further investigated from the induction of cytokine production of DC2.4 cells. Fig. 3-5B shows production of IFN-γ and IL-10, which respectively activate Th1 responses and Th2 responses [44], from DC2.4 cells during 4 h incubation with liposomes containing varying TRX contents. Compared to the untreated DC2.4 cells, which only slightly produced these cytokines, the liposome-treated cells produced much greater amounts of cytokines. Especially, the cells treated with the liposomes containing 20-30% of TRX induced a high level of cytokine production. The result suggests that these liposomes have the capability of activating DC2.4 cells for induction of Th1 and Th2 responses. In addition, the production of inflammatory cytokines TNF-α and IL-6, which are known to be indicators of DC activation [45], by DCs were measured. As Fig. 3-5C shows, production of these cytokines was enhanced with increasing TRX content in the liposomes. Results show that inclusion of cationic lipid onto the polymer-modified liposomes increases their capability of activating DCs.
**Fig. 3-5.** Activation of DC2.4 cells by treatment with liposomes. (A) Expression of MHC class I and CD80 molecules on DC2.4 cells treated with various liposomes at 0.5 mM lipid concentration for 4 h in the absence of serum and cultured for another 24 h in the presence of serum. As a positive control, cells were treated with 10 mg/mL LPS and 100 U/mL IFN-γ for 24 h. DC2.4 cells stained by anti-mouse MHC class I (H-2K^b/2D^b) biotinylated monoclonal antibodies of the indirect specificities followed by PE-conjugated streptavidin and FITC-labeled anti-CD80 monoclonal antibodies. Value in the upper right corner of each panel represents co-expression% of MHC class I and CD80. (B) IFN-γ and IL-10 production from DC2.4 cells treated with various liposomes at 0.5 mM lipid concentration for 4 h in the absence of serum. (C) TNF-α and IL-6 production from DC2.4 cells treated with various liposomes at 0.5 mM lipid concentration for 4 h in the absence of serum and cultured for another 24 h in the presence of serum. © 2014 Elsevier Ltd.
Next we investigated antigen presentation upon treatment with the OVA-loaded liposomes. DC2.4 cells were treated with OVA-loaded liposomes. Then the treated DCs were co-cultured with T cells of two kinds that respectively recognize the MHC class I/OVA derive peptide complexes and MHC class II/OVA-derived peptide complexes: CD8-OVA1.3 and OT4H.1D5 cells. These MHC class restricted OVA-derived peptide presentations were evaluated by measuring IL-2 secretion from these T cells when co-cultured with the liposome-treated DC2.4 cells (Fig. 3-6). As depicted in Fig. 3-6A, a high level of IL-2 secretion was induced from CD8-OVA1.3 cells by co-culturing with the OVA-loaded liposomes, compared to those co-cultured with free OVA-treated DCs, indicating that the OVA-loaded liposomes derived the MHC class I molecule-mediated presentation more efficiently than free OVA. However, no difference was observed among liposomes with different cationic lipid contents. A similar enhancement of IL-2 secretion was observed for OT4H.1D5 cells co-cultured with the OVA-loaded liposomes in comparison to those co-cultured with free OVA-treated DCs (Fig. 3-6B), indicating that the OVA-loaded liposomes have a higher capability of inducing the MHC class II-mediated presentation than free OVA has. The OVA-loaded liposomes with 20-30 mol% TRX showed slightly higher capability of inducing the OVA peptide presentation among these liposomes with different TRX contents. These results indicate that these liposomes can engender induction of the antigen presentation through both MHC class I and MHC class II molecules and that liposomes with high cationic lipid contents might have higher ability to induce antigen presentation through MHC class II molecules. These cationic lipid-incorporated liposomes were taken up by the cells efficiently and delivered OVA molecules both in endosomes/lysosomes and in cytosol of DCs (Figs. 3-3 and 3-4). Therefore, their excellent delivery performance might engender efficient antigen presentation via MHC class I and class II molecules.
Fig. 3-6. Activation of DC2.4 cells by treatment with liposomes. (A) Expression of MHC class I and CD80 molecules on DC2.4 cells treated with various liposomes at 0.5 mM lipid concentration for 4 h in the absence of serum and cultured for another 24 h in the presence of serum. As a positive control, cells were treated with 10 mg/mL LPS and 100 U/mL IFN-γ for 24 h. DC2.4 cells stained by anti-mouse MHC class I (H-2Kb/2Dd) biotinylated monoclonal antibodies of the indirect specificities followed by PE-conjugated streptavidin and FITC-labeled anti-CD80 monoclonal antibodies. Value in the upper right corner of each panel represents co-expression% of MHC class I and CD80. (B) IFN-γ and IL-10 production from DC2.4 cells treated with various liposomes at 0.5 mM lipid concentration for 4 h in the absence of serum. (C) TNF-α and IL-6 production from DC2.4 cells treated with various liposomes at 0.5 mM lipid concentration for 4 h in the absence of serum and cultured for another 24 h in the presence of serum. © 2014 Elsevier Ltd.

3. 3. 5. Induction of antigen-specific immune responses in vivo

We next examined the ability of the cationic lipid-incorporated liposomes for induction of antigen-specific immune responses in vivo. OVA-loaded liposomes of various types were administered twice subcutaneously to C57BL/6 mice. Seven days after the second immunization, whole blood was collected from immunized mice. OVA-specific IgG titer in serum was evaluated using ELISA (Fig. 3-7A). In the case of mice immunized with PBS, no OVA-specific IgG was detected from their sera. In contrast, immunization with OVA-loaded liposome provided production of OVA-specific IgG, indicating that administration of OVA-loaded liposomes induced OVA-specific immune responses. Similar levels of OVA-specific IgG production were observed for mice treated with liposomes with 0-20% TRX, but the liposomes with 30 mol% TRX induced higher level of OVA-specific IgG production, suggesting that high contents of the cationic lipid in the liposome
might enhance antigen-specific IgG production. Production of IgG subclasses IgG1 and IgG2a is known to derive respectively from Th1 and Th2. Their titer ratio is used to obtain information related to the activation of cellular immunity and humoral immunity [29, 46, 47]. Therefore, we measured their titer ratio to estimate the balance of cellular immunity and humoral immunity mediated by the liposomes. As portrayed in Fig. 3-7B, mice treated with the liposomes containing higher TRX contents exhibited higher IgG2a/IgG1 ratios, suggesting that cationic lipid inclusion in the liposomes induced Th1-dominant immune responses. Considering that the liposomes with high cationic lipid contents delivered OVA molecules into both cytosol and endosome/lysosome efficiently (Figs. 3-3 and 3-4), which activated DCs to secrete Th1-type cytokines (Figs. 3-5B and C), such properties of the cationic lipid-incorporated liposomes might engender the Th1-derived immune response rather than the Th2-derived immune response.

![Graph showing IgG titer and IgG2a/IgG1 ratio](image)

**Fig. 3-7.** Serum OVA-specific IgG titer (A) and IgG2a/IgG1 ratio (B) of C57BL/6 mice immunized with OVA-loaded MGlu-HPG liposomes containing various TRX content mol% twice at a week intervals. Blood samples were collected at 7 days after the second immunizations. *p < 0.01. © 2014 Elsevier Ltd.

We further confirmed the activation of OVA-specific immunity by measuring the IFN-γ secretion from splenocytes of the mice immunized with the OVA-loaded liposomes. The splenocytes were collected from the immunized mice and were cultured with OVA for 5 days. Then, the concentration of IFN-γ secreted from the splenocytes in the medium was measured using ELISA (Fig. 3-8). Splenocytes of the mice immunized with the liposomes containing 0-10% TRX secreted a low level
of IFN-γ, suggesting that their ability to recognize OVA might be low. However, splenocytes derived from the mice immunized with the liposomes containing 20-30% TRX exhibited a much higher level of IFN-γ secretion during incubation with OVA, which is indicative of their high responsivities to the antigen OVA. Because these splenocytes cultured in the absence of OVA only slightly secreted IFN-γ, observed IFN-γ secretion was induced by their specific response to OVA. Considering that the immunization with the TRX-rich liposomes generated antigenspecific Th1 cells effectively (Fig. 3-7), the Th1 cells of the splenocytes might produce IFN-γ responding to OVA.

![Graph showing IFN-γ concentration vs TRX content](image)

**Fig. 3-8.** *In vitro* stimulation of splenocytes from mice immunized with OVA-loaded liposomes. Splenocytes (2×10⁶/2 mL) isolated from OVA-loaded liposome-immunized C57BL/6 mice were incubated with or without 50 µg/mL of OVA for 5 days. IFN-γ secretion in the supernatant was detected by ELISA. Splenocytes were collected at 7 days after the second immunizations. © 2014 Elsevier Ltd.

3.3.6 Therapeutic effect

Finally, we investigated the effect of cationic lipid inclusion on therapeutic effects of the MGlue-HPG-modified liposome-mediate immunization for mice with OVA-expressing tumors (Fig. 3-9). Mice were first inoculated with OVA-expressing murine T lymphoma E.G7-OVA cells. Then OVA-loaded liposomes containing varying TRX contents were administered subcutaneously into the
tumor-bearing mice on 5 and 12 days after tumor cell inoculation. Then the tumor volumes were monitored (Fig. 3-9). When mice were administered PBS, the tumor volume increased rapidly. However, the mice administered the OVA-loaded liposomes exhibited decreased tumor volume after day 12, indicating that administration of the OVA-loaded liposomes induced OVA-specific immunity, which attacked tumor cells strongly. For the immunization with the liposomes containing 0-10 mol% TRX, tumor suppression persisted until day 18, after which the tumors grew again. In contrast, the mice administered liposomes containing 20-30 mol% TRX showed shrinkage of tumor volumes for longer periods, although the tumors started to grow after day 25. Because these liposomes have high capability for the induction of antigen-specific Th1-dominant immune responses (Figs. 3-7 and 3-8), it is likely that the OVA-specific immunity induced by these liposomes strongly attacked the tumor cells, thereby significantly shrinking the tumor burden.

An adjuvant such as MPLA was generally used to enhance the immune response of vaccines [48]. For our previous study, we incorporated MPLA into the MGlu-HPG-modified liposomes for efficient induction of immune responses [15]. We compared the capability of inducing antitumor immunity among MPLA-incorporated liposomes and TRX-incorporated liposomes loaded with OVA (Fig. 3-10). Although the tumor volume increased significantly with time for mice injected with PBS (Fig. 3-10A), those treated with MGlu-HPG-modified adjuvant-free liposomes showed significant tumor growth suppression (Fig. 3-10B). The MGlu-HPG-modified TRX-incorporated liposomes exhibited further tumor growth suppression, although one mouse showed a higher extent of tumor growth (Fig. 3-10C). The MPLA-incorporated liposomes showed even stronger tumor suppressive effects than the TRX-incorporated liposomes, but their tumor suppressive effects were not so different. This result suggests that their activities for tumor-suppressive immune induction are comparable (Figs. 3-10C and D). Considering that MPLA are obtained from bacteria, MPLA might have variations from the viewpoints of the biological activities and possibility of unexpected side effects originating from their structural variety and molecular purity. Therefore, the fact that the incorporation of the synthetic molecule TRX into the MGlu-HPG liposomes derived comparable antitumor immunity with
MPLA incorporated liposomes is noteworthy. Cationic lipid inclusion into the pH-sensitive fusogenic liposomes might be an efficient method for the production of efficient antigen delivery systems for cancer immunotherapy.

![Graphs showing tumor volume over time](image)

**Fig. 3-9.** Antitumor effect induced by subcutaneous administration with OVA-loaded liposomes with or without MPLA. Each line indicates the tumor volume of individual mouse. C57BL/6 mice were inoculated with E.G7-OVA (1×10⁶/mouse) and were treated with PBS (A), MGlu-HPG-modified liposomes without TRX (B) and with 30 mol% TRX (C), and MGlu-HPG-modified liposomes with MPLA (D) on 5 and 12 days after the tumor cell inoculation. All treated groups contained four mice. © 2014 Elsevier Ltd.

### 3.4. Conclusion

This study investigated the influence of inclusion of cationic lipid TRX into pH-sensitive fusogenic polymer-modified liposomes on their performance as antigen delivery systems. Results showed that the TRX inclusion improved their pH-sensitive membrane destabilizing ability in the weakly acidic pH region. Additionally, TRX introduction into the polymer-modified liposomes changed their performance for antigen delivery. Liposomes with higher TRX contents exhibited enhanced association to DCs and delivered their contents into both endosomes/lysosomes and cytosol of DCs, although the liposomes without TRX delivered their contents mostly into cytosol. In addition,
the TRX inclusion provides the capability for DC activation to the polymer-modified liposomes, resulting in efficient induction of Th1 cells and cytokine production from DCs treated with the liposomes. Results show that administration of these liposomes to mice induced antigen-specific immune responses, which caused marked therapeutic effects on tumor-bearing mice. Therefore, the cationic lipid-incorporated pH-sensitive liposomes can engender production of potent antigen delivery systems, which can contribute to the establishment of efficient cancer immunotherapy.

3.5. References


Chapter 4: pH-Sensitive polymer-modified liposome-based immunity-inducing system: effect of inclusion of cationic lipid and CpG-DNA

4. 1. Introduction

Recent advances in immunology and biotechnology have produced efficient therapeutic approaches based on human immune systems. Especially because of the success of immune checkpoint inhibitors such as ipilimumab and nivolumab, cancer immunotherapy is nearly regarded as a fourth standard cancer therapy [1]. Immune checkpoint inhibitors have revealed clearly that immune systems can attack and eliminate cancer cells, with roles played mainly by cytotoxic T lymphocytes (CTLs). Establishing more effective cancer immunotherapy requires an effective CTL induction system and canceling of immunosuppressive effects in tumor microenvironments using immune checkpoint inhibitors. Adoptive T cell transfer therapy, which is the treatment by administration of cancer-specific T cells cultured ex vivo, has been studied for such purposes [2]. Another strategy for the induction of cancer-specific CTLs is the utilization of dendritic cells (DCs). DCs can induce cancer-specific CTLs via presentation of the endogenous antigen mediated by major histocompatibility complex (MHC) class I molecules [3]. In general, exogenous antigen is presented by MHC class II to induce helper T lymphocytes (Th), which assist the CTL-mediated cellular immune responses or B cell-mediated antibody production. Therefore, the delivery of exogenous antigen into cytosol is necessary to regard the antigen as an endogenous antigen and to induce MHC class-I-mediated antigen presentation. Therefore, the cytoplasmic delivery system of exogenous antigen to DCs is necessary to induce antigen-specific CTLs for the establishment of efficient cancer immunotherapy.

Cytoplasmic delivery systems of various types have been reported in the literature [4]. Among them, lipid-based antigen delivery carriers are particularly useful because they can induce membrane fusion or destabilization of endosomal membrane and can achieve cytoplasmic delivery of antigen. Typical examples of cytoplasmic delivery systems using lipid-based carrier are viral fusogenic
protein-incorporated liposomes such as Sendai virus-derived fusogenic protein-introduced liposomes [5] or Virosome, influenza virus fusogenic protein (hemagglutinin)-incorporated liposomes [6]. Sendai virus-derived fusogenic protein-introduced liposomes generated fusion with plasma membrane and directly delivered the antigenic protein into cytosol of DCs [7]. Virosome induces fusion with endosomal membrane because hemagglutinin changes its conformation under weakly acidic pH in endosomes. Also, exposed hydrophobic moiety in hemagglutinin is inserted to the endosomal membrane, thereby causing the adjacency of virosome to the endosomal membrane. Sendai virus-derived fusogenic protein-introduced liposomes and Virosome could induce antigen-specific CTLs but they might induce unexpected immunity-related effects derived from viral components. Therefore, cytoplasmic delivery systems without viral components are sought, such as liposomes modified with synthetic molecules or peptides having fusogenic activity. We previously reported synthetic fusogenic polymers using carboxylated poly(glycidol) derivatives and these polymer-modified liposomes for the cytoplasmic delivery of contents [8–11]. Carboxylated poly(glycidol) derivatives change their properties from hydrophilic to hydrophobic after protonation of carboxylic acid groups under weakly acidic pH and destabilize lipid membrane. Modification of egg yolk phosphatidylcholine (EYPC) liposomes with these polymers produces liposomes having pH-responsive fusogenic ability [8]. Especially, 3-methylglutarylated hyperbranched poly(glycidol) (MGlut-HPG, Fig. 1) showed excellent membrane disruptive ability under acidic pH because of its bulky three-dimensional structures [8]. MGlut-HPG-modified liposomes delivered ovalbumin (OVA) or its CTL epitope peptide used as model antigens to cytosol of DCs and induced OVA-specific CTL responses [8, 9].

In the antigen presentation process, DCs present antigen to T cells via MHC molecules (first signal), activate T cells by co-stimulatory molecules (second signal), and cytokines (third signal) [12, 13]. These signals are important for the induction of antigen-specific immune response. If antigen presentation occurs without the second signal, then immune tolerance (anergy of T cells) is induced [14]. Furthermore, cytokines play crucially important roles of controlling immunity and deciding the
T cell subtype [15]. Activated state (matured) DCs express high levels of MHC molecules and co-stimulatory molecules; moreover, they produce specific cytokines [16]. Therefore, efficient antigen carriers require not only cytoplasmic delivery performance but also activation ability of DCs to induce DC maturation, which is designated as an adjuvant function.

Adjuvant function is crucially important for vaccines to control immune response through DC activation [17]. Alum has been the most famous and general adjuvant since the 1920s. A vaccine composed of antigen and alum can induce antigen-specific antibody response [18]. However, because alum has no ability to induce CTL response [18], synthetic adjuvant molecules from neither bacterial nor viral components are necessary to activate CTL response. Reportedly, cationic lipids have adjuvant functions [19,20]. We recently reported that the incorporation of 3,5-didodecyloxybenzamidine (TRX, Fig. 4-1) as a cationic lipid to MGlu-HPG-modified liposomes promoted their activation property of DC [21]. TRX-incorporated liposomes induced high CTL response and antitumor effect against tumor-bearing mice [21]. However, its therapeutic effect remained lower than that of liposomes containing monophosphoryl lipid A, which is an adjuvant derived from bacterial lipopolysaccharide [21].

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs). DCs detect and distinguish viruses and bacteria invading a living body using TLRs. Then they induce appropriate immunity against pathogens [23]. CpG-DNA, a synthetic unmethylated CG sequence-containing oligonucleotide, is recognized by TLR9, which expresses in endosome of DCs [22]. In this study, CpG-DNA was introduced to pH-sensitive polymer (MGlu-HPG)-modified liposome-based antigen carrier to improve their adjuvant function (Fig. 4-1). CpG-DNA has an anionic phosphoester backbone [24]. Therefore, CpG-DNA is expected to complex with cationic lipid (TRX) on the liposomal membrane through electrostatic interaction. Here, two complexation methods of CpG-DNA to liposomes were investigated (Fig. 4-2): Pre-mix for which CpG-DNA was mixed with liposomal lipids when liposomes were formed and Post-mix for which CpG-DNA was mixed with
pre-formed liposomes. The CpG-DNA complexation method effects on the immunity-inducing activity of liposomes were examined in vitro and in vivo.

**Fig. 4-1.** Design of liposomal vaccine using pH-sensitive polymer (MGlul-HPG)-modified liposomes containing cationic lipid (TRX) and Toll-like receptor 9 ligand (CpG-DNA) for efficient antigen delivery and activation of dendritic cells. These liposomes deliver not only double stimulation molecules (CpG-DNA and TRX) to endosomes but also antigen to cytosol of DCs. Matured DCs present antigen to T lymphocytes through MHC class I molecule and co-stimulatory molecule and cytokine production promote the activation of cytotoxic T lymphocytes.

**Fig. 4-2.** Preparation schemes of antigen-loaded liposomes used in this study.
4. 2. Materials and methods

4. 2. 1. Materials

EYPC was kindly donated by NOF Co. (Tokyo, Japan). 3,5-Didodecyloxybenzamidine hydrochloride (TRX) were kindly donated by Terumo Corp., Ltd. (Kanagawa, Japan). Lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). OVA, bovine serum albumin (BSA) and MPLA were purchased from Sigma (St. Louis, MO.). Triton X-100 were obtained from Tokyo Chemical Industries Ltd. (Tokyo, Japan). Quant-iT Oligreen ssDNA reagent was obtained from Molecular Probes (Oregon, USA). ODN 1826 (CpG-DNA: 5’-TCCATGACGTTCTGACGTT-3’) and Tween20 were obtained from Nacalai Tesque, Inc., (Kyoto, Japan). 3-Methylglutarylated hyperbranched poly(glycidol) with polymerization degree of 60 (MGlue-HPG) was prepared as previously reported [8]. The ratios of hydroxyl units, MGlue units and decyl amide units for MGlue-HPG was 9/80/11, as estimated using $^1$H NMR [8].

4. 2. 2. Cell culture

DC2.4 cells, which were an immature murine DC line, were provided from Dr. K. L. Rock (Harvard Medical School, USA) and were grown in RPMI-1640 (Nacalai Tesque) supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine (Wako), 100 mM MEM nonessential amino acid (Nacalai Tesque), 50 μM 2-mercaptoethanol (2-ME, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C [25]. E.G7-OVA, which is a chicken egg OVA gene-transfected murine T lymphoma and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) [26].
4.2.3. Animals

Female C57BL/6 mice (H-2b, 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.

4.2.4. Preparation of liposome

To a dry, thin membrane of EYPC and TRX (total lipids; $1.25 \times 10^{-5}$ mol) and MGlù–HPG (lipids/polymer = 7/3, w/w) was added 500 µL of OVA (4 mg/mL) phosphate buffered saline (PBS) (pH 7.4) and the mixture was sonicated for 2 min using a bath–type sonicator. 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 centrifuged with the speed of 55,000 rpm for 2 h at 4 °C twice to remove free OVA and CpG-DNA. For CpG-DNA inclusion to liposomes, two complexation methods were examined. In the case of Pre-mix, mixed thin membrane was dispersed by mixture of OVA/CpG-DNA (2.5, 5, 7.5 g/mol lipid) in PBS. Lipid concentration and OVA encapsulation were determined by Test-Wako C (Wako Pure Chemical Industries, Ltd) and Coomassie (Bradford) Protein assay reagent (Thermo-Scientific). CpG-DNA amounts in liposomes were determined by Quant-iT Oligreen ssDNA assay as following procedure. Lipid dispersion was mixed with Triton-X 100 (0.2% vol) and Oligreen ssDNA assay reagent in fluorescence microtiter plate. Microplate was excited at 480 nm and fluorescence emission intensity was detected at 520 nm using Microplate Reader (SH-8000 CORONA ELECTRIC Co., Ltd.).

4.2.5. Dynamic light scattering and zeta potential

Diameters and zeta potentials of the liposomes (0.1 mM lipids) in 0.1 mM phosphate aqueous solution 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.
4.2.6. Cellular uptake of liposome and CpG-DNA

The DC2.4 cells (1 × 10^5 cells) cultured for 2 days in a 12-well plate were washed with Hank’s balanced salt solution (HBSS) and then incubated in culture medium. The liposomes which lipids were substituted by Rh-PE (0.6 mol%) or the liposomes containing FITC-CpG-DNA were added gently to the cells and incubated for 4 h at 37 °C. The cells were washed with HBSS three times, and then the detached cells using trypsin were applied to a flow cytometer (CytoFlex, Beckman Coulter, Inc).

4.2.7. Intracellular behavior of liposomes

The FITC-CpG-DNA-incorporated liposomes containing Rh-PE were prepared as described above except that a mixture of polymer and lipids containing Rh-PE (0.6 mol%) was dispersed in PBS containing FITC-CpG-DNA (5 g/mol lipid). DC2.4 cells (2 × 10^5 cells) cultured 2 days in 35-mm glass-bottom dishes were washed with HBSS, and then incubated in serum-free RPMI-1640 medium (1 mL). The FITC-CpG-DNA-loaded liposomes (0.1 mM of lipid concentration, 5 g/mol lipid of CpG-DNA concentration, total volume was 2 mL) 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. In the case of staining cellular acidic compartments, LysoTracker Red or LysoTracker Green (Invitrogen) was used. Confocal laser scanning microscopic (CLSM) observation of these cells was performed using LSM 5 EXCITER (Carl Zeiss Co. Ltd.). Co-localization analysis was performed with LSM Software ZEN 2009 (Carl Zeiss Co. Ltd.).

4.2.8. Cytokine production from DC2.4 cells treated with liposomes

The DC2.4 cells (3 × 10^5 cells) cultured for 2 days in a 6-well plate were washed with HBSS, and then incubated in serum-free RPMI-1640 medium (2 mL). Cytokine (TNF-α and IL-12) production in supernatants of DC2.4 cells treated with liposomes was measured using an enzyme-linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd.) according to the manufacture’s instruction.
4. 2. 9. Analysis of liposome-treated DC phenotype

The DC2.4 cells (3 × 10^5 cells) cultured for 2 days in a 6-well plate were washed with HBSS, and then incubated in serum-free RPMI-1640 medium (2 mL). The OVA-loaded liposomes (0.1 mM of lipid and 1 μg/mL CpG-DNA concentration, 1 mL) were added gently to the cells and incubated for 6 h at 37 °C. The cells were washed with HBSS three times and cultured for 20 h. Cell phenotype was confirmed by a flow cytometric analysis. Briefly, 10^6 cells in 100 μL of staining buffer (PBS containing 0.1% BSA and 0.01% sodium azide) were incubated for 30 min on ice with the anti-FcγRII/III monoclonal antibody (eBioscience, 2.4G2) to block nonspecific binding of the subsequently used antibody reagents. The cells were re-suspended in 100 μL of staining buffer and incubated for 30 min on ice, using the manufacturer’s recommended amounts of biotinylated antibodies: anti-mouse H-2Kb/Db (BD Pharmingen, 28-8-6) and FITC-labeled anti-CD80 (abcam, 16-10A1). The cells were then re-suspended in 100 μL of staining buffer containing 10 μL of R-Phycoerythrin (PE)-conjugated streptavidin (Sigma), and nonspecific binding was measured using PE-conjugated streptavidin alone. After incubation for 30 min on ice, 10,000 events of the stained cells were analyzed for surface phenotype, using a flow cytometer (CytoFlex, Beckman Coulter, Inc). Between all incubation steps, cells were washed three times with staining buffer.

4. 2. 10. In vitro cytokine release measured by ELISA

50 μg of OVA-loaded liposomes or PBS were subcutaneously injected into the right backs of the mice under anesthesia twice at a week intervals. After a week from second immunization mice were sacrificed and splenocytes were suspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-ME. Splenocytes (2 × 10^6 in 2 mL) were cultured with or without 50 μg/mL of OVA for 5 days. After incubation, the concentration of IFN-γ was measured using murine IFN-γ ELISA development kit (PeproTech, London, UK) according to the manufacture’s instruction.
4. 2. 11. 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 with isoflurane. On days 5 and 12, 50 μg of OVA-loaded liposomes with or without MPLA (4 g/mol lipid) were subcutaneously injected into the right backs of the mice under anesthesia with isoflurane. 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.

4. 2. 12. Statistical analysis

Student's \( t \)-test or Tukey-Kramer-test were performed in the statistical evaluation of the results (Figs. 4-3, 4, 6, 7, 8).

4. 3. Results and discussion

4. 3. 1. Preparation of cationic lipid-and CpG-DNA-introduced liposomes

This study investigated the inclusion of both cationic lipids and CpG-DNA to pH-sensitive polymer-modified liposomes for the preparation of an efficient antigen delivery system. Figure 4-2 presents a summary of the preparation scheme of antigen-loaded liposomes used for this study. A mixed thin membrane composed of EYPC and MGlu-HPG was dispersed in PBS containing OVA. Liposome suspension was extruded through a 100 nm polycarbonate membrane. Then it was purified by ultracentrifugation. This liposome was designated as Lip. TRX-Lip was prepared from mixed thin membrane composed of EYPC, TRX, and MGlu-HPG according to the same procedure. Two complexation methods of CpG-DNA were examined: Pre-mix for which a mixed thin membrane with or without TRX was dispersed in a mixture of OVA/CpG-DNA solution, designated respectively as Pre-mix TRX+ and Pre-mix TRX−. The CpG-DNA contents in liposomes were ascertained using an ssDNA assay kit (Fig. 4-3). Figure 4-3 shows that the CpG-DNA contents in Pre-mix TRX+ were
significantly greater than those of Pre-mix TRX-. More than 80% of CpG-DNA in feed complexed with TRX-containing liposomes, whereas the complex efficiency of Pre-mix TRX- was less than 13%. These results demonstrate that CpG-DNA binds efficiently to cationic lipids on the liposomal membrane via electrostatic interaction. Another method for CpG-DNA complexation is Post-mix for which the same amount of CpG-DNA in Pre-mix TRX+ was added to pre-formed Lip and TRX-Lip, which are designated respectively as Post-mix TRX- and Post-mix TRX+. They were used without further purification (Fig. 4-2).

The particle size and ζ-potential of each liposome at pH 7.4 were evaluated using DLS and electrophoretic light scattering (Table 4-1). All liposomes were approximately 100 nm, which corresponds to the pore size of the polycarbonate membrane used for extrusion. All liposomes showed negative values of zeta potentials, which indicates that the liposome surface was covered by MGluc-HPG having many carboxyl groups. The TRX-containing liposomes exhibited more negative zeta potentials than liposomes without TRX, which suggests that the introduction of cationic lipid increased the amounts of MGluc-HPG polymers on the surface of liposome via electrostatic interactions. That result is consistent with those presented in earlier reports of the literature [21]. The inclusion of CpG-DNA to liposomes only slightly affected the size and zeta potentials of each liposome without CpG-DNA.

![Fig. 4-3. The amounts of CpG-DNA in Pre-mix liposomes with (closed symbols) or without (open symbols) TRX.](image)
4.3.2. Cellular association of cationic lipid- and CpG-DNA-introduced liposomes

Next, the cellular association of liposomes to dendritic cells was examined. For 4 h, DC2.4 cells were treated with rhodamine lipid-incorporated liposomes. Then, cellular fluorescence was measured using flow cytometric analysis. Figure 4-4A shows that liposomes with TRX (TRX-Lip, Pre-mix TRX+) exhibited over 10 times higher cellular fluorescence than those of liposomes without TRX (Lip, Pre-mix TRX−). Reportedly, more anionic nanoparticles are taken up more efficiently by dendritic cells or macrophages by the recognition of scavenger receptors on these cells, which are receptors to recognize anionic surface of apoptotic cells or aged erythrocytes [27, 28]. Liposomes with TRX showed much lower zeta potentials than those of liposomes without TRX (Table 1). Therefore, liposomes with TRX might be recognized efficiently by scavenger receptors on DC2.4 cells, which is consistent with results of our earlier study [21]. The inclusion of CpG-DNA to Lip or TRX-Lip caused the reduction of cellular association of liposomes to some extent, which suggests that CpG-DNA on the liposome surface might interrupt the interaction of carboxylate of MGlü-HPG with scavenger receptors.

Cellular association of CpG-DNA was investigated using FITC-labeled CpG-DNA (Fig. 4B). Compared with free CpG-DNA solution, Pre-mix TRX+ showed much higher FITC fluorescence, indicating that CpG-DNA was delivered efficiently by Pre-mixed liposomes. In contrast, both Post-mix TRX−/+ showed lower fluorescence intensity than that of free CpG-DNA, which suggests that

<table>
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<tr>
<th>Liposome</th>
<th>Mean diameter (nm)</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip</td>
<td>97 ± 6</td>
<td>-18 ± 6</td>
</tr>
<tr>
<td>Pre-mix (TRX−)</td>
<td>100 ± 3</td>
<td>-19 ± 5</td>
</tr>
<tr>
<td>Post-mix (TRX−)</td>
<td>88 ± 6</td>
<td>-11 ± 1</td>
</tr>
<tr>
<td>TRX-Lip</td>
<td>110 ± 6</td>
<td>-63 ± 4</td>
</tr>
<tr>
<td>Pre-mix (TRX+)</td>
<td>108 ± 8</td>
<td>-65 ± 3</td>
</tr>
<tr>
<td>Post-mix (TRX+)</td>
<td>109 ± 10</td>
<td>-60 ± 5</td>
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the existence of liposomes with negative charges (Table 4-1) might suppress the cellular association of CpG-DNA molecules.

Fig. 4-4. (A) Relative fluorescence intensity of DC2.4 cells treated with 0.6 mol% Rh-PE labeled liposome. Cells were incubated for 4 h at 0.5 mM of lipids and 2.5 μg/mL of CpG-DNA in the absence of serum. (B) Relative fluorescence intensity of DC2.4 cells treated with FITC-CpG-DNA complexed liposome. Cells were incubated with various liposomes for 4 h 0.1 mM of lipids 0.5 μg/mL of FITC-CpG-DNA in the absence of serum.

4.3.3 Intracellular distribution of cationic lipid- and CpG-DNA-introduced liposomes

TLR9 exists at endosomal lumen of immunocompetent cells. Therefore, precise delivery of CpG-DNA molecules to the inside of endosomes might cause efficient activation of immunocompetent cells. Therefore, the intracellular distribution of CpG-DNA delivered by liposomes was investigated next. For 4 h, DC2.4 cells were treated with FITC-CpG-DNA solution or FITC-CpG-DNA-introduced liposomes. The DC2.4 cells were then observed using CLSM (Fig. 4-5A). For cells treated with CpG-DNA solution, most of the FITC fluorescence was observed from the cell periphery, which suggests that most of the CpG-DNA adsorbed onto the cell surface and that their internalization efficiency was quite low. For cells treated with Pre-mix TRX−, red punctate fluorescence and green fluorescence were observed from the same intracellular locations, indicating that liposomes and CpG-DNA were internalized to cells. However, their fluorescence was quite low because the cellular
association of liposomes and CpG-DNA contents in liposomes were low (Figs. 4-3 and 4A). In contrast, cells treated with Pre-mix TRX+ showed much stronger red and green fluorescence than those of Pre-mix TRX-. According to the co-localization analysis of FITC fluorescence derived from CpG-DNA and rhodamine fluorescence derived from liposome, over 80% of FITC pixels in the region of interest (ROI, dashed white lines in the CLSM image) co-localized with rhodamine pixels (Fig. 4-5B), which indicates that Pre-mix TRX+ efficiently delivered both liposome and CpG-DNA to inside of cells. For more detailed evaluation of intracellular distribution of liposomes and CpG-DNA, cells treated with Pre-mix TRX+ were stained with LysoTracker (Fig. 4-5C). Figure 4-5C demonstrates that rhodamine and FITC-CpG-DNA fluorescence derived from Pre-mix TRX+ overlapped respectively with LysoTracker green and LysoTracker red. Their co-localization efficiency in the ROI was higher than 80% (Fig. 4-5D). These results suggest that Pre-mix TRX+ delivered CpG-DNA to endosomes in DC2.4 cells. Therefore, Pre-mix TRX+ is expected to stimulate DCs effectively via interaction with TLR9.

Intracellular delivery performance of CpG-DNA by “Post-mix” liposomes was also examined (Fig. 4-5A). In the case of cells treated with Post-mix TRX−, red fluorescence was located within cells, but most of the green fluorescence was observed from the cell periphery as it was in the case of free CpG-DNA solution-treated cells. This result suggests that liposomes and CpG-DNA were taken up independently by cells in the case of Post-mix TRX−. It is particularly interesting that in the case of cells treated with Post-mix TRX+, most of the green fluorescence located within cells, unlike the case of Post-mix TRX−. To elucidate the internalization mechanism of CpG-DNA by Post-mix TRX+, time-dependence of the intracellular distribution of CpG-DNA and liposomes was evaluated (Fig. 4-S1). Figure 4-S1 shows that green and red fluorescence was observed only slightly at an earlier stage (30 min of incubation time). After 1-h incubation, strong red fluorescence was observed from inside of cells. Moreover, the green fluorescence almost overlapped with the red fluorescence. After 4-h incubation, both red and green fluorescence were observed from inside the cells, but a part of the green fluorescence was located at a different site from that of red fluorescence. These results suggest
that CpG-DNA might form a complex with liposomes in the culture medium and be internalized. Eventually, it is released from liposomes. To investigate the complex formation with CpG-DNA and liposomes, Lip or TRX-Lip and CpG-DNA were incubated for 10 min or 4 h and ultracentrifuged. Then the amounts of CpG-DNA in the supernatant and the precipitated liposomes were measured using an ssDNA assay kit (Fig. 4-S2). According to Figure 4-S2, about 60% of CpG-DNA was detected from the liposome fraction after 4 h-incubation with TRX-Lip, whereas almost all CpG-DNA molecules were detected from the supernatant in the case of Lip. These results indicate that CpG-DNA can bind to TRX-Lip via electrostatic interaction in Post-mix TRX+. After application to culture medium, some CpG-DNA molecules might start to bind to TRX-Lip. Then, CpG-DNA/TRX-Lip complexes might internalize to cells. Therefore, Post-mix TRX+ can promote the internalization of CpG-DNA to dendritic cells. The co-localization efficiency of FITC with rhodamine was also evaluated between Pre-mix TRX+ and Post-mix TRX+ (Fig. 4-5B). Figure 5A shows that the ROI was set only to the inside of cells, as shown by dashed white lines in the CLSM images. Pre-mix TRX+ showed higher than 80% co-localization efficiency, as described above. In contrast, Post-mix TRX+ exhibited low co-localization efficiency compared with Pre-mix TRX+. MGlu-HPG-modified liposomes with TRX (TRX-Lip) are destabilized in response to weakly acidic pH inside of endosomes, as described in reports of earlier studies [21]. However, because CpG-DNA molecules in Pre-mix TRX+ might bind tightly to liposomal membrane via electrostatic interaction, CpG-DNA molecules might exist in endosomes with liposomes even after pH-responsive destabilization of liposomes. In contrast, the interaction between CpG-DNA and liposomes in Post-mix TRX+ might be low. Therefore, CpG-DNA molecules were released from endosomes when the endosomal membrane was destabilized by TRX-Lip. Such differences in the intracellular distribution of CpG-DNA inside of dendritic cells might cause the different activation profiles of dendritic cells, as described below.
Fig. 4-5. (A) CLSM images of DC2.4 treated with FITC-CpG-DNA or various liposomes. Cells were incubated with 0.6 mol% Rh-PE-labeled liposomes for 4 h at 0.1 mM of lipids and 0.5 μg/mL of FITC-CpG-DNA in the absence of serum. (B) Co-localization analysis of ROI indicated with white dashed-line in Figure 4-5A. (C) CLSM images of “Pre-mix TRX+”-treated DC2.4 stained by LysoTracker Green (left) or Red (right). (D) Co-localization analysis of the region of interest (ROI) indicated with white dashed-line in Figure 4-5C.

Fig. 4-S1. CLSM images of DC2.4 treated with “Post-mix TRX+” for 0.5 h, 1 h, 2, and 4 h. Cells were incubated with 0.6 mol% Rh-PE-labeled liposomes at 0.1 mM of lipids and 0.5 μg/mL of FITC-CpG-DNA in the absence of serum. Scale bar represents 10 μm.
Fig. 4-S2. Cationic lipid-inclusion to liposomes increased the CpG-DNA binding to the liposomes. MGlU-HPG-modified liposomes (0.5 mM lipids) with or without TRX were incubated with CpG-DNA solution (5 g/mol lipids) for 10 min or 4 h on the ice bath and then, liposome suspension was ultracentrifuged. The amounts of CpG-DNA in supernatant (open bars) or precipitation (closed bars, liposome fraction) were determined by ssDNA assay kit.

4.3.4. Activation of dendric cells by cationic lipid- and CpG-DNA-introduced liposomes

Next, the activation of dendritic cells by liposomes was evaluated in the viewpoints of cytokine production from DCs or upregulation of surface marker molecules. Figure 4-6 depicts the cytokine production from DC2.4 cells treated with CpG-DNA or various liposomes. Compared with cells with no treatment, cells treated with CpG-DNA solution produced high levels of TNF-α, which indicates that DC2.4 cells were activated by CpG-DNA, but no production of IL-12 was observed. Lip-treated cells also produced TNF-α, but various amounts of CpG-DNA inclusion to Lip by Pre-mix method (Pre-mix TRX−) did not affect the TNF-α production from cells. In addition, IL-12 production was not detected by these liposomes under experimental conditions. Compared with Lip and Pre-mix TRX−, TRX-Lip induced high levels of both TNF-α and IL-12 production, which is consistent with results of our earlier study [21]. Furthermore, inclusion of CpG-DNA to TRX-Lip by Pre-mix method strongly promoted TNF-α and IL-12 production but not by Post-mix method. Considering the difference in intracellular distribution of CpG-DNA between Pre-mix TRX+ and Post-mix TRX+.
(Fig. 4-5A), Pre-mix TRX+, which mainly delivered CpG-DNA to endosomes, might induce the activation of cells through TLR9 in endosomes more efficiently than Post-mix TRX+, which delivered CpG-DNA not only to endosomes but also to cytosol (Figs. 4-5A and S1), resulting in higher production of cytokines. IL-12 from DCs is the determinant factors of the differentiation of naive Th into Th1 [29]. Therefore, Pre-mix TRX+ is expected to promote cellular immune response through Th1 induction.

**Fig. 4-6.** TNF-α (A) and IL-12 (B) productions from DC2.4 cells treated with liposomes for 24 h in the absence of serum. Lipid and CpG-DNA concentrations were 0.5 mM and 2.5 μg/mL, respectively. After incubation, cell medium
were collected and centrifuged, and then cytokine levels in the supernatant were measured by ELISA. * p < 0.05. ** p < 0.01.

Surface marker molecules such as MHC classes I, II, and co-stimulatory molecules (CD80) play important roles in antigen presentation. Maturated DCs highly express MHC and co-stimulatory molecules and lead the activation of antigen-specific immune responses. Therefore, the expression of these surface markers on DC2.4 cells treated with CpG-DNA or various liposomes was analyzed using immunofluorescence staining (Fig. 4-7). The treatment of CpG-DNA solution affected the expression of MHC and CD80 molecules only slightly. Cells treated with Lip showed a slight increase of MHC class I molecules but not of CD80 molecules, which indicates that MGl-HPG liposomes with no adjuvants (Lip) were unable to activate the dendritic cells fully. Compared with Lip, TRX-Lip promoted the expression of both MHC and CD80 molecules. This result indicates that TRX induced maturation of DCs, as reported previously [21]. CpG-DNA-introduced liposomes (Pre-mix TRX+ and Post-mix TRX+) also induced strong maturation of DC2.4 cells, but no significant difference exists between TRX-Lip, Pre-mix, and Post-mix, except for CD80 expression. Figure 4-7C shows that Post-mix showed high expression of CD80 compared with Pre-mix. As shown in Fig. 4-S1, CpG-DNA molecules in Post-mix TRX+ first bound to liposomes. Then they were delivered not only to endosomes but also to cytosol. The cytosol contains inflammasome of NALP3, which is known as a cytosolic DNA sensor [30]. Therefore, Post-mix TRX+ might activate dendritic cells not only by TLR9 in endosome but also NALP3 in cytosol. Such multiple stimulations might induce the high expression of CD80 molecules by Post-mix TRX+. Therefore, it is likely that methods of CpG-DNA inclusion to liposomes (Pre-mix or Post-mix) strongly affect the activation properties of dendritic cells, which might be attributed to the intracellular distribution of CpG-DNA.
Immunofluorescent staining of DC2.4 treated with CpG-DNA or various liposomes. Cells were incubated with liposomes for 6 h at 0.1 mM of lipid concentration and 0.5 μg/mL of CpG-DNA concentration in the absence of serum. Then cells were incubated with cell culture medium containing 10% fetal bovine serum for 20 h. After incubation, surface molecules of cells were stained using specific antibodies. * \( p < 0.05 \).

4. 3. 5. In vivo immune responses of cationic lipid- and CpG-DNA-introduced liposomes

Next, in vivo immune response induced by liposomes was investigated. Various liposomes containing OVA were administered subcutaneously to mice. At 7 days after immunization, splenocytes were collected and cultured in vitro in the presence of OVA for 5 days. IFN-γ production from splenocytes during the 5-day culture was measured using ELISA (Fig. 4-8). IFN-γ, which is known as Th1 cytokine, is important for induction and activation of cellular immunity. Splenocytes from mice treated with PBS only slightly produced IFN-γ irrespective to OVA concentration used in vitro culture. In contrast, in the cases of liposome-treated mice, IFN-γ production increased depending on OVA concentration during in vitro culture. These results indicate that OVA-specific cellular immune responses were induced in spleen by the administration of liposomes. Compared with Pre-mix TRX− and TRX-Lip, Pre-mix TRX+ and Post-mix TRX+ exhibited significantly strong cellular immune responses. This result suggests the importance of co-delivery of CpG-DNA and cationic lipids for induction of efficient cellular immune responses. Furthermore, Post-mix TRX+ induced the highest production level of IFN-γ unlike the in vitro results in cytokine production (Fig. 4-6). Considering the result in high expression of CD80 molecules by Post-mix TRX+ (Fig. 4-7C), high
expression of co-stimulatory molecules might be the most important for induction of in vivo cellular immune responses.

![Graph showing IFN-γ concentration in different groups](image)

**Fig. 4-8.** In vitro stimulation of splenocytes from mice immunized with 50 µg OVA-loaded and 0 or 1 µg CpG-DNA-complexed liposomes at days 7 and 14. 7 days after second immunization, splenocytes (4×10^6/2 mL) isolated from immunized C57BL/6 mice were incubated with or without 50 µg/mL, 25 µg/mL of OVA for 120 h. IFN-γ production in the supernatant was measured by ELISA.

4.3.6. Cancer immunotherapeutic effect by cationic lipid- and CpG-DNA-introduced liposomes

Therapeutic effects by liposomes on tumor-bearing mice were investigated. The E.G7-OVA cells, which are OVA-expressing tumor cells, were injected to mice. At 5 and 12 days after tumor inoculation, liposomes of various types were administered subcutaneously to mice. Then, tumor growth was monitored. Figures 4-9A and 9B depict a comparison of the immunotherapeutic effects of TRX-Lip, Pre-mix TRX−, and Pre-mix TRX+. Compared with PBS-treated mice, all mice treated with these liposomes showed a decrease of tumor volumes, indicating that OVA-specific cellular
immunity induced by these liposomes efficiently killed E.G7-OVA tumor cells. TRX-Lip and Pre-mix TRX− showed almost identical antitumor effects, which might be attributed to the almost identical cellular immune responses of these liposomes (Fig. 4-8). Among these liposomes, Pre-mix TRX+ exhibited the strongest antitumor effect and survival rate: the tumor volume of mice treated with Pre-mix TRX+ started to decrease 3 days after first immunization and disappeared once. Figures 4-9C and 9D compare the antitumor effects of Pre-mix TRX+ and Post-mix TRX+. As shown in Fig. 4-9C, Post-mix TRX+ showed stronger therapeutic effects than that of Pre-mix TRX+, which might reflect higher cellular immune response by Post-mix TRX+, as presented in Fig.4-8. In these experiments, separate immunization of TRX-Lip and CpG-DNA solution at different sites was also examined as a comparison of Post-mix TRX+, which injects TRX-Lip and CpG-DNA solution at same sites immediately after mixing. Figures 4-9C and 9D both show that separate delivery of TRX-Lip and CpG-DNA produced lower antitumor effects and survival rates than those of Post-mix TRX+. This result suggests that the co-delivery of antigen-loaded liposomes and adjuvants to DCs existing in same region is necessary for the induction of effective cellular immunity.

Finally, therapeutic effects for more progressive tumors were investigated (Figs. 4-9E and 9F). Liposomes were administered at Day 9 when tumor volumes reached approximately 500 cm$^3$, which is eight times larger than at Day 5. In these experiments, Lip, TRX-Lip, and Post-mix TRX+ were administered to tumor-bearing mice. Their therapeutic effects were evaluated. The tumor volumes of Lip-treated mice decreased to some degree, but increased again from Day 25. Both TRX-Lip and Post-mix TRX+ showed decreased tumor volume from earlier timing (Day 13) than Lip did (Day 20). In addition, Post-mix TRX+ showed stronger antitumor effects and prolongation of the survival of mice, which is consistent with Figs. 4-9A and 9B. Therefore, the simultaneous delivery of CpG-DNA, cationic lipids and antigen by Post-mix TRX+ is an effective strategy for induction of cancer-specific cellular immune responses.
Fig. 4-9. (A-D) Antitumor effects induced by subcutaneous administration with OVA-loaded various liposomes. C57BL/6 mice were immunized on days 5 and 12 with PBS (closed diamonds), TRX-Lip (closed circles), Pre-mix TRX— (open triangles), Pre-mix TRX+ (closed triangles), Post-mix TRX+ (closed squares), TRX-Lip and CpG-DNA injected different sites (open diamonds). Black arrows indicated the day of subcutaneous administration. Change in tumor volume of mice (A, C) was monitored after E.G7-OVA cells ($1 \times 10^6$ cells/mouse) inoculation. All treated groups contained four mice. The amounts of OVA and CpG-DNA administered were 50 $\mu$g and 1 $\mu$g per mouse, respectively. (B, D) Kaplan-Meier curves for (A) and (C), respectively. (E, F) Antitumor effects for late stage tumor induced by subcutaneous administration with OVA-loaded various liposomes. C57BL/6 mice were immunized on days 9 and day 16 with PBS (closed diamonds), Lip (open circles), TRX-Lip (closed circles), Post-mix TRX+ (closed squares). Change in tumor volume of mice (E) was observed after E.G7-OVA cells ($1 \times 10^6$ cells/mouse) inoculation. (F) Kaplan-Meier curves for (E).
4. 4. Conclusion

This study investigated the immunity-inducing performance of CpG-DNA- and cationic lipid-introduced pH-sensitive polymer-modified liposomes. Two complexation methods of CpG-DNA to liposomes were compared: Pre-mix and Post-mix. Cationic lipid inclusion to liposomes promoted not only the binding of CpG-DNA to liposomes but also internalization of CpG-DNA. Both Pre-mix and Post-mix with cationic lipids activated dendritic cells efficiently *in vitro*. Especially, Post-mix promoted the expression of co-stimulatory molecules, which is important for efficient antigen presentation. Post-mix induced higher cellular immune responses and antitumor effects on tumor-bearing mice than those of Pre-mix. Therefore, Post-mix method is suitable to prepare antigen carriers with high immunity-inducing effects for effective cancer immunotherapy.

4. 5. References


Chapter 5: pH-Sensitive polymer-liposome-based antigen delivery systems potentiated with interferon-γ gene lipoplex for efficient cancer immunotherapy

5.1. Introduction

Establishment of cancer immunotherapy, which is a treatment to activate cancer-specific immune responses, is eagerly anticipated as a promising next-generation cancer therapeutic modality because of its high selectivity to target cancer cells and a low risk of side effects [1-3]. Antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages are known to play a central role in the induction of immunity. Therefore, these cells are regarded as targets for delivery of tumor-associated antigens to induce cancerspecific immune responses. Actually, APCs can induce and activate antigen-specific immune responses of two kinds, humoral immunity and cellular immunity, depending on the delivery routes of antigenic molecules [4-9]. Once exogenous antigens such as proteins are taken up by APCs via endocytosis, these molecules are degraded to peptide fragments in endosomes and/or lysosomes. Then the peptide fragments are carried on major histocompatibility complexes (MHC) class II molecules, which engenders antigen presentation to CD4-positive T cells. These CD4-positive T cells differentiate to various types of helper T cells such as Th1 cells and Th2 cells, both of which induce humoral immunity [8,9]. In contrast, endogenous antigenic proteins which exist in cytosol of APCs are degraded by proteasomes and bind to MHC class I molecules, which engenders antigen presentation to CD8-positive T cells. The CD8-positive T cells differentiate to cytotoxic T lymphocytes (CTLs), which can attack the antigen-expressing cancerous cells or virus-infected cells [8,9]. To achieve effective cancer immunotherapy, induction and activation of cancer-specific CTLs are crucial because CTL-based cellular immunity directly eliminates the antigen-expressing cancer cells. Therefore, efficient antigen carriers are necessary to deliver antigenic molecules into the cytosol of APCs for efficient induction of antigen-specific cellular immune responses.

Delivery systems of various kinds including virus-based particles, polymeric nanoparticles,
nanogels, and liposomes reportedly achieve cytoplasmic delivery of exogenous antigens into DCs and induction of immune responses [10-18]. Among them, pH-sensitive liposomes might be of particular importance because of their ability to deliver contents into cytosol via membrane fusion with endosome, which contains a weakly acidic environment [16-19]. We have reported on efficient cytoplasmic delivery using liposomes modified with pH-sensitive polymers based on nontoxic poly(glycidol)s or biodegradable polysaccharides such as 3-methylglutarylated poly(glycidol) (MGluPG) (Fig. 5-1) and 3-methylglutarylated dextran [20-23]. These polymers become fusogenic under weakly acidic conditions. Surface modification of stable liposomes with these polymers provides them with pH-sensitive fusion ability. Moreover, these liposomes achieved efficient delivery of contents such as calcein, pyranine, and ovalbumin (OVA) into cytosol of cells of various kinds including CV1, HeLa and mouse DC-derived DC2.4 cells [20-22]. Especially, surface modification of egg yolk phosphatidylcholine (EYPC) liposomes with MGluPG generated pH-sensitive liposomes with excellent cytoplasmic delivery performance [21]. Recently, we used these pH-sensitive polymer-modified liposomes such as MGluPG-modified liposomes for antigenic protein delivery and showed that administration of the liposomes encapsulating antigenic protein OVA induced OVA-specific immunity [24]. Indeed, the target-specific cellular immunity derived by the OVA-loaded liposome administration caused suppression of OVA-expressing E.G7-OVA tumor, but regrowth of the tumor was observed [24]. Despite the excellent ability of polymer-modified liposomes for antigen delivery into DC cytosol, no strong tumor suppressive effect was attained, indicating that the antigen delivery function alone is insufficient for the antigen-delivery system to derive efficient antigen-specific cellular immunity [23, 24].

In general, bioactive molecules of various kinds such as cytokine are involved in DC-mediated activation of target-specific cellular immunity [25]. Among them, interferon (IFN)-γ is an effective molecule for the activation of immune response. IFN-γ is secreted from immune cells of various kinds, such as Th1 cells, CTLs, NK cells and DCs, and activates cellular immune responses strongly by promoting antigen-presentation on MHC molecules of DCs [26,27]. Therefore, for the efficient
activation of target-specific cellular immunity, the combination of efficient delivery of antigen into DC's cytosol and induction of IFN-γ might be an effective approach.

Actually, IFN-γ is known to show short half-life in the body and to induce significant side effects when administered systemically [28]. Therefore, instead of IFN-γ molecule, IFN-γ-encoding gene has been used for the local production of IFN-γ by introducing the gene into immune cells or cancerous cells of diseased tissues [28-32]. To date, viral vectors of various kinds based on adenovirus, adenoassociated virus, and retrovirus have been used to deliver IFN-γ-encoding gene in vivo [28-32]. However, non-viral vectors with low immunogenicity and no pathogenicity might be preferred for gene delivery considering that virus-based gene vectors might entail risks of unexpected immune responses derived from viral components [33, 34]

Therefore, this study examines development of a new type of antigen-specific immunity activation system that achieves efficient activation of cellular immunity through the combination of antigen delivery and IFN-γ gene delivery using antigen-loaded pH-sensitive MGlup-PG-liposomes and IFN-γ gene-containing lipoplexes (Fig. 5-1). We pursued two approaches for the co-delivery of antigen and IFN-γ gene using hybrid complexes formed by the mixing of the MGlup-PG-liposomes and lipoplexes (Fig. 5-1A) [35-37] or a combination of liposome delivery and lipoplex delivery without complexation, which is termed as combination delivery (Fig. 5-1B). Synergetic effects of co-delivery of antigen and IFN-γ gene with these systems on the induction of target-specific cellular immunity were investigated.
Fig. 5-1. Design of co-delivery system of antigen and cytokine gene for induction of efficient antitumor immunity.

(A) Co-delivery of antigen and gene using hybrid complexes of IFN-γ gene-loaded lipoplexes and antigen-loaded pH-sensitive polymer-modified liposomes. (B) Combination delivery of IFN-γ gene-loaded lipoplexes and antigen-loaded pH-sensitive polymer-modified liposomes. Subcutaneously administered complexes or liposomes delivered antigen and IFN-γ gene into cytosol of same dendritic cells by membrane fusion responding to endosomal acidic pH. These dendritic cells activate cytotoxic T lymphocytes (CTLs) concomitantly with the induction of CTLs, which results in effective induction of antitumor immunity. © 2015 Elsevier Ltd.
5. 2. Materials and methods

5. 2. 1. Materials

EYPC and L-dioleoyl phosphatidylethanolamine (DOPE) were kindly donated by NOF Co. (Tokyo, Japan). 3,5-Dipentadecyloxybenzamidine hydrochloride (TRX) was kindly donated by Terumo Corp., Ltd. (Kanagawa, Japan). OVA, bovine serum albumin (BSA), 3-O-desacyl-40-monophosphoryl lipid A (MPLA), and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, USA). Tween20, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), mitomycin-C, and glucose were obtained from Nacalai Tesque, Inc., (Kyoto, Japan). Dilauroyl phosphatidyl choline (DLPC) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Pyranine was obtained from Tokyo Chemical Industries Ltd. (Tokyo, Japan). p-Xylene-bis-pyridinium bromide (DPX) was from Molecular Probes (Oregon, USA). FITCOVA was prepared by reacting OVA (10 mg) with FITC (11.8 mg) in 0.5 M NaHCO₃ (4 mL, pH 9.0) at 4 °C for three days and subsequent dialysis [22]. Plasmid DNA pCMV-IFN-γ (Fig. 5-S1) was constructed by inserting cDNA of murine interferon-g (mIFN-γ) into pcDNA3.1/mic-His (-) (Invitrogen) as described previously [38]. The mIFN-γ cDNA was obtained by PCR using primers designed to amplify the particular nucleotide sequence as described previously [39]. Plasmid DNA pEGFP-C1 was obtained from Clontech (PaloAlto, CA, USA). All plasmid DNAs were obtained using a KURABO QuickGene SP Kit. Fluorescence labeling of pDNA was performed using Mirus Label IT Nucleic Acid Labeling Reagents (Madison, USA) according to manufacturer's instruction. MGlupG was prepared using poly(glycidol) with polymerization degree of 76 as previously reported [21,22]. Composition of MGlupG was estimated to be [hydroxyl unit]/[MGlup unit]/[decyl amide] ratio of 4/84/12 using ¹HNMR (Scheme 5-1).
Fig. 5-S1. Structure of pCMV-IFN-\(\gamma\) (A) and nucleotide and predicted amino acid sequences of mIFN-\(\gamma\) cDNA (B). Sequences in parentheses at 5’ and 3’ ends are respectively Xhol and BamHI restriction sites. ACC is Kozak sequence. Underlined part is signal sequence. © 2015 Elsevier Ltd.

Scheme 5-1. Structure of MGlu-PG for modification of liposomes. © 2015 Elsevier Ltd.
5. 2. 2. Cell culture

DC2.4 cells, which were an immature murine DC line, were provided from Dr. K. L. Rock (Harvard Medical School, USA) and were grown in RPMI-1640 (Nacalai Tesque) supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine (Wako), 100 mM MEM nonessential amino acid (Nacalai Tesque), 50 μM 2-mercaptoethanol (2-ME, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C [40]. 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) [41].

5. 2. 3. Animals

Female C57BL/6 mice (H-2b, 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.

5. 2. 4. Preparation of MGlue-PG-liposomes

MGluePG-liposomes were prepared as previously reported [21, 22]. Briefly, a dry thin membrane of a mixture of EYPC (5 mg) and MGlue-PG (2.14 mg) was dispersed in PBS containing 4 mg/mL of OVA (pH 7.4, 1 mL) using a vortex mixer and was further hydrated by five cycles of freeze-and-thaw. The liposome suspension was extruded through a polycarbonate membrane with a pore size of 50 nm. The liposome suspension was centrifuged with the speed of 95,000 rpm for 2 h at 4 °C twice to remove free OVA from the OVAloaded liposomes, and then were dispersed in 5 mM Hepes and 5 wt% glucose solution (pH 7.4, 1 mL). Pyranine-loaded MGlue-PGliposomes were prepared as described above except that a mixture of polymer and lipid was 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 [21,22,42].
5. 2. 5. Hybrid complexes of lipoplex and MGLu-PG-liposomes

Hybrid complexes were prepared as previously reported [36,37]. Briefly, to a dry thin membrane of a mixture of TRX, DLPC, DOPE (0.13 μmol, 0.13 μmol, 0.26 μmol), 5 wt% glucose solution (1 mL) was added and sonicated for 2 min using a bath-type sonicator to afford a cationic liposome suspension. Plasmid DNA pCMV-IFN-γ or pEGFP-C1 (1 μg) dissolved in 5 mM Hepes and 5 wt% glucose solution (pH 7.4) (25 μL), was added to the liposome suspension (100 μL) for 10 min in an ice bath to afford a lipoplex with the cationic lipid-DNA phosphate charge ratio (N/P ratio) of 4. Then, a given volume (20-60 μL) of MGLuPG-liposome suspension (0.228 mM of lipids) was added to the lipoplex suspension and incubated for 10 min in an ice bath. Mixing ratio of lipoplexes and MGLuPG-liposomes were represented as C/P ratio defined as follows:

\[
C/P \text{ ratio} = [\text{Carboxyl groups of MGLu-PG}] / [\text{Phosphate groups of DNA}]
\]

Liberation of pDNA from lipoplexes was evaluated using electrophoretic analysis. Hybrid complexes with varying C/P ratios were electrophoresed on 0.6 wt% agarose gel in 40mMTris, 20mM sodium acetate, and 2mMEDTA buffer (pH 8.0) containing 1 mg/mL ethidium bromide at 100 V for 30 min. The ethidium bromidestained bands were visualized using a LAS-1000UVmini (Fujifilm, Japan) and analyzed with Science Lab2003 Multi Gauge software (Fujifilm, Japan).

For in vivo experiments, the concentration of each sample was increased as follows: a mixture of TRX, DLPC, and DOPE (respectively 2.24 μmol, 2.24 μmol, and 4.48 μmol) was dispersed in 5 wt% glucose solution (0.65 mL). pCMV-IFN-γ (40 μg) dissolved in 5 mM Hepes and 5 wt% glucose solution (pH 7.4) (100 μL) was added to the liposome suspension (150 μL) and kept for 10 min in an ice bath to afford lipoplexes. Then, 69 mL of MGLuPG-liposome suspension (2.66 mM of lipids) was added to the lipoplex suspension and incubated for 10 min in an ice bath to afford a hybrid complexes with C/P ratio of 2.
5.2.6. Dynamic light scattering and zeta potential

Diameters and zeta potentials of the liposomes (0.1 mM lipids), lipoplex (2 μg pCMV-IFN-γ), and hybrid complex (2 μg pCMV-IFN-γ) in 5 wt% glucose solution were measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd, Worcestershire, UK). Data were obtained as an average of more than three measurements on different samples.

5.2.7. Cellular association of liposomes, lipoplexes and hybrid complexes

The pDNA-containing lipoplexes and hybrid complexes were prepared as described above except for using FITC-pCMV-IFN-γ. DC2.4 cells (1 × 10^5 cells) cultured for 2 days in 12-well plates were washed with Hank's balanced salt solution (HBSS), and then incubated in culture medium (1 mL). Hybrid complexes (0.5 mg pDNA) with various C/P ratios were added gently to the cells and incubated for 4 h at 37 °C. As a comparison, OVA-loaded MGlU-PG-liposomes corresponding to each C/P ratio (0, 3.2 and 6.4 mM of lipid concentration, 0.5 mL) and lipoplexes (0.5 μg pDNA) were separately added to the cells. After the incubation, the cells were washed with HBSS three times and fluorescence intensity of these cells was determined by a flow cytometric analysis (EPICS XL, Beckman Coulter, Inc).

5.2.8. Intracellular behavior of liposomes, lipoplexes and complexes

The FITC-OVA-loaded MGlU-PG-liposomes and Rhodamine (Rh)-pDNA-containing lipoplexes and their hybrid complexes (C/P ratio of 4) were prepared as described above except for using FITC-OVA and Rh-pCMV-IFN-γ. DC2.4 cells (2 × 10^5 cells) cultured for 2 days in 35-mm glass-bottom dishes were washed with HBSS, and then incubated in culture medium (2 mL). The FITC-OVA-loaded MGlU-PG-liposomes (0.2 mM of lipid concentration, 2 mL), or lipoplex (2 μg pDNA), and hybrid complex (2 μg pDNA) 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 and then cellular acidic compartments were stained by LysoTracker Blue (Invitrogen). Confocal laser scanning microscopic (CLSM) analysis of these cells was performed using LSM 5 EXCITER (Carl Zeiss Co. Ltd.).
5. 2. 9. Transfection of cells and cytokine ELISA

The DC2.4 cells ($3 \times 10^5$ cells) cultured for 2 days in 6-well plates were washed with HBSS, and then incubated in culture medium (2 mL). Hybrid complexes (2 μg pCMV-IFN-γ) with various C/P ratios were added gently to the cells and incubated for 4 h at 37°C. As a comparison, OVA-loaded MGLuPG-liposomes (0.5 mM of lipid concentration, 1 mL) and pEGFP-C1-containing lipoplexes (2 μg pDNA) were added to the cells. After the incubation, cells were washed with HBSS three times and cultured for 24 h in 2 mL of culture medium. At 24 h, supernatants of cultured cells were collected for measurements of IFN-γ using an enzyme-linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd.) according to the manufacturer’s instruction.

5. 2. 10. Treatment of tumor-bearing mice

E.G7-OVA cells ($1 \times 10^6$ cells) were subcutaneously inoculated into the left backs of C57BL/6 mice under anesthesia with isoflurane. On days 5 and 12, OVA (20 μg)-loaded MGLu-liposomes with MPLA (4 g/mol lipid), pCMV-IFN-γ (40 μg)-containing lipoplexes and their hybrid complexes (C/P ratio of 2) were subcutaneously injected into the right backs of the mice under anesthesia with isoflurane. For the combination delivery, the liposomes and the lipoplexes were administered into the right backs of mice without complexation. For the separate delivery, the liposomes were administered into the right backs of mice and the lipoplexes were administered intratumorally. 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 2500 mm$^3$. All treated groups contained four mice.

5. 2. 11. CTL assay

CTL assay was performed as reported previously [24,43]. E.G7-OVA cells ($1 \times 10^6$ cells) were subcutaneously inoculated into the left backs of C57BL/6 mice under anesthesia. On day 5, OVA (20 μg)-loaded MGLu-liposomes with MPLA (4 g/mol lipid), or combination of liposomes and pCMV-IFN-γ (40 μg)-containing lipoplexes were subcutaneously injected into the right backs of the mice
under anesthesia with isoflurane. On day 10, splenocytes were suspended in RPMI-1640 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 effector cells to target cells ratio of 8.0, which was defined as E/T ratio, using a lactate dehydrogenase (LDH) cytotoxicity detection assay (Takara Biomedicals, Tokyo, Japan). E.G7-OVA or EL4 cells were used as target cells.

5. 2. 12. Immunohistochemical analysis

E.G7-OVA cells (1 × 10^6 cells) were subcutaneously inoculated into the left backs of C57BL/6 mice under anesthesia. On days 5 and/or 12, 20 µg of OVA-loaded MGlupG-liposomes with MPLA (4 g/mol lipid), or combination of the MGlupG-liposomes and 40 µg of pCMV-IFN-γ-containing lipoplexes were subcutaneously injected into the right backs of the mice under anesthesia with isoflurane. On days 7, 10, and 13, tumor of each mouse was excised for immunohistochemistry.

For immunofluorescence staining, tumor tissues were frozen immediately after embedding in Tissue-Tek OCT (Sakura Finetek). Tumor tissues were sectioned into 10 mm slices using a cryomicrotome (CM3050S, Leica) and mounted on glass slides. The sections were incubated with PBS containing 10% goat serum (The Jackson Laboratory) for 15 min at room temperature to block the non-specific binding of antibody. Subsequently, the sections were incubated with rat polyclonal PE-anti mouse CD8 (1:1000 dilution, eBioscience) at room temperature for 1 h, followed by incubated for 45 min with F(ab′)2 fragment of goat polyclonal Cy3-anti rat IgG (1:500 dilution, Jackson immune research) at room temperature. Slides were mounted with Vectashield containing 4, 6-diamidino-2-phenylindole (DAPI, Vector Laboratories). CLSM analysis of these slides was performed using LSM 5 EXCITER (Carl Zeiss Co. Ltd.). For histopathology, excised tissues were fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin wax for hematoxylin and eosin (H&E) staining. Tumor tissues were sectioned into 5 mm slices and mounted on glass slides
followed by H&E staining using DRS-601 Slide stainer (Sakura Finetek). Microscopic analysis of these slides was performed using BX40 microscope (Olympus).

5.3. Results and Discussion

5.3.1. Preparation of OVA and IFN-γ gene carrier systems

In this study, we used MGLu-PG-modified EYPC liposomes and TRX-based cationic liposomes respectively as carriers of antigenic protein OVA and IFN-γ gene (Fig. 5-1). The effect of their combination on induction of antigen-specific immune response was investigated.

We have already shown that the mixing of MGLuPGliposomes and TRX-based lipoplexes in aqueous solutions produced their hybrid complexes through electrostatic interaction [35-37]. The diameters and zeta potentials of the liposomes, lipoplexes and hybrid complexes are presented in Table 5-1. The lipoplexes must have a positively charged surface for the complex formation with negatively charged MGLu-PG-liposomes. Therefore, we prepared the lipoplexes with the N/P ratio of 4.0. In fact, the lipoplexes were estimated as small (ca. 130 nm) particles with the zeta potential of 39 mV. MGLu-PG-liposomes showed mean diameters of around 70 nm and zeta potentials of around −46 mV, which was derived from negatively charged MGLu-PG chains fixed on the liposome surface. The morphology for MGLu-PG-liposomes and the lipoplexes was investigated using transmission electron microscopy (Fig. 5-S2). Spherical vesicular structures with size of 50-70 nm and spherical multi-layered structures with size of 100-200 nm were observed for MGLuPG-liposomes and the lipoplexes, respectively, which are consistent with the results of DLS (Table 5-1).
Table 5-1. Sizes and ζ-potential of liposomes, lipoplexes, and hybrid complexes with various C/P ratios

<table>
<thead>
<tr>
<th>C/P ratio</th>
<th>Mean diameter (nm)</th>
<th>PDI</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGluPG liposome</td>
<td>–</td>
<td>73.3 ± 46</td>
<td>0.08</td>
</tr>
<tr>
<td>Lipoplex</td>
<td>0</td>
<td>128.1 ± 5.1</td>
<td>0.16</td>
</tr>
<tr>
<td>Hybrid complex</td>
<td>2</td>
<td>158.1 ± 10.2</td>
<td>0.34</td>
</tr>
<tr>
<td>Hybrid complex</td>
<td>4</td>
<td>219.1 ± 15.0</td>
<td>0.38</td>
</tr>
<tr>
<td>Hybrid complex</td>
<td>6</td>
<td>1078.3 ± 76.5</td>
<td>0.54</td>
</tr>
</tbody>
</table>

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Fig. 5-S2. Transmission electron microscopic (TEM) images of OVA-loaded MGlu-PG liposomes (A) and IFN-γ gene-containing lipoplexes (N/P ratio of 4) (B). Scale bars represent 200 nm. TEM analysis was performed using JEM-2000FEX II (JEOL Ltd, Japan). Samples were stained using 2% phosphotungstic acid aqueous solution (pH 7.4). © 2015 Elsevier Ltd.

The lipoplexes and the MGluPG-liposomes were mixed at varying molar ratios of the MGluPG carboxyl groups to DNA phosphate groups of the lipoplexes (C/P ratios) for the generation of their hybrid complexes with various compositions. Their diameters and zeta potentials are also presented in Table 5-1. With increasing C/P ratio, their sizes increased and their zeta potentials decreased, suggesting that positively charged lipoplexes were covered with negatively charged MGluPG-liposomes. When these liposomes and lipoplexes were mixed at the C/P ratio of 6.0, large aggregates were formed, probably because charge neutralization through their complexation might decrease hydration of their surface and decrease the colloidal stability of the hybrid complex particles.

To evaluate the effect of MGluPG-liposome complexation on the stability of lipoplexes, we examined the liberation of pDNA from the lipoplexes by electrophoretic analysis (Fig. 5-2A and B).
Liberation of pDNA from the lipoplexes was observed only slightly after mixing of MGlupG-liposomes at C/P ratios of 0-5.0, which indicates that binding of negatively charged liposomes destabilizes the lipoplexes only slightly during their complex formation. We also investigated whether the MGlupG-liposomes retain their contents or not during the complex formation with lipoplexes. First, MGlupG-liposomes loaded with water-soluble fluorescence dye pyranine were prepared. The release of pyranine from the liposomes was monitored in the absence or presence of the lipoplex (Fig. 5-2C). Irrespective of the presence or absence of the lipoplexes, pyranine release from the liposomes was fundamentally the same, indicating that liposome integrity was maintained through interaction with the lipoplexes. These results indicate that the hybrid complexes were formed without marked destabilization of the component liposomes and lipoplexes.

Fig. 5-2. (A, B) Electrophoretic analysis of hybrid complexes with N/P ratio of 4 and various C/P ratios. Percent of free pDNA was plotted against C/P ratios (B). (C) Percent release of pyranine from MGlupG-modified liposomes (circle) or hybrid complexes prepared by mixing of pyranine-loaded MGlupG-modified liposomes and lipoplexes (square) at pH 7.4. Lipid concentration was $2 \times 10^{-5}$ M. © 2015 Elsevier Ltd.
5. 3. 2. Co-delivery of antigen and gene into dendritic cells

We compared the co-delivery of antigen and gene into DCs between a combination of the MGLuPG-liposomes and the lipoplexes and their hybrid complexes. First, we examined the delivery of pDNA mediated by the hybrid complexes using pDNA labeled with FITC. The lipoplexes were prepared using FITC-labeled pDNA and mixed with MGLuPG-liposomes at C/P ratios of 2 and 4, which produced hybrid complexes with high colloidal stability (Table 5-1). DC2.4 cells were incubated with these hybrid complexes as well as the parent lipoplexes (C/P ratio of 0); then the intensity of FITC fluorescence for the treated cells was measured using a flow cytometer (Fig. 5-3). Cellular association of hybrid complexes tended to decrease slightly with increasing C/P ratio. The positive charge of the lipoplex surface decreased concomitantly with increasing C/P ratios (Table 5-1). Therefore, the hybrid complexes might not interact with the cells so effectively as with the lipoplexes through electrostatic interaction. Nevertheless, the hybrid complexes might exhibit levels of cellular association that are comparable to those of the parent lipoplexes, probably because scavenger receptors of DCs recognized negatively charged MGLuPG chains of the hybrid complexes [37, 44, 45]. Alternatively, MGLuPG-liposomes and the lipoplexes were added to DC2.4 cells at the corresponding C/P ratios without mixing. As presented in Fig. 5-3, the FITC fluorescence intensity of the treated cells was almost constant, irrespective of the C/P ratio, indicating that the existence of MGLuPG-liposomes did not interfere with the cellular association of the lipoplexes.
Fig. 5-3. Mean fluorescence intensity of DC2.4 cells treated with FITC-labeled pDNA-loaded hybrid complexes with various C/P ratios (closed bars) for 4 h in the presence of 10% FBS. Also, MGluPG-modified liposomes and FITC-labeled pDNA-loaded lipoplexes at corresponding C/P ratios were added without pre-mixing to DC2.4 cells (open bars). pDNA concentration was 0.5 μg/mL. Lipid concentration were 3.2 and 6.4 μM for C/P ratio of 2 and 4, respectively. Mean fluorescence intensity after subtraction of the background autofluorescence (0.3 a.u.) is shown. © 2015 Elsevier Ltd.

We further examined co-delivery of antigenic protein and pDNA into DCs mediated by the liposomes, lipoplexes, and their hybrid complexes using FITC-labeled OVA and Rh-labeled pDNA. We prepared MGluPG-liposomes loaded with FITC-OVA, the lipoplexes containing Rh-pDNA and their hybrid complexes and incubated DC2.4 cells with them. After the incubation, the cells were stained with LysoTracker Blue reagent to visualize late endosomes and lysosomes; then the cells were observed with CLSM (Fig. 5-4). The cells treated with the lipoplexes showed red Rh-pDNA fluorescence in the cells and a large fraction of the fluorescent dots were overlapped with LysoTracker Blue fluorescence, which were observed as pale purple-colored dots in the merged image. This fact suggests that the lipoplexes were taken up by DCs via endocytosis and that they are located mainly in endosomes and/or lysosomes. The cells treated with the MGluPG-liposomes exhibited both dotted fluorescence and diffuse fluorescence of FITC-OVA, which suggests that OVA molecules were present in both endosomes and lysosomes as well as the cytosol of the cells. After internalization through endocytosis, the MGluPG-liposomes might destabilize or fuse with endosomes and
lysosomes, resulting in transfer of FITC-OVA molecules into cytosol [21,22]. When these liposomes and lipoplexes were added to the cells simultaneously without pre-mixing, we observed all dotted FITC-OVA fluorescence, diffuse FITC-OVA fluorescence, and dotted Rh-pDNA fluorescence, indicating that the liposomes and lipoplexes respectively delivered OVA and pDNA into the cells without interfering with their delivery functions. When these liposomes and lipoplexes were added to the cells after their pre-mixing, the cells again displayed these Rh-pDNA fluorescence and FITC-OVA fluorescence as observed with the liposome and lipoplex-treated cells. This observation indicates that the hybrid complexes also delivered pDNA and OVA into DC2.4 cells as these liposomes and lipoplexes achieved.

Fig. 5-4. Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with lipoplexes containing Rh-labeled pDNA (red), MGl-PG-modified liposomes containing FITC-OVA (green), combination of these lipoplexes and liposomes, and their hybrid complexes (C/P ratio of 4) for 4 h at 37 °C in the presence of 10% FBS. Cellular acidic compartments were stained using LysoTracker Blue. © 2015 Elsevier Ltd.
5.3.3. IFN-γ production of dendritic cells treated with IFN-γ-gene-cotaining complex

As described above, the lipoplexes and the hybrid complexes efficiently delivered pDNA containing IFN-γ gene into DC2.4 cells. Therefore, we examined whether the transfected cells actually produce IFN-γ or not. DC2.4 cells were treated with the lipoplexes and the hybrid complexes with C/P ratios of 2 or 4. The pDNA contains gene coding IFN-γ with the signal peptide sequence. Therefore, IFN-γ produced in DC2.4 cells are secreted from the cells. Consequently, IFN-γ concentration in the medium of the transfected cells was evaluated by ELISA (Fig. 5-5). The medium of the cells treated with the MGlu-PG-liposomes or EGFP-gene-containing lipoplexes contained negligible levels of IFN-γ, whereas the IFN-γ concentration in the medium of the cells treated with the lipoplexes containing IFN-g gene was greatly increased during 24 h incubation after transfection. This fact indicates that the IFN-γ gene delivered by the lipoplexes was expressed in the DC2.4 cells and that it engenders efficient IFN-γ production. Compared to the lipoplexes, the hybrid complexes exhibited somewhat lower ability of IFN-γ production and the ability tended to decrease concomitantly with increasing C/P ratio.

As Fig. 5-4 shows, significant difference is not observed in colocalization of pDNA and lysosomes between the lipoplex-treated cells and the hybrid complex-treated cells and, for both cases, pDNA molecules existing in the cytosol were observed (Fig. 5-S3). Taking into account that the hybrid complexes showed lower amounts of cellular association (Fig. 5-3), the hybrid complex-treated cells might contain less amounts of pDNA in the cytosol, compared to the lipoplexes-treated cells. In addition, considering that pDNA molecules may be associated with components of the lipoplexes or hybrid complexes after escaping from endosomes, their characteristic differences derived from their associated components may affect their intracellular behaviors and IFN-γ gene expression efficiency, although their intracellular behaviors are currently to be elucidated. A quite high level of IFN-γ was produced with the hybrid complexes with the C/P ratio of 2. Therefore, we used them in the following experiments.
Fig. 5-5. IFN-γ production from DC2.4 cells treated with OVA-loaded MGluePG-modified liposomes, pCMV-IFN-γ-loaded lipoplexes (C/P ratio = 0) and hybrid complexes (C/P ratio = 2 or 4) for 4 h and cultured for another 24 h in the presence of 10% FBS. Lipoplexes containing pEGFP-C1 were also examined as a control. © 2015 Elsevier Ltd.

Fig. 5-S3. Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with lipoplexes or hybrid complexes (C/P ratio of 4) containing Rh-labeled pDNA (red) for 4 h at 37°C in the presence of 10% FBS. Cellular acidic compartments were stained using LysoTracker Blue. Arrowheads indicate pDNA in cytosol. Scale bar represents 10 μm. © 2015 Elsevier Ltd.

5.3.4. Therapeutic effects of co-delivery of antigen and gene on tumor-bearing mice

We investigated the synergetic effects of co-delivery of antigen and IFN-γ gene on the induction of antitumor immunity using a combination of MGlue-PG-liposomes and the lipoplexes or their hybrid complexes. Mice were first inoculated with OVA-expressing murine T lymphoma E.G7-OVA cells. Then IFN-γ gene-containing lipoplexes, MGlue-PG-liposomes encapsulating OVA, and their hybrid
complexes were injected subcutaneously into tumor-bearing mice on 5 and 12 days after tumor cell inoculation. Then the tumor volumes were monitored (Fig. 5-6A). When mice were administered with PBS, the tumor volume increased rapidly. In addition, a similar rapid increase of tumors was observed for mice administered with the lipoplexes, indicating that delivery of IFN-γ gene using the lipoplexes was ineffective. In contrast, administration of the MGLu-PG-liposomes induced a significant decrease of tumor volume after day 12, indicating that administration of the OVA-loaded MGLu-PG-liposomes induced OVA-specific immunity, which attacks the OVA-expressing E.G7-OVA tumor cells, as reported previously [24]. The administration of the hybrid complexes also induced significant tumor suppression. However, their antitumor effect was almost equal to that of the OVA-loaded MGLuPG-liposomes administration (Fig. 5-6A). The mice survival was prolonged only slightly compared to the mice treated with the MGLu-PG-liposome administration (Fig. 5-6D).

Co-delivery of antigen and IFN-γ gene by combination of the MGLu-PG-liposomes and the lipoplexes without their hybrid formation by pre-mixing was also examined (Fig. 5-6B and E). These liposomes and lipoplexes were injected subcutaneously around the same site of the opposite side of the tumor. Then tumor growth was monitored (Fig. 5-6B). Tumors of mice treated with the combination of these liposomes and lipoplexes decreased around day 12 and disappeared around day 18 after their administration, as was the case with the MGLu-PG-liposome administration. Whereas regrowth of tumor was observed for the mice treated with the MGLu-PG-liposomes, no tumor regrowth was observed for the mice treated with the combination of these liposomes and lipoplexes. All mice treated with the combination administration were cured and survived over 60 days (Fig. 5-6E). This result indicates that much stronger antitumor effects were induced by the combination administration than the MGLuPG-liposome administration.

For comparison, administration of the MGLu-PG-liposomes and the lipoplexes at distant sites of the tumor-bearing mice were examined further (Fig. 5-6C and F). The MGLu-PG-liposomes were administered at opposite sides of the tumor. Lipoplexes were administered into the tumor and the tumor growth was monitored. In contrast to administration by a combination of these liposomes and
lipoplexes, their administration at distant sites exhibited tumor regrowth, which occurred also for MGl-u-PG-liposome administration, suggesting the importance of their injection at the same site of mice to obtain their synergetic effect for strong tumor suppression. When the lipoplexes containing IFN-γ gene and the OVA-loaded MGl-u-PG-liposomes are administered at the same site, DCs existing there might take up the particles and migrate to same draining lymph node. There, MHC class I-mediated antigen presentation might be induced with the MGl-u-PG-liposomes [18,24]. The activation of antigen-specific CTLs might be promoted concomitantly by secretion of IFN-γ from these same and/or nearby DCs existing in the same draining lymph node, resulting in induction of strong antitumor immune responses (Fig. 5-6B and C). In contrast, when these lipoplexes and liposomes are injected at distant sites, DCs existing at each site might take up each particle. Additionally, they might migrate to different lymph nodes, where effective activation of cellular immunity through synergy of antigen presentation and IFN-γ secretion might not occur.

In comparison of administration between the combination of the MGl-u-PG-liposomes and the lipoplexes and their hybrid complexes, the former led to much stronger tumor suppression. As described above, a higher level of IFN-γ gene expression was induced in DC2.4 cells treated with the lipoplexes than in those with the hybrid complexes (Fig. 5-5). Therefore, efficient IFN-γ secretion of DCs treated with the lipoplexes might result in the efficient cellular immune response. In addition, considering that the hybrid complexes were larger than these lipoplexes and liposomes (Table 5-1), uptake of the hybrid complexes might be less efficient than these component particles, which also might result in the lower suppression of tumors in mice.
Fig. 5-6. Antitumor effects induced by co-delivery of antigen and IFN-γ gene to tumor-bearing C57BL/6 mice. Change in tumor volumes (A-C) and survival (%) (D-F) were shown for the mice treated with hybrid complexes (closed squares), combination of OVA-loaded MGLu-liposomes and IFN-γ gene-containing lipoplexes without complexation (closed circles) or separate injection of OVA-loaded MGLu-liposomes and IFN-γ gene-containing lipoplexes at different sites (open triangles). Schematic illustrations of each administration route was shown in the figure. Data for administration of PBS (open squares), the OVA-loaded MGLu-liposomes (closed triangles) and the IFN-γ gene-containing lipoplexes (open circles) were also given. Independent experiments were performed to estimate the effect of the hybrid complexes (A, D), combination (B, D) or separate injection (C, E) for the OVA-loaded MGLu-liposomes and the IFN-γ gene-containing lipoplexes. The hybrid complexes with C/P ratio of 2 were used. © 2015 Elsevier Ltd.
5. 3. 5. Induction of antigen-specific CTLs in spleen

We estimated the induction of the antigen-specific CTL in spleen to confirm the activation of cellular immunity in mice by administration through a combination of the OVA-loaded MGlu-PG-liposomes and the IFN-γ gene-containing lipoplexes. For this study, OVA-specific CTLs were obtained by correcting splenocytes from E.G7-OVA tumor-bearing mice immunized with these liposomes and lipoplexes and subsequent stimulation with mitomycin C-treated E.G7-OVA cells. Then their capabilities to induce cellular lysis against EL4 cells and E.G7-OVA cells were measured (Fig. 5-7).

Compared to the immunization with the MGlu-PG-liposomes, the immunization with the combination of these liposomes and lipoplexes showed a high level of cellular lysis against E.G7-OVA cells. Indeed, the same CTLs showed a marginal level of cytotoxicity to OVA-non-expressing EL4 cells, indicating that administration through the combination of the MGluPG-liposomes and lipoplexes induced the antigen-specific CTLs more effectively than administration of the MGlu-PG-liposomes.

![Graph showing induction of antigen-specific cytotoxic T lymphocytes in spleen.](image)

**Fig. 5-7.** Induction of antigen-specific cytotoxic T lymphocytes in spleen. E.G7-OVA cells (1 × 10^6 cells/mouse) were inoculated into the left backs of C57BL/6 mice. On day 5, PBS, OVA-loaded MGlu-liposomes, or combination of OVA-loaded MGlu-liposomes and the IFN-γ gene-containing lipoplexes were administered into right backs of mice. On day 10, splenocytes were collected and cultured with mitomycin C-treated E.G7-OVA cells for 5 days. Cytotoxic activity was measured by a LDH assay at E/T ratio of 8. E.G7-OVA cells (closed bars) and EL4 cells (open bars) were used as target cells. Each point represents means ± SD (n = 3). © 2015 Elsevier Ltd.
5.3.6. Histopathological analysis of tumor tissues

Finally, immunohistochemical analysis was conducted to detect CTLs in the tumor tissues. Tumors were dissected from mice treated with PBS, OVA-loaded MGlut-PG-liposomes, and a combination of these liposomes and lipoplexes at various times. Tumor sections were stained with CD8-specific antibody and its secondary antibody labeled with Cy3; then they were observed with CLSM (Fig. 5-8A). For tumors of the PBS-treated mice, CTLs was not observed at any time between day 7 and day 13. For tumors of the MGlut-PG-liposome-treated mice, only a few CTLs were observed on days 7 and 10, but many red fluorescence dots appeared in the tumor section on day 13, which indicates that many CTLs infiltrated into the tumor tissue, corresponding to the tumor cell death that occurred at around this time (Fig. 5-6). For tumors of the mice treated with the combination of the liposomes and lipoplexes, marked CTL was observed in the tumor even on day 10, which means that efficient CTL infiltration took place within 5 days after the immunization.

In general, the T-cell responses are known to be induced in 4-5 days after immunization. In fact, antigen-specific T cells were observed on the 5th day from the administration of antigen-loaded liposomes at spleen (Fig. 5-7). Therefore, it is likely that the antigenspecific T cells were activated, migrated to the tumor sites and attacked the antigen-expressing tumor cells in 5 days after coadministration of antigen-loaded liposomes and lipoplexes. Immunization through the liposome-lipoplex combination might cause the induction of OVA-specific CTLs and subsequent clonal expansion of the CTLs, resulting in the efficient CTL infiltration in the tumor within the short period. The efficient infiltration of CTLs in the tumor tissues was also observed on day 13. This result indicates that immunization with the liposome-lipoplex combination strongly activates target-specific CTLs, which enabled the efficient infiltration of CTLs into the tumor tissues for earlier and longer periods after immunization.

Tumor sections were analyzed further using H&E staining (Fig. 5-8B). For tumors of mice immunized with the OVA-loaded MGlut-PG liposomes, a fraction of tumor cells lost nuclei and were damaged, but most cells appeared to be healthy on day 10. However, on day 13, damaged cells, such
as apoptotic cells with deformed nuclei (Fig. 5-8B-b) and denucleated necrotic cells (Fig. 5-8B-c), became readily apparent, indicating that the tumor cells were damaged and killed effectively during day 10-13. For tumors of mice immunized with the liposome-lipoplex combination, many damaged cells were observed even on day 10. On day 13, a part of the tumor displayed severely damaged cells on day 13 (Fig. 5-8B-d), but most parts of the tumor consisted of fibrotic tissues and fibroblast-like cells (Fig. 5-8B-e), which might be a healing process for normal tissue after tumor cell death. These results indicate that immunization with the liposome-lipoplex combination delivery led to tumor cell death at the early stage of the treatment and rapid progress of therapeutic processes.

![Immunofluorescence analysis of tumor sections](image)

Fig. 5-8. (A) Immunofluorescence analysis of tumor sections from mice subcutaneously administered with PBS, OVA-loaded MGluliposomes, or combination of OVA-loaded MGluliposomes and the IFN-γ gene-containing lipoplexes on days 5 and/or 12. CD8 positive cells in tumor sections were stained using anti-mouse CD8 antibody and Cy3-anti-rat IgG as a secondary antibody (Red). Cellular nucleus were stained by DAPI (Blue). © 2015 Elsevier Ltd.
Fig. 5-8. (B) H&E staining for tumor sections from mice subcutaneously administered with PBS, OVA-loaded MGlu-liposomes, or combination of OVA-loaded MGlu-liposomes and the IFN-γ gene-containing lipoplexes on days 5 and/or 12. Magnified images for regions (a-e) in the middle images are shown in the bottom. © 2015 Elsevier Ltd.

5.4. Conclusion

This study presented an efficient strategy for the potentiation of pH-sensitive MGlu-PG-liposome-based antigen carriers to achieve efficient induction of tumor-specific immunity using IFN-γ gene
lipoplexes. Results demonstrated that both the antigenic protein (OVA) and IFN-γ gene-containing pDNA into DC2.4 were delivered in two ways by the combination of antigen-loaded MGlU-PG-liposomes and the IFN-γ gene-containing lipoplexes and their hybrid complexes, although the hybrid complexes showed lower transfection activities than the parent lipoplexes did. The immunization of tumor-bearing mice with the combination of the MGlU-PG-liposomes and lipoplexes achieved more efficient tumor-specific immune responses than the immunization with the hybrid complexes because both efficient antigen-presentation for specific CTL induction and efficient IFN-γ production were induced simultaneously for DCs of the same regional lymph node. Consequently, the strong target-specific immunity induced by the immunization caused the disappearance of tumors in mice, which survived for more than 60 days without tumor regrowth. Therefore, the combination of the pH-sensitive MGlU-PG-liposome-mediated and lipoplex-mediated antigen and IFN-γ gene delivery systems might be a promising strategy for efficient cancer immunotherapy.

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Chapter 6: Dextran derivative-based pH-sensitive liposomes for cancer immunotherapy

6. 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 glutaric 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-methylglutaric anhydride. The resultant pH-sensitive dextran derivatives (MGlu-attached dextran: MGl-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 (Fig. 6-1) have been investigated.

![Fig. 6-1. 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. © 2014 Elsevier Ltd.](image-url)
6. 2. Materials and methods

6. 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) fluorescein isothiocyanate (FITC), dextran having molecular weight of 40,000 (Dex$_{40k}$), and 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].

6. 2. 2. Synthesis of dextran derivatives

3-Methyl-glutarylated dextran (MGl-Dex) was prepared by reaction of dextran with 3-methylglutaric anhydride. For MGl$_{56}$-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 MGl-Dex polymers were also synthesized by reaction of dextran with various amounts of 3-methylglutaric anhydride by the same procedure. $^1$H NMR for MGl-Dex (400 MHz, D$_2$O + NaOD): δ 0.9 (s, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 1.9-2.3 (br, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 3.5-4.0 (br, glucose 2H, 3H, 4H, 5H, 6H), 5.0 (br, glucose 1H).
As anchor moieties for fixation of MGlue-Dex onto liposome membranes, 1-aminodecane was combined with carboxyl groups of MGlue-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 MGlue units to decyl amide units for MGlue-Dex polymers were estimated using $^1$H NMR. $^1$H NMR for MGlue-Dex-C$_{10}$ (400 MHz, D$_2$O + NaOD) : $\delta$ 0.8-0.9 (br, -CO-NH-CH$_2$-(CH$_2$)$_8$-CH$_3$), 0.9 (s, -CO-CH$_2$-CH(CH$_3$)$\cdot$-CH$_2$-$\cdot$), 1.2-1.4 (br, -CO-NH-CH$_2$-(CH$_2$)$_8$-CH$_3$), 1.9-2.3 (br, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-$\cdot$), 3.2 (br, -CO-NH-CH$_2$-(CH$_2$)$_8$-CH$_3$), 3.5-4.0 (br, glucose 2H, 3H, 4H, 5H, 6H), 5.0 (br, glucose 1H).

6. 2. 3. Titration

To 10 mL of an aqueous solution of each polymer (carboxylate concentration: 3.0 × 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).

6. 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.

6. 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 mM 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.

6. 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].

6. 2. 7. Animals

Female C57BL/6 mice (H-2b, 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.

6. 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.
6.2.9. 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 were obtained as an average of more than three measurements on different samples.

6.2.10. 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 25mM phosphate solution (pH 7.4). Release of pyranine from liposome was measured as previously reported [12, 13, 24]. Liposomes encapsulating pyranine (lipid concentration: $2.0 \times 10^{-5}$ 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

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

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 Triton X-100 (final concentration: 0.1%).

6.2.11. Intracellular behavior of liposome

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 ($3 \times 10^5$ 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.

6. 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 in liposomes was 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 ml of substrate solution was added. The plates were allowed to stand for 10 min at room temperature, and 100 ml 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].

6. 2. 13. In vitro cytokine release measured by enzyme-immunosorbent assay (ELISA)

On days 7 and 14, 100 μg of OVA loaded in liposomes was 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) were 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-labeled rabbit antimouse 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 mL, 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 microplate reader (Wallac1420, Perkin Elmer). The concentration of IFN-γ in the samples was evaluated on the basis of standard curves.

6.2.14. 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 mg of OVA loaded in liposomes was 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).
6. 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 in liposomes was 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 2500 mm^3. All treated groups contained four mice.

6. 3. Results and discussion

6. 3. 1. Characterization of dextran derivative

Several kinds of pH-sensitive dextran derivatives (MGlu-Dex) with different contents of MGluc 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 MGluc units for fixation of dextran derivatives onto liposome membrane (Scheme 6-1). The obtained dextran derivatives were characterized using 1H NMR. Fig. 6-2 depicts 1H NMR spectra of Dex_70k, MGluc_76-Dex_70k, and MGluc_56-Dex_70k-C10. In comparison of spectra for Dex_70k, which was used as a starting material, (Fig. 6-2A) and for MGluc_76-Dex_70k, (Fig. 6-2B), which was the product, introduction of MGluc residues to dextran was confirmed from the existence of new peaks corresponding to MGluc residues (0.9 ppm, 2-2.3 ppm) in Fig. 6-2B. From the integration ratio of peaks of MGluc residues to those of dextran backbone (3.5-4.0 ppm), 76% of hydroxyl groups of dextran were estimated as combined with MGluc residues. Similarly, from the integration ratio between dextran backbone, MGluc residues, and decyl groups (0.9-1.5 ppm), decyl-amidated MGluc residues and MGluc 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\(_{70k}\)-C10. Compositions of dextran derivatives prepared in this study are presented in Table 6-1.

![Scheme 6-1](image)

**Scheme 6-1.** Synthetic route of pH-sensitive dextran derivatives. © 2014 Elsevier Ltd.

![Fig. 6-2](image)

**Fig. 6-2.** \(^1\)H NMR spectra of (A) Dex\(_{70k}\), (B) MGlu\(_{76}\)-Dex\(_{70k}\), and (C) MGlu\(_{56}\)-Dex\(_{70k}\)-C10 in D\(_2\)O/NaOD. © 2014 Elsevier Ltd.

**Table 6-1.** Composition of dextran derivatives

<table>
<thead>
<tr>
<th>Polymer</th>
<th>-OH(mol%)</th>
<th>-MGlu(mol%)</th>
<th>-Anchor(mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGlu(<em>{13})Dex(</em>{70k})</td>
<td>87</td>
<td>13</td>
<td>—</td>
</tr>
<tr>
<td>MGlu(<em>{34})Dex(</em>{70k})</td>
<td>66</td>
<td>34</td>
<td>—</td>
</tr>
<tr>
<td>MGlu(<em>{52})Dex(</em>{70k})</td>
<td>52</td>
<td>48</td>
<td>—</td>
</tr>
<tr>
<td>MGlu(<em>{76})Dex(</em>{70k})</td>
<td>24</td>
<td>76</td>
<td>—</td>
</tr>
<tr>
<td>MGlu(<em>{56})Dex(</em>{70k})</td>
<td>15</td>
<td>85</td>
<td>—</td>
</tr>
<tr>
<td>MGlu(<em>{56})Dex(</em>{40k})</td>
<td>34</td>
<td>66</td>
<td>—</td>
</tr>
<tr>
<td>MGlu(<em>{72})Dex(</em>{500k})</td>
<td>28</td>
<td>72</td>
<td>—</td>
</tr>
<tr>
<td>MGlu(<em>{52})Dex(</em>{70k})_C(_{10})</td>
<td>68</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>MGlu(<em>{56})Dex(</em>{70k})_C(_{10})</td>
<td>37</td>
<td>56</td>
<td>7</td>
</tr>
<tr>
<td>MGlu(<em>{77})Dex(</em>{70k})_C(_{10})</td>
<td>19</td>
<td>77</td>
<td>4</td>
</tr>
<tr>
<td>MGlu(<em>{56})Dex(</em>{40k})_C(_{10})</td>
<td>43</td>
<td>55</td>
<td>6</td>
</tr>
<tr>
<td>MGlu(<em>{56})Dex(</em>{500k})_C(_{10})</td>
<td>36</td>
<td>58</td>
<td>6</td>
</tr>
</tbody>
</table>

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Acid-base titration of MGlu-Dex was done (Fig. 6-3). 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 MGlu13-Dex70k, MGlu48-Dex70k, and MGlu76-Dex70k. In contrast, MGlu76-Dex70k, MGlu66-Dex40k and MGlu72-Dex500k, 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 pKa for these MGlu-Dex were determined from their titration curves. They are listed in Table 6-2.

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 6-2. MGluc13-Dex70k showed no precipitation throughout the experimental pH region, probably because this polymer has a hydrophilic nature even after all MGluc 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 MGluc unit% of MGluc-Dex, indicating that polymers with higher content of MGluc-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.

Table 6-2. pKa and precipitation pH for various dextran derivatives

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pK\text{a}</th>
<th>precipitation pH</th>
<th>Degree of protonation at precipitation pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGluc13-Dex70k</td>
<td>5.30</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>MGluc48-Dex70k</td>
<td>5.81</td>
<td>4.3</td>
<td>0.92</td>
</tr>
<tr>
<td>MGluc76-Dex70k</td>
<td>6.63</td>
<td>4.5</td>
<td>0.98</td>
</tr>
<tr>
<td>MGluc66-Dex40k</td>
<td>6.26</td>
<td>4.5</td>
<td>0.95</td>
</tr>
<tr>
<td>MGluc72-Dex500k</td>
<td>6.24</td>
<td>4.5</td>
<td>0.95</td>
</tr>
</tbody>
</table>

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]. Fig. 6-4 depicts the \(I_1/I_3\) ratio of pyrene fluorescence in the buffer dissolving various polymers as a function of pH. In buffers dissolving MGluc13-Dex70k and MGluc35-Dex70k, 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 6-2).

**Fig. 6-4.** 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$_{44}$-Dex$_{70k}$ (closed circles), MGlu$_{76}$-Dex$_{70k}$ (closed diamonds), and MGlu$_{85}$-Dex$_{70k}$ (closed squares) dissolved in 30 mM sodium acetate and 120 mM NaCl solution of varying pHs. Concentrations of polymers and pyrene were 0.25 mg/mL and 1 mM, 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. © 2014 Elsevier Ltd.

### 6.3.2. 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 6-3). 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.

### Table 6-3. Particle sizes and zeta potentials of liposomes

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>96.5 ± 1.0</td>
<td>-18.6 ± 1.1</td>
</tr>
<tr>
<td>MGluc26-Dex70k-C10</td>
<td>103.1 ± 0.4</td>
<td>-49.5 ± 1.1</td>
</tr>
<tr>
<td>MGluc56-Dex70k-C10</td>
<td>109.3 ± 4.3</td>
<td>-46.5 ± 2.1</td>
</tr>
<tr>
<td>MGluc77-Dex70k-C10</td>
<td>104.8 ± 1.6</td>
<td>-40.7 ± 0.7</td>
</tr>
<tr>
<td>MGluc58-Dex40k-C10</td>
<td>142.7 ± 0.3</td>
<td>-54.0 ± 1.5</td>
</tr>
<tr>
<td>MGluc55-Dex500k-C10</td>
<td>150.9 ± 3.3</td>
<td>-54.1 ± 1.3</td>
</tr>
</tbody>
</table>

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. 6-5). Fig. 6-5A 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 polymermodified 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. 6-5B as a function of pH. MGluc26-Dex70k-C10-modified liposomes enhanced content release below pH 5.5, although enhancement was observed below pH 6.5 MGluc26-Dex70k-C10-modified and MGluc26-Dex70k-C10-modified liposomes. Because pKa and hydrophobicity of the MGluc-Dex tends to increase concomitantly with increasing MGluc residues on the polymer chains (Table 6-2 and Fig. 6-4), the MGluc-Dex with higher MGluc residue contents become protonated at higher pH and acquired a hydrophobic character, which enables strong interaction with the liposome membranes. This result
indicates that MGlru 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 (Fig. 6-5C, D). 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 MGlru unit content on the dextran backbone, but is not influenced by the chain length of the polymer backbone.

Fig. 6-5. pH-Sensitive contents release behaviors of dextran derivative-modified liposomes. Time courses (A) and pH-dependence (B) for pyranine release from liposomes modified with MGlru26-Dex70k-C10 (triangles), MGlru56-Dex70k-C10 (circles), or MGlru77-Dex70k-C10 (diamonds), and unmodified (squares) liposomes. Open and closed symbols in (A) express at pH 7.4 and at pH 5.0, respectively. Percent release after 30 min-incubation was shown in (B). Time courses (C) and pH-dependence (D) for pyranine release from liposomes modified with MGlru58-Dex40k-C10 (diamonds), or
6.3.3 Liposome-mediated OVA delivery to DCs

Next, we examined the capability of these dextran derivativemodified 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. 6-6). As presented in Fig. 6-6A, 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 MGl-Dex also exhibited punctate fluorescence of Rh-PE but diffuse fluorescence of FITC-OVA was visible in the cells (Fig. 6-6B-F), 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. 6-5), 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, MGl-Dex-modified liposomes were shown to generate fusion activity at weakly acidic pH (Fig. 6-S1). Therefore, it is likely that, after internalization into the cell through endocytosis, MGl-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 MGlul residue contents (Fig. 6-6B-D) 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 (Fig. 6-6C, E, F). 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 MGlul-Dex liposomes
displayed not only diffuse fluorescence but also punctate fluorescence (Fig. 6-6B-F). 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 liposometreated cells using flow cytometry (Fig. 6-7). The MGlu-Dexmodified 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 MGlu26-Dex70k-C10, MGlu56-Dex70k-C10, and MGlu77-Dex70k-C10, and unmodified liposomes (Fig. 6-7A), the liposomes having MGlu56-Dex70k-C10 exhibited much more efficient association to DCs than those without polymers or with MGlu26-Dex70k-C10. This result underscores the importance of MGlu residues for efficient cellular association of the liposomes. However, the liposomes with MGlu77-Dex70k-C10 exhibited lower cellular association than MGlu56-Dex70k-C10 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. 6-7B). MGlu residues attached onto the long polymer chains might be recognized more efficiently by the cellular receptors.
Fig. 6-6. Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with Rhodamine-PE-labeled and FITC-OVA-loaded EYPC liposomes without polymer (A) or modified with MGlu<sub>26</sub>-Dex<sub>70k</sub>-C<sub>10</sub> (B), MGlu<sub>55</sub>-Dex<sub>70k</sub>-C<sub>10</sub> (C), MGlu<sub>77</sub>-Dex<sub>70k-C10</sub> (D), MGlu<sub>55</sub>-Dex<sub>40k-C10</sub> (E), or MGlu<sub>55</sub>-Dex<sub>500k-C10</sub> (F) for 4 h at 37 °C in serum-free medium. Bar represents 10 μm. © 2014 Elsevier Ltd.
6. 3. 4. Liposome-mediated induction of antigen-specific immunity  

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. 6-8A). 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. 6-7).

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. 6-8B shows, administration of MGlu_{56}-Dex_{70k}-C_{10}-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}-C_{10}-modified liposomes. This result suggests that MGlu_{56}-Dex_{70k}-C_{10}-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.

![Graph](image)

**Fig. 6-8.** 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 MGl-Dex-C10-modified liposomes loaded with OVA. OVA-specific IgG titers were measured by ELISA at 12 days after the second administrations. © 2014 Elsevier Ltd.

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. 6-9). Compared with the splenocytes of the free OVA- or OVA-loaded unmodified liposome-immunized mice, those of mice treated with OVA-loaded MGl-Dex liposomes exhibited higher production of IFN-γ responding to OVA. Especially, treatment with OVA-loaded MGl56-Dex70k-C10-modified liposomes generated splenocytes with the highest production of IFN-γ. Combined with the results of Fig. 6-8, MGl56-Dex70k-C10-modified 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. Fig. 6-10 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 MGlut-Dex liposomes induced a much stronger level of CTL responses. These splenocytes only slightly exhibited cytotoxicity against EL4 cells. This result indicates that these MGlut-Dex liposomes can induce antigen-specific CTLs efficiently. Despite a difference in antigen-specific Th1 cell induction ability between MGlut26-Dex70k-C10-liposomes and MGlut56-Dex70k-C10-liposomes (Figs. 6-8 and 6-9), 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.

![Fig. 6-9. IFN-γ production of splenocytes of OVA-immunized mice.](image)

Splenocytes were isolated from C57BL/6 mice 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. © 2014 Elsevier Ltd.
OVA-specific CTL induction in spleen of tumor-bearing mice after 7 days from subcutaneous immunization with unmodified liposomes (squares), MGluc26-Dex70k-C10-modified liposomes (triangles), and MGluc56-Dex70k-C10-modified liposomes (diamonds). Cytotoxic activity was measured at various effector 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). © 2014 Elsevier Ltd.

6. 3. 5. Therapeutic effect of liposome-mediated immunization

We examined the therapeutic effects of immunization with MGluc-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. 6-11). First, we examined tumor-suppressive effects of the liposomes having MGluc-Dex with different MGluc contents. As Fig. 6-11A 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 MGluc26-Dex70k-C10-modified liposomes and MGluc56-Dex70k-C10-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. 6-11C). Comparison of tumor-suppressive
effects between these MGlul-Dex-liposomes revealed that MGlul56-Dex70k-C10-liposomes showed somewhat higher antitumor effect than that of MGlul26-Dex70k-C10-liposomes. This result might have derived from the induction of stronger Th1 responses by the MGlul56-Dex70k-C10-liposomes (Figs. 6-8 and 9).

We also compared the efficacy for tumor-suppression between the OVA-loaded liposomes having MGlul-Dex with different chain lengths (Fig. 6-11B and D). Despite a large difference in the polymer chain lengths, both MGlul58-Dex40k-C10-modified liposomes and MGlul55-Dex500k-C10-modified liposomes exhibited marked tumor regression. As described above, chain lengths of MGlul-Dex covering liposomes only slightly affect their abilities for cellular association (Fig. 6-7) and cytoplasmic delivery of antigen (Fig. 6-6). Results show that chain lengths of MGlul-Dex only slightly influence the tumorsuppressive effects of MGlul-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, MGlul-Dex liposomes exhibited comparable performance to poly(glycidol)-based pH-sensitive liposomes for the induction of tumor-suppressive immunity.
Fig. 6-11. 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 volumes were 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), MGIu56-Dex70k-C10-modified liposomes (triangles), and MGIu56-Dex70k-C10-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) MGIu56-Dex70k-C10-modified liposomes (circles) MGIu53-Dex40k-C10-modified liposomes (squares), and MGIu53-Dex50k-C10-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. © 2014 Elsevier Ltd.
6.4. 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.

6.5. References


Chapter 7: Bioactive polysaccharide-based pH-sensitive polymers for cytoplasmic delivery of antigen and activation of antigen-specific immunity

7.1. Introduction

Induction of cancer-specific immunity is crucially important to achieve immunotherapy. Especially, cell-mediated immune response (cellular immunity) is regarded as the most effective immune response to eliminate tumor cells directly [1-3]. For the induction of cellular immunity, the delivery of antigen to cytosol of antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs), and antigen presentation mediated by major histocompatibility complex (MHC) class I molecules are necessary [4-7]. Efficient cytoplasmic delivery carriers of antigen are necessary for the induction of cellular immunity and the establishment of cancer immunotherapy.

To date, various antigen cytoplasmic delivery systems have been developed such as polymeric nanoparticles, polymeric micelles, nanogels, and liposomes [8-14]. Among them, liposomes are useful as antigen carriers because of their biocompatibility and their ease of introducing functionality such as cellular targeting ability and pH-responsive content release properties [13,14]. In fact, pH-sensitive liposomes have been prepared using a mixture of nonbilayer forming lipids and amphiphiles having carboxylic acid or by the modification of virus-derived fusogenic proteins, pH-sensitive peptide, and pH-sensitive polymers [14-17]. These pH-sensitive liposomes induce destabilization of the endosomal membrane or membrane fusion with endosomal membranes in intracellular acidic environments (endosomes or lysosomes), which achieve the cytoplasmic delivery of contents [14-17].

We previously reported cytoplasmic delivery of antigen using liposomes modified with carboxylated poly(glycidol)s, which have a backbone resembling biocompatible poly(ethylene glycol) [18-21]. 3-Methyl glutarylated poly(glycidol)-modified liposomes delivered model antigenic protein, ovalbumin (OVA) into cytosol of DCs by membrane fusion with endosomal membrane
responding to acidic pH, and induced MHC class I-mediated antigen presentation [22]. Subcutaneous administration of these liposomes induced OVA-specific cellular immunity and therapeutic effects on tumor-bearing mice [23]. As a biodegradable pH-sensitive polymer, 3-methyl glutarylated dextran (MGlu-Dex), which has a polysaccharide backbone and pH-responsive MGlue side chain, was synthesized [24]. MGlu-Dex-modified liposomes also achieved the cytoplasmic delivery of OVA and the induction of cellular immunity [24].

To induce stronger cellular immunity, antigen delivery systems require functions not only for delivery of antigen into DC cytosol but also to activate DCs, which is designated as an adjuvant function. Typically, toll like receptor (TLR) ligands such as CpG-DNA, poly(I:C), and monophosphoryl lipid A (MPLA), which activate APCs via interaction with TLRs expressing in these cells, were introduced to antigen carriers as adjuvant molecules [25-29]. We have also combined immunity-activating molecules or systems such as MPLA mentioned above, cationic lipids, and IFN-γ-encoding gene delivery systems with pH-sensitive polymer-modified liposomes to enhance the cellular immune responses [30,31]. Actually, inclusion of these molecules or systems improved their ability to induce cellular immunity and antitumor effects [26,29-31]. However, these antigen delivery systems have complicated compositions because antigen delivery and activation of immune response are achieved by each functional molecule. Therefore, antigen carriers that can achieve both antigen delivery and activation by a single functional molecule are sought.

In this study, the inclusion of both antigen delivery function and adjuvant function to antigen carriers was attempted using intrinsically bioactive polysaccharide-based pH-sensitive polymers. For this purpose, curdlan and mannan were chosen as base polymers for the preparation of pH-sensitive polymers with DC activation ability (Fig. 7-1). Curdlan and mannan are microorganism-derived bioactive polysaccharides that are recognized, respectively, by surface receptors of macrophages or DCs such as Dectin-1 and Dectin-2 [32-34]. Recognition by these lectins leads to the activation of APCs and to the promotion of secretion of various cytokines, thereby activating immune response (Fig. 7-1). pH-Sensitive moieties (MGlu units) were introduced to these polysaccharides. Their
abilities to deliver antigen to DCs, to activate DCs, and to induce antigen-specific immune responses were investigated and compared with those of conventional pH-sensitive polysaccharide (MGl-u-Dex).

Fig. 7-1. Design of liposomes modified with bioactive polysaccharide derivatives for induction and activation of antigen-specific immunity. These liposomes are taken up by dendritic cells 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 the induction of cellular immunity. Liposomes modified with bacteria-derived polysaccharide derivatives, MGl-u-Curd or MGl-u-Man are also recognized via Dectin-1 and Dectin-2, respectively, which causes the promotion of Th1 cytokine production and the activation of cellular immunity. © 2017 Elsevier Ltd.
7.2. Materials and methods

7.2.1. Materials

Egg yolk phosphatidylcholine (EYPC) was kindly donated by NOF Co. (Tokyo, Japan) for preparation of liposome. Lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA) for fluorescence labeling of liposomal membrane. 3-Methylglutaric anhydride, curdlan from Alcaligenes faecalis, mannan from Saccharomyces cerevisiae for synthesis of pH-sensitive polymers, OVA, MPLA, fluorescein isothiocyanate (FITC), p-Xylene-bis-pyridinium bromide (DPX) for preparation of liposomes, and dextran sulfate sodium salt from Leuconostoc spp. for the evaluation of cellular association of liposomes, were purchased from Sigma (St. Louis, MO.). 1-Aminodecane for synthesis of pH-sensitive polymers, pyranine and Triton X-100 for preparation of liposomes 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) for synthesis of pH-sensitive polymers. 1,10-Dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) was from Life Technologies for fluorescence labeling of liposomal membrane. 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 [23]. pH-sensitive dextran derivatives (MGlu-Dex) were prepared as previously reported [24].

7.2.2. Synthesis of polysaccharide derivatives

3-Methyl-glutarylated curdlan (MGlu-Curd) was prepared by reaction of curdlan with 3-methylglutaric anhydride. For MGlu$_{65}$-Curd, curdlan (1.0 g, 6.2 mmol of OH groups) and LiCl (1.0 g) were dissolved in $N,N$-dimethylformamide (20 mL) and 9.0 equiv. of 3-methylglutaric anhydride (7.27 g, 56.7 mmol) was added to the solution. The mixed solution was kept at 100 °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-Curd polymers and
MGlu-Man were also synthesized by reaction of curdlan or mannan with various amounts of 3-
methylglutaric anhydride by the same procedure. $^1$H NMR for MGlu-Curd (400 MHz, D$_2$O+NaOD):
$\delta$ 0.9 (s, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 1.9 – 2.3 (br, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 3.5 – 4.0 (br, glucose 2H, 3H, 4H, 5H, 6H), 5.0 (br, glucose 1H). $^1$H NMR for MGlu-Man (400 MHz, D$_2$O+NaOD):
$\delta$ 0.9 (s, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 1.9 – 2.3 (br, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 3.5 – 4.2 (br, glucose 2H, 3H, 4H, 5H, 6H), 5.0 – 5.3 (br, glucose 1H).

As anchor moieties for fixation of MGlu-Curd and MGlu-Man onto liposome membranes, 1-
aminodecane was combined with carboxy groups of MGlu-Curd or MGlu-Man. Each polymer was
dissolved in water around pH 7.4, and 1-aminodecane (0.1 equiv. to hydroxy group of polymer) was
reacted to carboxy groups of the polymer using DMT-MM (0.1 equiv. to hydroxy group of polymer)
at room temperature for 6 h with stirring. The obtained polymers were purified by dialysis in water.
The ratios of MGlu units to decyl amide units for polymers were estimated using $^1$H NMR. $^1$H NMR
for MGlu-Curd-C$_{10}$ (400 MHz, D$_2$O+NaOD): $\delta$ 0.8 - 0.9 (br, -CO-NH-CH$_2$-(CH$_2$)$_8$-CH$_3$), 0.9 (s, -
CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 1.2 – 1.4 (br, -CO-NH-CH$_2$-(CH$_2$)$_8$-CH$_3$), 1.9 – 2.3 (br, -CO-CH$_2$-
CH(CH$_3$)-CH$_2$-), 3.2 (br, -CO-NH-CH$_2$-(CH$_2$)$_8$-CH$_3$), 3.5 – 4.0 (br, glucose 2H, 3H, 4H, 5H, 6H), 5.0
(br, glucose 1H). $^1$H NMR for MGlu-Man-C$_{10}$ (400 MHz, D$_2$O+NaOD): $\delta$ 0.8 - 0.9 (br, -CO-NH-
CH$_2$-(CH$_2$)$_8$-CH$_3$), 0.9 (s, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 1.2 – 1.6 (br, -CO-NH-CH$_2$-(CH$_2$)$_8$-CH$_3$), 1.9 –
2.3 (br, -CO-CH$_2$-CH(CH$_3$)-CH$_2$-), 3.2 (br, -CO-NH-CH$_2$-(CH$_2$)$_8$-CH$_3$), 3.5 – 4.2 (br, glucose 2H, 3H, 4H, 5H, 6H), 5.0 – 5.3 (br, glucose 1H). According to the GPC analysis for polymers on a system
equipped with a column (Waters, Ultrahydrogel Linear) with differential refractive index detection
(Jasco RI-930) using acetate buffer (pH 6.6) as the eluent, there is no peak derived from low-
molecular weight impurities (data not shown).

7. 2. 3. Titration

To 10 mL of an aqueous solution of each polymer (carboxylate concentration: 3.0 x 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 1.0 M HCl and pH of the resultant solution was measured using an automated titration instrument (AUT-701, DKK-TOA Corporation, Tokyo, Japan).

7.2.4. Precipitation pH

Precipitation pH of polymer was determined by measuring the optical density of aqueous polymer solutions (0.05 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.

7.2.5. Cell culture

DC2.4 cell, which is an immature murine DC line, was provided from Dr. K. L. Rock (Harvard Medical School, USA) and were grown in RPMI-1640 supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine, 100 mM nonessential amino acid, 50 μM 2-mercaptoethanol (2-ME, Gibco) and antibiotics at 37 °C [35]. E.G7-OVA, which is a chicken egg OVA gene-transfected clone of C57BL/6 mice-derived T lymphoma and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) [36].

7.2.6. 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 centrifuged with the speed of 55,000 rpm for 2 h at 4 °C twice 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. Loading efficiency of OVA to liposomes was determined and summarized in Table 7-S1.

<table>
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<th>Liposome</th>
<th>Loading efficiency of OVA (%)</th>
<th>OVA (g) / mol lipids**</th>
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<tr>
<td>Unmodified</td>
<td>52.7 ± 4.6</td>
<td>168 ± 15</td>
</tr>
<tr>
<td>MGlut29-Curd-C10</td>
<td>44.3 ± 6.8</td>
<td>142 ± 23</td>
</tr>
<tr>
<td>MGlut6-Man-C10</td>
<td>44.7 ± 10</td>
<td>143 ± 33</td>
</tr>
<tr>
<td>MGlut56-Dex-C10</td>
<td>35.1 ± 3.4</td>
<td>112 ± 11</td>
</tr>
</tbody>
</table>

* Determined by 100 x (OVA (g) in liposome / OVA (g) in feed).
** The concentrations of lipids and OVA were determined using Wako phospholipids C (Wako Pure Chemical Inds. Ltd.) and Coomassie (Bradford) protein assay kit (Pierce Biotechnology Inc.), respectively.

7.2.7. Dynamic light scatteering and zeta potential

Diameters and zeta potentials of the liposomes (0.1 mM of lipid concentration) 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.

7.2.8. 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) and liposome suspension was purified using a sepharose 4B column. Release of pyranine from liposome was measured as previously reported [20,24,37]. Liposomes encapsulating pyranine (lipid concentration: 2.0 × 10^{-5} M) were added to PBS of varying pH 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, FP-6200). The percent release of pyranine from liposomes was defined as

Release (%) = (F_f - F_i) / (F_f - F_i) × 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%).

7. 2. 9. Cellular association of liposomes and inhibition assay

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 PBS. DC2.4 cells (1.5 × 10^5 cells) cultured for 2 days in 12-well plates were washed with Hank’s balanced salt solution (HBSS), and then incubated in serum-free RPMI medium (0.5 mL). The Rh-PE-labeled liposomes (1 mM lipid concentration, 0.5 mL) 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. Fluorescence intensity of these cells was determined by a flow cytometric analysis (EPICS XL, Beckman Coulter, Inc). For inhibition assay, free dextran sulfate, curdlan and mannan at various concentrations were pre-incubated to cells for an hour before the incubation of Rh-PE-labeled liposomes for 4 h. Rhodamine fluorescence of each liposome was measured and cellular fluorescence shown in Fig. 7-6 was corrected using liposomal fluorescence intensity.

7. 2. 10. Intracellular behavior of liposomes

The FITC-OVA-loaded liposomes containing DiI were prepared as described above except that a mixture of polymer and lipid containing DiI (0.6 mol%) was dispersed in PBS containing FITC-OVA (4 mg/mL). DC2.4 cells (3 × 10^5 cells) cultured for 2 days in 35-mm glass-bottom dishes were washed with HBSS, and then incubated in serum-free RPMI medium (1 mL). The FITC-OVA-loaded liposomes (1 mM lipid concentration, 1 mL) 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.). Each liposome showed almost same fluorescence intensity for DiI and FITC. All CLSM images in Fig. 7-8 were taken at same sensitivity setting.
7.2.11. Cytokine production from cells treated with polymers or liposomes

The DC2.4 cells (1.5 × 10^5 cells) cultured for 2 days in 12-well plates were washed with HBSS, and then incubated in serum-free RPMI medium (0.5 mL). Polysaccharide derivatives (2 mg/mL, 0.5 mL) or liposomes with/without MPLA (4 g/mol lipid) (1 mM lipid concentration, 0.5 mL) were added gently to the cells and incubated for 24 h at 37 °C. After the incubation, supernatants of cultured cells were collected for measurements of TNF-α, IL-10 and/or IL-12 using an enzyme-linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd.) according to the manufacturer’s instruction.

7.2.12. In vivo cytokine release measured by ELISA and CTL assay

Female C57BL/6 mice (H-2b, 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. On days 7 and 14, 100 μg of OVA-loaded liposomes were subcutaneously injected into the right backs of the mice under anesthesia with isoflurane. On day 21, 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 various concentrations of OVA solution (0, 25, 50 μg/mL) for 5 days. After the incubation, the concentration of IFN-γ was measured using murine IFN-γ ELISA development kit (Peprotech, London, UK) according to the manufacturer’s instruction. Splenocytes were also 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 effector cells to target cells (E.G7-OVA) ratio of 25, which was defined as E/T ratio, using a lactate dehydrogenase (LDH) cytotoxicity detection assay (Takara Biomedicals, Tokyo, Japan).

7.2.13. 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 with isoflurane. On days 5 and 15, 100 µg of OVA-loaded liposomes with or without MPLA (4 g/mol lipid) 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 2500mm³. All treated groups contained 8 to 12 mice.

7. 2. 14. Statistical analysis

Tukey-Kramer method was employed in the statistical evaluation of the results in Figs. 7-6, 7-7, 7-9-12, 7-S6 and 7-S7. Survival analysis using Log-rank test was performed in Fig. 7-12.

7. 3. Results and discussion

7. 3. 1. Characterization of copolymer and copolymer-modified liposomes

Curdlan derivatives (MGlut-Curd) and mannan derivatives (MGlut-Man) with different contents of MGlut groups as pH-sensitive moiety were synthesized by reacting curdlan or mannan with various amounts of 3-methylglutaric anhydride (Fig. 7-2). Decyl (C₁₀) groups were further introduced to MGlut-Curd or MGlut-Man by reaction of decylamine with carboxylic acid groups of MGlut units for fixation of these polymers onto liposome membrane (Fig. 7-2). The obtained curdlan derivatives and mannan derivatives were characterized using ¹H NMR. Fig. 7-3A, C respectively depict ¹H NMR spectra of curdlan, MGlut₄₃-Curd, and MGlut₄₁-Curd-C₁₀. In comparison of spectra for curdlan (Fig. 7-3A) and for MGlut₄₃-Curd (Fig. 7-3B), introduction of MGlut groups to curdlan was confirmed from the existence of new peaks corresponding to MGlut groups (0.9 ppm, 2-2.3 ppm) in Fig. 7-3B. From the integration ratio of peaks of MGlut residues to those of sugar backbone (3.5-4.0 ppm), 43% of hydroxy groups of curdlan were estimated as combined with MGlut residues. Similarly, from the integration ratio between sugar backbone, MGlut residues, and decyl groups (0.9-1.5 ppm), decylamidated MGlut residues and MGlut residues were found to be combined to 4% and 41% of
hydroxy groups of curdlan, respectively, in products obtained using the reaction of MGl\(_{43}\)-Curd and decylamine, which is designated as MGl\(_{41}\)-Curd-C\(_{10}\). Mannan derivatives were evaluated using the same procedure (Fig. 7-3D, F). Compositions of curdlan derivatives and mannan derivatives prepared for this study are shown in Table 7-1.

**Fig. 7-2.** (A) Synthetic route for polysaccharide derivatives having carboxylic acid groups and alkyl chains as anchor units to liposomal membrane. (B) Structures of polysaccharides used in this study. © 2017 Elsevier Ltd.

**Fig. 7-3.** \(^1\)H NMR spectra of (A) curdlan, (B) MGl\(_{43}\)-Curd, (C) MGl\(_{41}\)-Curd-C\(_{10}\), (D) mannan, (E) MGl\(_{74}\)-Man, and (F) MGl\(_ {65}\)-Man-C\(_{10}\) in D\(_2\)O/NaOD. © 2017 Elsevier Ltd.
Table 7-1. Compositions of Polysaccharide Derivatives

<table>
<thead>
<tr>
<th>Polymer</th>
<th>-OH (mol%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>-MGluc (mol%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>-Anchor (mol%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGluc&lt;sub&gt;32&lt;/sub&gt;-Curd</td>
<td>68</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;42&lt;/sub&gt;-Curd</td>
<td>57</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;45&lt;/sub&gt;-Curd</td>
<td>35</td>
<td>65</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;31&lt;/sub&gt;-Curd</td>
<td>19</td>
<td>81</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;43&lt;/sub&gt;-Man</td>
<td>57</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;74&lt;/sub&gt;-Man</td>
<td>26</td>
<td>74</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;9&lt;/sub&gt;-Dex&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91</td>
<td>9</td>
<td>-</td>
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<tr>
<td>MGluc&lt;sub&gt;35&lt;/sub&gt;-Dex&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;55&lt;/sub&gt;-Dex&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35</td>
<td>65</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;21&lt;/sub&gt;-Curd-C&lt;sub&gt;10&lt;/sub&gt;</td>
<td>15</td>
<td>85</td>
<td>-</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;31&lt;/sub&gt;-Curd-C&lt;sub&gt;10&lt;/sub&gt;</td>
<td>76</td>
<td>21</td>
<td>3</td>
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<tr>
<td>MGluc&lt;sub&gt;41&lt;/sub&gt;-Curd-C&lt;sub&gt;10&lt;/sub&gt;</td>
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<td>41</td>
<td>4</td>
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<tr>
<td>MGluc&lt;sub&gt;59&lt;/sub&gt;-Curd-C&lt;sub&gt;10&lt;/sub&gt;</td>
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<td>59</td>
<td>6</td>
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<tr>
<td>MGluc&lt;sub&gt;77&lt;/sub&gt;-Curd-C&lt;sub&gt;10&lt;/sub&gt;</td>
<td>17</td>
<td>77</td>
<td>6</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;36&lt;/sub&gt;-Man-C&lt;sub&gt;10&lt;/sub&gt;</td>
<td>60</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;55&lt;/sub&gt;-Man-C&lt;sub&gt;10&lt;/sub&gt;</td>
<td>29</td>
<td>65</td>
<td>6</td>
</tr>
<tr>
<td>MGluc&lt;sub&gt;56&lt;/sub&gt;-Dex-C&lt;sub&gt;10&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27</td>
<td>56</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by <sup>1</sup>H NMR.
<sup>b</sup> Synthesized as previously reported [24].
<sup>c</sup> MGluc<sub>X</sub>-Curd refers to the polymer with X mol% of MGluc groups. MGluc<sub>Y</sub>-Curd-C<sub>10</sub> refers to the polymer with Y mol% of MGluc groups and decyl (C<sub>10</sub>) groups as indicated mol% in a column in –Anchor (mol%).

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In our earlier study, the contents of MGluc residues on dextran derivatives affected their protonation state because of proximity effects between carboxylic acid groups [24]. Therefore, acid-base titration of MGluc-Curd and MGluc-Man was conducted (Fig. 7-4). MGluc-Curd and MGluc-Man changed their protonation states depending on pH in neutral and weakly acidic regions. However, the protonation profiles of MGluc-Curd having various MGluc contents were almost identical, different from the results found for dextran derivatives [24]. Curdlan is known to form a triple helix structure in the alkaline aqueous solution. The secondary hydroxyl groups form mutual hydrogen bonding inside of the helix. Other hydroxy groups exist outside of the helix [38]. The effects of MGluc unit introduction on helix formation of curdlan were evaluated by complexation with Congo red, as previously reported [39].
As shown in Fig. 7-S1A, the absorption spectra of Congo red changed in the presence of curdlan or curdlan derivatives. The maximum absorption wavelength ($\lambda_{\text{max}}$) shifted from 487 nm (free Congo red) to 515 nm in the presence of curdlan (Fig. 7-S1B), which indicates that Congo red forms the complex with curdlan, which takes a helical structure [38-40]. In the cases of MGl蔺-Curd with Congo red, the extent of red shift decreased concomitantly with increasing MGl蔺 unit contents (Fig. 7-S1B), suggesting that the helix structure of curdlan was dissociated by the introduction of MGl蔺 units to the OH groups of curdlan. However, $\lambda_{\text{max}}$ for MGl蔺77-Curd with Congo red remained higher than that of free Congo red, indicating that even MGl蔺-Curd having high MGl蔺 unit contents partially takes the helical structure that can form a complex with Congo red. Although the detailed structure of MGl蔺-Curd in the aqueous solution is unclear at present, such a unique conformation of curdlan derivatives might affect the density of carboxylic acid groups, thereby producing the same protonation profiles and almost identical pKa values irrespective of MGl蔺 residue contents (Table 7-2). MGl蔺43-Man and MGl蔺74-Man also exhibited almost identical protonation profiles (Fig. 7-4). Mannan has $\alpha$ (1, 6)-linked backbone and $\alpha$ (1, 2)-linked and $\alpha$ (1, 3)-linked branches (Fig. 7-2B). MGl蔺43-Man and MGl蔺74-Man might have almost equal density of carboxylic acid groups in their branches, resulting in almost equal pKa values. In the cases of MGl蔺-Curds, protonation was promoted strongly at around pH 5.0, which might result from the formation of aggregation [41]. To confirm the formation of aggregation, the optical density of MGl蔺-Curd and MGl蔺-Man was measured. The solutions of MGl蔺-Curd and MGl蔺-Man were transparent at neutral pH, indicating that these polymers were soluble in water. In contrast, these solutions suddenly became turbid under a certain pH, which is defined as the precipitation pH at which these polymers change their characteristics from hydrophilic to hydrophobic and lose their water solubility. As shown in Table 7-2, the respective values of precipitation pH of MGl蔺65-Curd and MGl蔺81-Curd are 4.8 and 5.0, which correspond to the region in which protonation was promoted in titration curves (Fig. 7-4). In addition, degrees of protonation were 0.94-0.98 at around precipitation pH, indicating that most of the carboxylic acid groups must be protonated for aggregation of these polymers. Compared with MGl蔺-
Curd, MGlul43-Man showed lower precipitation pH. The solution of MGlul43-Man was transparent under experimental conditions. These results indicate that MGlul-Curd has more hydrophobic properties than MGlul-Man does, probably because of their helical structures (Fig. 7-S1).

![Fig. 7-4. Acid-base titration curves for (A) MGlul50-Curd (open diamonds), MGlul65-Curd (gray triangles), MGlul81-Curd (closed squares), and (B) MGlul43-Man (closed diamonds), and MGlul74-Man (open squares). © 2017 Elsevier Ltd.](image)

![Fig. 7- S1. (A) Absorption spectra for Congo red/curdlan derivative complexes in 0.2 M Na3PO4 solution (pH 12). (B) Effect of MGlul unit contents on the maximum absorption wavelength of Congo red/curdlan derivatives. The](image)
concentrations of Congo red and curdlan derivatives were 18 µM and 36 mM (as a glucose residue concentration), respectively. © 2017 Elsevier Ltd.

Table 7-2. pKa and precipitation pH for polysaccharide derivatives

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pKa</th>
<th>Precipitation pH</th>
<th>Degree of protonation at precipitation pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGlu24-Curd</td>
<td>5.85</td>
<td>4.4</td>
<td>0.98</td>
</tr>
<tr>
<td>MGlu24-Curd</td>
<td>5.78</td>
<td>4.8</td>
<td>0.94</td>
</tr>
<tr>
<td>MGlu24-Curd</td>
<td>5.96</td>
<td>5.0</td>
<td>0.95</td>
</tr>
<tr>
<td>MGlu24-Man</td>
<td>6.40</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>MGlu24-Man</td>
<td>6.53</td>
<td>3.8</td>
<td>0.99</td>
</tr>
<tr>
<td>MGlu24-Dex</td>
<td>6.26</td>
<td>4.5*</td>
<td>0.95*</td>
</tr>
</tbody>
</table>

* Previously reported [24].

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7. 3. 2. Preparation of polysaccharide derivative-modified liposomes

Polysaccharide derivative-modified liposomes were prepared by dispersion of mixed thin film composed of EYPC and polysaccharide derivatives having anchor moieties in aqueous solution containing OVA. Then, the liposome suspension was extruded through polycarbonate membranes with pore size of 100 nm and purified by ultracentrifugation. The size and zeta potential of obtained liposomes were evaluated (Table 7-3). Compared with diameters of unmodified liposomes (157 nm), polysaccharide derivative-modified liposomes showed smaller diameters. After ultracentrifugation, liposome pellet in centrifugation tube was redispersed in PBS. Liposome size might be changed during this redispersion process depending on their surface properties. Polysaccharide derivative-modified liposomes which have many carboxylates on the liposome surface might be re-dispersed more efficiently than unmodified liposomes. Zeta potentials were decreased by modification of polysaccharide derivatives, which indicates that the liposome surfaces are covered with carboxylated polysaccharide derivatives. The liposomes modified with MGlu- Curd-C10 or MGlu-Dex-C10 exhibited highly negatively charged values of around −50 mV, whereas MGlu-Man-C10-modified liposomes showed low zeta potentials. This might result from insufficient exposure of carboxylic acids on the liposomal surface because of steric hindrance derived from their branched structures (Fig. 7-2B).
The pH-sensitivity of polysaccharide derivatives was evaluated using polymer-modified liposomes. Fluorescence dye pyranine and quencher DPX were encapsulated into liposomes and pH-responsive release behaviors were examined (Fig. 7-5). At neutral pH, all liposomes retained their contents tightly. However, in the weakly acidic pH region (pH 6.5-5.0, which corresponds to early or late endosomes), content release was observed in polysaccharide derivative-modified liposomes, indicating that polysaccharide derivatives became hydrophobic and destabilized the liposomal membrane at these levels of pH. Compared with MGluer-Man-C_{10}-modified liposomes, MGluer-Curd-C_{10}-modified liposomes induced significant content release at narrow pH region, which might reflect the difference of hydrophobicity between MGluer-Curd and MGluer-Man in a weak pH region (Table 7-2). Moreover, the protonation of MGluer-Curd was promoted at around pH 5.0 (Fig. 7-4), which might rapidly change MGluer-Curd from hydrophilic to hydrophobic and which destabilizes liposomal membrane efficiently by hydrophobized MGluer-Curds. In our earlier study, MGluer-Dex with higher amounts of MGluer unit induced content release at higher pH regions because of pKa increase [24]. However, MGluer-Curd-C_{10}-modified liposomes showed similar pH-sensitivity irrespective of the difference of MGluer unit contents, except for MGluer_{77}-Curd-C_{10}, which reflects that these MGluer_{43}-Curd and MGluer_{65}-Curd showed almost equal pKa values (Table 7-2). In addition, the hydrophobicity of MGluer-Curd-C_{10} was evaluated using pyrene fluorescence (Fig. 7-S2) [42]. MGluer-Curd-C_{10} formed hydrophobic domains at a weakly acidic pH region, which causes the liposomal membrane disruption.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Hydrodynamic size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>157 ± 4</td>
<td>−11.6 ± 5</td>
</tr>
<tr>
<td>MGluer_{21}-Curd-C_{10}</td>
<td>109 ± 3</td>
<td>−46.9 ± 5</td>
</tr>
<tr>
<td>MGluer_{31}-Curd-C_{10}</td>
<td>121 ± 6</td>
<td>−47.3 ± 4</td>
</tr>
<tr>
<td>MGluer_{50}-Curd-C_{10}</td>
<td>131 ± 2</td>
<td>−50.5 ± 1</td>
</tr>
<tr>
<td>MGluer_{77}-Curd-C_{10}</td>
<td>121 ± 4</td>
<td>−46.5 ± 2</td>
</tr>
<tr>
<td>MGluer_{90}-Man-C_{10}</td>
<td>123 ± 3</td>
<td>−20.0 ± 2</td>
</tr>
<tr>
<td>MGluer_{95}-Man-C_{10}</td>
<td>100 ± 1</td>
<td>−31.7 ± 2</td>
</tr>
<tr>
<td>MGluer_{96}-Dex-C_{10}a</td>
<td>109 ± 4</td>
<td>−46.5 ± 2</td>
</tr>
</tbody>
</table>

* As previously reported [24].
presented in Fig. 7-5A. MGlu\textsubscript{77}-Curd-C\textsubscript{10} showed slightly higher hydrophobicity than MGlu\textsubscript{41}-Curd-C\textsubscript{10} and MGlu\textsubscript{59}-Curd-C\textsubscript{10} at pH 7.0-6.0, which reflects the pH-responsiveness of MGlu\textsubscript{77}-Curd-C\textsubscript{10}-modified liposome at a higher pH region than those of MGlu\textsubscript{41}-Curd-C10-modified and MGlu\textsubscript{59}-Curd-C\textsubscript{10}-modified liposomes.

**Fig. 7-5.** pH-sensitive contents release behaviors of curdlan (A) or mannan (B) derivative-modified liposomes. Pyranine release after 30 min-incubation from liposomes modified with MGlu\textsubscript{21}-Curd-C\textsubscript{10} (closed diamonds), MGlu\textsubscript{41}-Curd-C\textsubscript{10} (closed circles), MGlu\textsubscript{59}-Curd-C\textsubscript{10} (closed squares), MGlu\textsubscript{77}-Curd-C\textsubscript{10} (closed triangles), MGlu\textsubscript{56}-Man-C\textsubscript{10} (open squares), MGlu\textsubscript{65}-Man-C\textsubscript{10} (open circles), and without polymer (open diamonds) were shown. Lipid concentrations were $2.0 \times 10^{-5}$ M. Each point is the mean ± SD (n = 3). © 2017 Elsevier Ltd.

**Fig. 7-S2.** pH-Dependence of $I_1/I_3$ of pyrene fluorescence in the absence of polymers (closed diamonds) or the presence of MGlu\textsubscript{41}-Curd-C\textsubscript{10} (closed circles), MGlu\textsubscript{59}-Curd-C\textsubscript{10} (closed squares), and MGlu\textsubscript{77}-Curd-C\textsubscript{10} (closed triangles)
dissolving in 30 mM sodium acetate and 120 mM NaCl solution of varying pH. 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. © 2017 Elsevier Ltd.

7.3.3 Interaction of polysaccharide derivative-modified liposomes with DCs

Dendritic cells have various lectins that recognize specific polysaccharides or oligosaccharides derived from bacteria [34]. Therefore, the effects of polysaccharide structures on the cellular association of polysaccharide derivative-modified liposomes were examined. Fig. 7-6 depicts a comparison of fluorescence from the cells treated with Rh-PE-labeled liposomes modified with or without polysaccharide derivatives. For MGlú-Curd-C\(_{10}\)-modified liposomes, the cellular association changed depending on MGlú unit contents. MGlú\(_{59}\)-Curd-C\(_{10}\) showed the highest cellular association, which was 13 times higher than that of unmodified liposomes. For MGlú-Man-C\(_{10}\)-modified liposomes, the cellular association was low, probably because low zeta potential of these liposomes suppressed interaction with scavenger receptors on dendritic cells, which recognize anionic molecules (Table 7-3). In comparison with the backbone structure, MGlú\(_{59}\)-Curd-C\(_{10}\)-modified liposomes exhibited higher cellular association than MGlú\(_{65}\)-Man-C\(_{10}\)-modified and MGlú\(_{56}\)-Dex-C\(_{10}\)-modified liposomes. These results show that both MGlú unit contents and backbone structures are important to obtain liposomes with high affinity to DCs.

To reveal the cellular association mechanism of these liposomes, inhibition assay was performed using dextran sulfate, curdlan, and mannan, which are the respective inhibitors for scavenger receptors, Dectin-1, and Dectin-2 [32,33,43]. Fig. 7-7A, B depict the effects of inhibitors on the cellular association of MGlú-Curd-C\(_{10}\)-modified liposomes. Cellular association of MGlú-Curd-C\(_{10}\)-modified liposomes was strongly suppressed in the presence of dextran sulfate (Fig. 7-7A). Reportedly, cellular association of carboxylated polymer-coated liposomes or nanoparticles to macrophages and DCs is inhibited by dextran sulfate because these nanoparticles are recognized by scavenger receptors, which are receptors on macrophages and DCs for the recognition of anionic
molecules or surface such as aged erythrocytes or apoptotic cells [44]. Therefore, the result of strong inhibition by dextran sulfate indicates that MGlu-Curd-C\textsubscript{10}-modified liposomes have high affinity to scavenger receptors on DC2.4 cells. The presence of curdlan also suppressed the cellular association of MGlu-Curd-modified liposomes to some degree but not suppressed the cellular association of MGlu-Dex-modified liposomes, indicating the recognition of MGlu-Curd by Dectin-1 (Fig. 7-7B). However, the cellular association of liposomes modified with MGlu-Curd having high MGlu contents was not suppressed by curdlan, indicating that the modification of high amounts of MGlu unit to curdlan interrupts the interaction of curdlan backbone with Dectin-1. Cellular association of MGlu-Man-C\textsubscript{10}-modified liposomes were also inhibited by dextran sulfate (Fig. 7-7C), suggesting that MGlu-Man-C\textsubscript{10}-modified liposomes also internalized to the cells via interaction with scavenger receptors. In contrast, the presence of free mannan showed no effect to the cellular association of MGlu-Man-C\textsubscript{10}-modified liposomes irrespective of the MGlu unit contents (Fig. 7-7D). This result indicates that the modification of MGlu units to mannan completely suppresses the interaction of mannan derivatives with Dectin-2. Combined with these results, high performance of MGlu-Curdmodified liposomes in cellular association might result from the efficient recognition of these liposomes by both scavenger receptors and Dectin-1.

Next, the intracellular antigen delivery performance of polysaccharide derivative-modified liposomes was examined. FITC-labeled model antigenic protein, OVA-loaded liposomes containing DiI as a fluorescence lipid were applied to DC2.4 cells. Then intracellular distributions of OVA and DiI (liposome) were observed using CLSM (Fig. 7-8, 7-S3). For cells treated with unmodified liposomes, punctate red and green fluorescence was observed within cells even in the case of high concentration of liposomes and FITC-OVA (Fig. 7-S3), indicating that both liposomes and FITC-OVA were trapped in endosomes or lysosomes. In the cases of cells treated with polysaccharide derivative-modified liposomes, punctate red and green fluorescence was also observed. However, some parts of the green fluorescence were diffused throughout cells, except to the nucleus. The colocalization efficiency of FITC fluorescence with DiI fluorescence was calculated from CLSM.
images (Fig. 7-S4). Liposomes modified with polysaccharide derivatives, especially MGlut21-Curd-C10, MGlut41-Curd-C10 and MGlut59-Curd-C10, showed much lower colocalizaion values than that of unmodified liposomes. These results indicate that polysaccharide derivatives became hydrophobic at weakly acidic pH inside of early or late endosomes and efficiently destabilized their own liposomal membrane and endosomal membrane, consequently delivering of FITC-OVA into cytosol of DCs. MGlut-Curd-C10-modified liposomes with 21, 41, 59% of MGlut unit showed excellent performance of cytoplasmic delivery of FITC-OVA, whereas MGlut77-Curd-C10-modified liposomes showed both punctate and diffused fluorescence of FITC-OVA (Fig. 7-8). MGlut77-Curd-C10-modified liposomes showed sharp content release below pH 6.5, which corresponds to the pH region of early endosomes in the cells (Fig. 7-5A). Therefore, MGlut77-Curd-C10-modified liposomes might release FITC-OVA within the early endosomes, but the pH in early endosomes was insufficient for MGlut77-Curd-C10 to induce destabilization of the endosomal membrane, resulting in observation of punctate green fluorescence inside of cells after 4 h incubation (Fig. 7-8). In contrast, MGlut-Curd-C10-modified liposomes with 21, 41, 59% of MGlut unit showed content release below at pH 6.0, which corresponds to the pH region of late endosomes in the cells (Fig. 7-5A). In addition, these polymers formed strong hydrophobic domains below pH 6.0 (Fig. 7-S2). Therefore, these polymers induced both content release from liposomes and endosomal membrane destabilization, resulting in efficient cytoplasmic delivery of FITC-OVA. In the case of MGlut-Man-C10-modified liposomes, cytoplasmic delivery performance was not so high, reflecting their moderate performance of contents released at pH 6.5-5.5 (Fig. 7-5B). These results indicate that the cytoplasmic delivery performance is affected by pH-sensitive content release performance, which is determined not only by the main chain structure, but also by the MGlut unit contents. The excess destabilization of endosomal membrane might cause the cytotoxicity. Therefore, the cytotoxicity of MGlut-Curd-C10-modified liposomes with high cytoplasmic delivery performance was examined (Fig. 7-S5). As shown in Fig. 7-S5, no cytotoxicity was observed under experimental conditions.
Fig. 7-6. Fluorescence intensity for DC2.4 cells treated with Rh-PE-labeled EYPC liposomes modified with or without polysaccharide derivatives having various amounts of MGl units. DC2.4 cells were incubated with liposomes (lipid concentration: 0.5 mM) for 4 h at 37 °C in serum free medium. Cellular auto fluorescence was corrected. *p < 0.05. **p < 0.01. © 2017 Elsevier Ltd.

Fig. 7-7. Inhibition of cellular association of liposomes modified with MGl21-Curd-C10 (closed diamonds), MGl41-Curd-C10 (closed circles), MGl59-Curd-C10 (closed squares), MGl77-Curd-C10 (closed triangles), MGl36-Man-C10 (open squares), MGl65-Man-C10 (open circles), and MGl56-Dex-C10 (closed inverted triangles). Various concentrations of free dextran sulfate (A, C), free curdlan (B) and free mannan (D) were incubated with DC2.4 cells for 1 h before sample
Relative fluorescence intensity was calculated as the ratios of the amount of association in the presence of ligands to that in the absence of ligands. *$p < 0.05$ and **$p < 0.01$ compared to the case in the absence of ligands. © 2017 Elsevier Ltd.

Fig. 7-8. Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with DiI-labeled and FITC-OVA-loaded liposomes modified with indicating polysaccharide derivatives or without polymer for 4 h at 37 °C in serum-free medium. Bar represents 10 μm. © 2017 Elsevier Ltd.

Fig. 7-S3. Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with DiI-labeled and FITC-OVA-loaded EYPC liposomes (lipid concentration was 3 mM and OVA concentration was 500 μg/mL) for 4 h at 37 °C in serum-free medium. Bar represents 10 μm. (A) Sensitivity for DiI and FITC was same with Figure 7-8. (B) Sensitivity for DiI was enhanced to clarify the location of liposomes. © 2017 Elsevier Ltd.
Fig. 7-S4. Colocalization of FITC fluorescence derived from FITC-OVA with DiI fluorescence calculated from CLSM images. © 2017 Elsevier Ltd.

Fig. 7-S5. The cytotoxicity of the OVA-loaded liposomes containing MPLA was assessed by WST-8 assay. The DC2.4 cells (5 x 10^4 cells) cultured for 2 days in 48-well plates were treated with liposomes at various concentrations of OVA for 24 h. Then, the culture medium was carefully replaced with 0.11 mL of fresh RPMI containing 10% FBS and 10 μL of WST-8 (5 mg/mL) was added to each well. After 2 h-incubation at 37 °C, the survived cells were determined by absorbance at 450 nm using Wallac 1420 ARVO SX multilabel counter (Perkin Elmer Life Sciences). OVA concentration of 50 μg/mL corresponds to 0.5 mM lipid concentration used for CLSM analysis. © 2017 Elsevier Ltd.
7.3.4. Activation of DCs by polysaccharide derivatives and liposomes

β-glucans and mannan are recognized by DCs or macrophages, which activates them [32-34]. In addition, curdlan- or mannan-modified poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles also activated the macrophages or dendritic cells [45, 46]. Therefore, we investigated whether carboxylated polysaccharides retain their capability to activate the immunocompetent cells, or not.

DC2.4 cells were incubated with MGlue unit-introduced curdlan, mannan and dextran without anchor moiety overnight. Then the production of Th1 cytokines such as TNF-α and IL-12 was measured. As presented in Fig. 7-9, cytokine production from the DC2.4 cells increased concomitantly with increasing MGlue unit contents in polysaccharides. These results demonstrate that DCs were activated by these polysaccharide derivatives. The introduction of MGlue units promoted their capability for activation. As described in previous reports of the related literature, macrophage activation by carboxylmethylated curdlan or curdlan sulfate was evaluated [32, 47]. Carboxylmethylated curdlan showed no activation property of APCs [32], although curdlan sulfate slightly increased cytokine production from macrophages [47]. Compared with those earlier studies, the activation properties of MGlue unit incorporated curdlan were quite high, probably because of the high density of carboxylic acid groups on polysaccharide main chain compared with curdlan sulfate (sulfate contents: 9.23% [47]). In addition, IL-12 production from the cells treated with curdlan and mannan derivatives having medium amounts of MGlue units was apparently higher than that of dextran derivatives (Fig. 7-9B). This result suggests the importance of backbone structures for the efficient activation of DCs. Actually, IL-12 is an important cytokine for the activation of cellular immune responses. Therefore, MGlue-Curd and MGlue-Man, of which backbones are bioactive polysaccharide, are expected to activate the cellular immune responses.
Fig. 7-9. (A) TNF-α and (B) IL-12 productions from DC2.4 cells treated with curdlan (diamonds), mannan (triangles) and dextran (squares) derivatives having various contents of MGlus groups (1 mg/mL) for 24 h *p < 0.05 and **p < 0.01 compared to the parent polysaccharide. © 2017 Elsevier Ltd.

Activation properties of polysaccharide derivative-modified liposomes were also examined (Fig. 7-10). Fig. 7-10A shows TNF-α production from DC2.4 cells treated with liposomes modified with or without MGlus-Curd-C10. Reflecting the results of the polymers themselves (Fig. 7-9), TNF-α production increased concomitantly with increasing MGlus unit contents in curdlan derivative-modified liposomes. Fig. 7-10B-D shows the effects of backbone structures on TNF-α, IL-10 and IL-12 production, respectively. Cytokine production from these polysaccharide derivative-modified liposomes was quite higher than that of cells treated with unmodified liposomes. There is no significant difference between the backbone structures of polysaccharides. IL-1β production, which indicates the activation of inflammasome, was also examined (Fig. 7-S6). As shown in Fig. 7-S6, IL-1β production was enhanced by carboxylated polysaccharide derivative-modified liposomes, especially MGlus-Curd and MGlus-Man-modified liposomes, indicating that carboxylated polysaccharide derivatives, especially bioactive curdlan or mannan derivatives might activate inflammasome. In these experiments, MPLA, which is a commonly used lipid type adjuvant [28, 29, 48], is introduced into the liposomal membrane, where its effect on cytokine production was also investigated. MPLA inclusion slightly promoted cytokine production from liposome-treated cells, but its effect was not significant, suggesting that DC2.4 cells might be fully activated by these
polysaccharide derivative-modified liposomes, irrespective of MPLA inclusion.

**Fig. 7-10.** Cytokine production from DC2.4 cells treated with polysaccharide derivatives-modified liposomes with (closed bars) or without MPLA (open bars) (0.5 mM lipid concentration) for 24 h. (A) Effect of MGluc group contents for MGluc-Curd-C_{10}-modified liposomes. (B-D) Effects of main chain structures and inclusion of MPLA. *p < 0.05. **p < 0.01. © 2017 Elsevier Ltd.

**Fig. 7-S6.** IL-1β production from DC2.4 cells treated with polysaccharide derivatives-modified liposomes without MPLA (0.5 mM lipid concentration) for 24 h. *p <0.01. © 2017 Elsevier Ltd.
7.3.5. Induction of \textit{in vivo} immune responses

Next, the induction of immune response \textit{in vivo} by polysaccharide derivative-modified liposomes was examined. OVA-loaded liposomes were administered subcutaneously to mice twice. Seven days after the second immunization, splenocytes were collected and stimulated in vitro for 5 days in the presence of OVA. Furthermore, IFN-\(\gamma\) secretion from splenocytes was measured using ELISA (Fig. 7-11A). In the cases of splenocytes from mice treated with polysaccharide derivative-modified liposomes, high amounts of IFN-\(\gamma\) were produced compared with the case of PBS. Production levels increased concomitantly with increasing OVA concentrations used for in vitro culture, indicating that OVA-specific Th1-dominant immune responses were induced by polysaccharide derivative-modified liposomes. Especially, M\text{Glu-Curd-C}_{10}-modified liposomes induced the highest production of IFN-\(\gamma\), which might result from efficient cellular association and cytoplasmic delivery of OVA into antigen-presenting cells by M\text{Glu-Curd-C}_{10}-modified liposomes (Figs. 7-6 and 7-8).

The CTL activity in the spleen was also evaluated. Splenocytes stimulated \textit{in vitro} for 5 days were co-cultured with E.G7-OVA cells, which are OVA-expressing tumor cells, as a target cell. Then, cytotoxic effects against E.G7-OVA cells were measured (Fig. 7-11B). Compared with splenocytes from PBS-treated mice, splenocytes from mice treated with polysaccharide derivative-modified liposomes showed higher cytotoxicity against E.G7-OVA cells, indicating that OVA-specific CTLs were induced efficiently by immunization of polysaccharide derivative-modified liposomes. Reflecting the results of IFN-\(\gamma\) secretion, M\text{Glu-Curd-C}_{10}-modified liposomes exhibited higher CTL response, suggesting that IFN-\(\gamma\) secretion from Th1 cells or CTLs activated cellular immune response efficiently.

As another strategy for induction of cellular immunity, mice were treated with the mixture of OVA solution and liposomes modified with polysaccharide derivatives and their immunity-inducing performance was evaluated (Fig. 7-S7). Unexpectedly, the mixture of OVA and polysaccharide derivative-modified liposomes also induced IFN-\(\gamma\) secretion from splenocytes as the case of OVA-loaded liposomes (Fig. 7-11A). Especially, M\text{Glu59-Curd-C}_{10}- and M\text{Glu65-Man-C10}-modified
liposomes induced much higher cellular immune responses than that of MGlu56-Dex-C10-modified liposomes. Polysaccharide derivative-modified liposomes could promote the production of Th1 cytokines from dendritic cells (Figs. 7-9 and 10). In addition, the transfer of OVA molecules to cytosol might be promoted if both OVA molecules and liposomes are taken up in the same endosomes. Cytoplasmic transfer of OVA and the production of Th1 cytokines by mixture of OVA and polysaccharide derivative-modified liposomes might induce the OVA-specific cellular immune responses. These unexpected results are promising because various antigenic proteins or peptides can be combined with polysaccharide derivative-modified liposomes by just mixing for induction of antigen-specific cellular immunity.

**Fig. 7-11.** (A) IFN-γ production from splenocytes of OVA-immunized mice. Splenocytes were isolated from C57BL/6 mice immunized with PBS (white bars), OVA encapsulated liposomes modified with MGlu59-Curd-C10 (gray bars), MGlu65-Man-C10 (striped bars) and MGlu56-Dex-C10 (closed bars) twice. Splenocytes (2 × 10^6/mL in 2 mL) were incubated with OVA (0, 25 or 50 μg/mL) for 5 days. Concentration of IFN-γ in the medium was measured by ELISA. (B) OVA-specific CTL induction in spleen of mice after 7 days from subcutaneous immunization with polysaccharide derivative-modified liposomes twice. Cytotoxic activity was measured at effecter cell/target cell (E/T) ratio of 25 using the LDH assay. Aliquots of OVA (100 μg) was administered per mouse. E.G7-OVA cells were used as target cells. Each plots shows the data from each mouse and bars represent the mean value. *p < 0.05. **p < 0.01. †p = 0.14. © 2017 Elsevier Ltd.
**Fig. 7-S7.** IFN-γ production from splenocytes of OVA-immunized mice. Splenocytes were isolated from C57BL/6 mice immunized with 100 µg of OVA solution (white bars) or OVA solution with liposomes modified with MGlu59-Curd-C10 (gray bars), MGlu65-Man-C10 (striped bars) and MGlu56-Dex-C10 (closed bars) twice. Splenocytes (2 × 10^6/mL in 2 mL) were incubated with OVA (0, 25 or 50 µg/mL) for 5 days. Concentration of IFN-γ in the medium was measured by ELISA. *p < 0.05. **p < 0.01. © 2017 Elsevier Ltd.

### 7.3.6. Therapeutic effect of polysaccharide derivative-modified liposomes

Finally, therapeutic effects on tumor-bearing mice by immunization with polysaccharide derivative-modified liposomes were investigated. E.G7-OVA cells were inoculated to mice at 5 days and 15 days after tumor cell inoculation, OVA-loaded liposomes were administered subcutaneously to mice. Then tumor growth and survival were monitored (Fig. 7-12). This experiment also examined the effects of MPLA inclusion to liposomes on their therapeutic effect. For mice treated with PBS, the tumor volume increased with time. However, in the cases of mice treated with polysaccharide derivative-modified liposomes, tumor volumes decreased at around 7 days after from immunization, indicating the induction of strong cellular immune responses against E.G7-OVA cells by these liposomes, as presented in Fig. 7-11B. In the absence of MPLA, MGlu-Curd-C10-modified liposomes showed higher tumor regression and more prolonged survival than either MGlu-Dex-C10-modified (p = 0.0533) or MGlu-Man-C10-modified liposomes (p = 0.0495) (Fig. 7-12A, C and Table 7-S2), which
might derive from the synergy of efficient intracellular delivery of OVA to DCs (Figs. 7-6 and 7-8), the activation of DCs (Fig. 7-9). In the presence of MPLA, all polysaccharide derivative modified liposomes exhibited much stronger antitumor effects than those of liposomes without MPLA. Most tumors nearly disappeared (Fig. 7-12B). Mice survival was also prolonged efficiently. 40-50% of mice became completely tumorfree during 60 days (Fig. 7-12D). In this case, MGlut-Man-C\textsubscript{10}-modified liposomes showed the strongest antitumor effects in the polysaccharide derivatives. This result suggests that MPLA is a suitable adjuvant to enhance the immunity-inducing effects of MGlut-Man-C\textsubscript{10}-modified liposomes. The combination of proper adjuvant with polysaccharide-modified liposomes might produce more efficient antigen delivery systems. It is noteworthy that, even in the absence of MPLA, MGlut-Curd-C\textsubscript{10}-modified liposomes showed strong tumor regression comparable to MPLA-containing liposomes (Fig. 7-12A). In addition, there is no significant difference in survival between MGlut-Curd-C\textsubscript{10}-modified liposome with or without MPLA (p = 0.364, Table 7-S2). Therefore, MGlut-Curd-C\textsubscript{10}-modified liposomes are beneficial as adjuvant-free antigen delivery systems.

7.4. Conclusion

This study developed bioactive polysaccharide-based pH-sensitive polymers. MGlut unit-introduced curdlan and mannan efficiently destabilized liposomal membrane at weakly acidic pH and promoted Th1 cytokine production from dendritic cells. MGlut-Curd-C\textsubscript{10}-modified or MGlut-Man-C\textsubscript{10}-modified liposomes were taken up efficiently by DCs mainly via scavenger receptors. They delivered model antigenic protein into cytosol of DCs. Subcutaneous administration of these liposomes to mice induced OVA-specific cellular immune responses and CTLs in spleen, resulting in the strong therapeutic effects on tumor-bearing mice. Therefore, bioactive polysaccharide-based pH-sensitive polymers and these polymer-modified liposomes are promising as potent antigen delivery systems to achieve both antigen delivery and activation of immune systems for cancer immunotherapy.
Fig. 7-12. Antitumor effects induced by subcutaneous administration of OVA-loaded liposomes without (A, C, open symbols) or with (B, D, closed symbols) MPLA. 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. MGlu_{65}-Dex-C_{10}-modified liposomes (squares), MGlu_{59}-Curd-C_{10} modified liposomes (diamonds), and MGlu_{65}-Man-C_{10}-modified liposomes (triangles) containing 100 µg of OVA were subcutaneously administered into the right backs of the mice twice on day 5 and day 15. Mice administered with PBS (circles) were used as controls to confirm the development of tumor. Typical results of tumor volume change using four mice were shown in Fig. 7-12A and B. Eight to twelve mice were used for each treated group. *p < 0.05 and **p < 0.01 compared to PBS-treated group. Results for Log-rank test is shown in Table 7-S2. © 2017 Elsevier Ltd.
### Table 7-S2. Survival Analysis by Log-Rank test for Figs 7-12

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</tr>
<tr>
<td>PBS vs MGl-Dex-liposome MPLA(-)</td>
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<td>PBS vs MGl-Man-liposome MPLA(-)</td>
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<tr>
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</tr>
<tr>
<td>PBS vs MGl-Dex-liposome MPLA(+)</td>
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*p* < 0.05, **p** < 0.01.

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### 7. 5. References


Chapter 8: General Conclusion

8. 1. Summary

In this thesis, the author explored the rational design of the immune-inducing system for cancer immunotherapy. In order to create the rational antigen carriers, the author designed carriers that deliver antigen into cytosol of dendritic cells and activate dendritic cells (adjuvant effect). Prepared systems were evaluated by in vitro and in vivo experiment. The results obtained in this work were discussed from the viewpoint of antigen carriers for cancer immunotherapy. The author constructed immune-inducing systems that liposomes modified with pH-sensitive polymers as cytosolic delivery function of antigen and cationic lipids, toll-like receptor ligands and gene encoding cytokine were introduced as adjuvant molecules. Furthermore, polysaccharides were chemically modified to obtain safer pH-sensitive polymer having both function of cytosolic delivery and adjuvant function. The main results obtained in this thesis were summarized below.

In Chapter 2, the author demonstrated 3-methyl-glutarylated hyperbranched poly(glycidol) (MGlu-HPG) as pH-sensitive polymer modified liposome delivered OVA-derived antigenic peptide into cytosol of DCs. OVA-I (SIINFEKL) and OVA-II (PSISQAVHAHAEEINEAPβA) bind to MHC class I and class II molecules of DCs. The liposome encapsulating the peptide was efficiently taken up by the mouse dendritic cell line, DC 2.4 cell and delivered the peptide to the cytosol of DC 2.4 cell. Subcutaneous administration of OVA-I peptide-encapsulated MGlu-HPG-modified liposome to mice bearing OVA-expressing T lymphoma, E.G7-OVA tumor induced stronger antitumor effect than administration of only OVA-I peptide. It was shown that the tumor suppressing effect was much lower when OVA-II-encapsulated MGlu-HPG modified liposome was administered. It was suggested that pH-sensitive polymer-modified liposomes containing OVA-I peptide are useful for effective cancer immunotherapy.

In Chapter 3, the author aimed for preparation of immune-inducing system that had both function of cytosolic antigen delivery and adjuvant effect. As an adjuvant molecules, cationic lipid, 3,5-
didodecyloxybenzamidine (TRX)-introduced MGlue-HPG modified liposome were prepared at various contents of TRX. TRX-inclusion improved their pH sensitivity at weakly acidic pH and association of liposomes with DC2.4 cells. TRX-incorporated liposomes delivered entrapped OVA not only to cytosol but also to endosome/lysosome. Treatment with cationic lipid-incorporated liposomes induced up-regulation of antigen presentation-involving molecules on DCs, the promotion of cytokine production, and antigen presentation via both MHC class I and II molecules. Especially, antigen presentation via MHC class II was promoted by cationic lipid inclusion, which might correspond to efficient endosome/lysosome delivery of OVA. Subcutaneous administration of OVA-loaded cationic lipid-incorporated liposomes induced antigen-specific antibody production in serum and Th1-dominant immune responses in the spleen. Furthermore, administration of the cationic lipid-incorporated liposomes to mice bearing E.G7-OVA tumor more significantly reduced the tumor volume than liposomes without cationic lipids. Therefore, cationic lipid inclusion into pH-sensitive polymer-modified liposomes, which can achieve both efficient antigen intracellular delivery and activation of DCs, is an effective approach to develop antigen carriers for efficient cancer immunotherapy.

In Chapter 4, to obtain more effective antigen carriers, TLR9 ligands (CpG-DNA) was additionally introduced into cationic lipid-incorporated pH-sensitive polymer-modified liposomes. The author examined the influence of CpG-DNA conjugation method on immunity induction function. One method is “Pre-mix”: mixed thin membrane was dispersed in mixture of OVA/CpG-DNA solution and another is “Post-mix”: CpG-DNA was added to pre-formed liposomes. Post-mix induced higher expression of CD80 molecule on DC2.4 cells, while lower production of cytokines than those of Pre-mix. Post-mix showed stronger antigen-specific immune response and antitumor effects in vivo than those of Pre-mix, suggesting that Post-mix is superior CpG-DNA complexation method to induce strong cancer immunity. In the case of Post-mix, CpG-DNA molecules were adsorbed on surface of liposomes, which might cause more effective delivery of CpG-DNA.
molecules to TLR9 existing in endosomes than Pre-mix which contains most of CpG-DNA inside of liposomes. Therefore, they are promising as antigen delivery carriers for cancer immunotherapy.

In Chapter 5, potentiation of pH-sensitive liposome-based antigen carriers with IFN-\(\gamma\) gene lipoplexes was attempted to achieve efficient induction of tumor-specific immunity. MGluPG-modified liposomes and cationic liposomes were used, respectively, for the delivery of antigenic protein OVA and IFN-\(\gamma\)-encoding pDNA. The MGluPG-modified liposomes and the cationic liposome-pDNA complexes (lipoplexes) formed hybrid complexes via electrostatic interactions after their mixing in aqueous solutions. The hybrid complexes co-delivered OVA and IFN-\(\gamma\)-encoding pDNA into DC2.4 cells as was the case of MGluPG-modified liposomes for OVA or the lipoplexes for pDNA. Both the lipoplexes and the hybrid complexes transfected DC2.4 cells and induced IFN-\(\gamma\) protein production, but transfection activities of the hybrid complexes were lower than those of the parent lipoplexes. Subcutaneous administration of hybrid complexes to mice bearing E.G7-OVA tumor reduced tumor volumes, which might result from the induction of OVA-specific CTLs. However, the hybrid complex-induced antitumor effect was the same level of the MGluPG-modified liposome-mediated antitumor immunity. In contrast, an extremely strong antitumor immune response was induced when these liposomes and lipoplexes without complexation were injected subcutaneously at the same site of tumor-bearing mice.

In Chapter 6, pH-sensitive dextran derivatives having 3-methylglutarilated residues (MGlu-Dex) were prepared by reacting dextran with 3-methyl-glutaric anhydride. Surface modification of liposomes with MGlu-Dex produced highly pH-sensitive liposomes that were stable at neutral pH but which were destabilized strongly in the weakly acidic pH region. MGlu-Dex-modified liposomes were taken up efficiently by DC2.4 cells and delivered entrapped OVA molecules into the cytosol. When MGlu-Dex-modified liposomes loaded with OVA were administered subcutaneously to mice, the antigen-specific humoral and cellular immunity was induced more effectively than in unmodified liposomes loaded with OVA. Furthermore, administration of MGlu-Dex-modified liposomes loaded with OVA to mice bearing E.G7-OVA tumor significantly suppressed tumor growth and extended
the mice survival. Results suggest that MGlu-Dex-modified liposomes are promising for the production of safe and potent antigen delivery systems that contribute to the establishment of efficient cancer immunotherapy.

In Chapter 7, bioactive polysaccharide-based pH-sensitive polymers were constructed to achieve not only cytoplasmic delivery of antigen but also activation of DCs. Curdlan and mannan were used as bioactive polysaccharides because they are known to activate DCs via their respective interactions with Dectin-1 and Dectin-2. Carboxylated curdlan and mannan promoted Th1 cytokine production from DCs, indicating the activation of DCs by these polysaccharide derivatives. These polymer-modified liposomes released their contents at weakly acidic pH and delivered OVA into cytosol of DCs. Subcutaneous administration of curdlan derivative-modified or mannan derivative-modified liposomes induced strong antigen-specific immune responses and stronger antitumor effects than those of liposomes modified with dextran derivative. Therefore, bioactive polysaccharide-modified liposomes that achieve both cytoplasmic delivery of antigen and activation of DCs are promising for cancer immunotherapy.

The author demonstrated rational immunity-inducing system for cancer immunotherapy, which have cytosolic antigen delivery function and adjuvant effect. Especially, the author demonstrated multiplexing the adjuvant functions potentiated immune-inducing activity of antigen carriers. The author wishes this study would be a meaningful contribution to practical cancer immunotherapy.
8. 2. Acknowledgments

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8. 3. List of Publications


