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# A liposome-based antigen delivery system using pH-sensitive fusogenic polymers for cancer immunotherapy

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ABSTRACT

Highly pH-sensitive liposomes that deliver antigenic molecules into cytosol through fusion with or destabilization of endosome were prepared by surface modification of egg yolk phosphatidylcholine/dioleoylphosphatidylethanolamine (1/1, mol/mol) liposomes with 3-methylglutarylated poly(glycidol) of linear (MGlu-LPG) or hyperbranched structure (MGlu-HPG). These polymer-modified liposomes were stable at neutral pH, but they became strongly destabilized below pH 6, which corresponds to the pH of endosome. These polymer-modified liposomes were taken up by murine dendritic cells (DCs) more efficiently than the unmodified liposomes were through an endocytic pathway. They introduced entrapped ovalbumin (OVA) molecules into cytosol. Subcutaneous or nasal administration of the polymer-modified liposomes loaded with OVA induced generation of OVA-specific cytotoxic T cells (CTL) much more effectively than the unmodified liposomes loaded with OVA. Furthermore, administration of the polymer-modified OVA-loaded liposomes to mice bearing E.G7-OVA tumor significantly reduced the tumor burden, although the OVA-loaded unmodified liposomes only slightly affected tumor growth. Results suggest that the polymer-modified liposomes with highly pH-sensitive destabilizing property are promising as antigen carriers for efficient cancer immunotherapy.



## 1. Introduction

Activation of immune systems for cancer therapy has received much attention. Dendritic cells (DCs), potent professional antigen-presenting cells, play an important role in activation of innate and adaptive immunity [1–3]. Therefore, efficient delivery of cancer-specific antigens into DCs is crucial for the establishment of effective cancer immunotherapy [4,5].

Two major routes exist for the presentation of antigen mediated by DCs. When exogenous antigenic proteins are taken up by DCs *via* endocytosis, these molecules are degraded to peptide fragments in lysosome and are presented on major histocompatibility complex (MHC) class II molecules, which engender induction of humoral immunity. In contrast, endogenous antigenic proteins existing in cytosol of DCs are degraded by proteasomes. Their derived peptide fragments are presented by MHC class I molecules, which engender activation of antigen-specific cytotoxic T lymphocytes (CTLs). It is generally considered that the induction of the tumor-specific CTLs is important to achieve efficient tumor immunotherapy because these CTLs attack the target cells directly and eliminate them effectively. Therefore, it is crucial to use a carrier system that delivers antigen into the cytosol of DCs for induction of effective cellular immunity, which exhibits an effective tumor-suppressive effect.

To date, antigen delivery systems of various types have been produced using nanoparticles for induction of antigen-specific CTLs. For example, poly(lactide-*co*-glycolide) (PLGA) nanoparticles were used for the cytoplasmic delivery of antigens [6]. γ-Poly(-glutamic acid)-based nanoparticles entrapped with antigenic ovalbumin (OVA) were also used for delivery of OVA into DCs [7–9]. In addition, dextran-based nanoparticles containing acid-labile acetal groups were used for delivery of OVA into DC and derived presentation of OVA-derived peptides *via* the MHC class I pathway [10,11]. These nanoparticles might be taken up by DCs *via* endocytosis and might cause transfer of antigenic peptides into cytosol during degradation in lysosomes, although the mechanism has not been elucidated to date. Efficient delivery of antigen into cytosol of DCs is a key factor for efficient antigen presentation on MHC class I molecules [11]. Therefore, to engender antigen presentation *via*

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MHC class I pathway efficiently, carrier systems should have functions that promote the transfer of antigen from endosome and/or lysosome into cytosol of DCs.

Membrane-based nanoparticles such as liposomes are regarded as good candidates for use as antigen delivery vehicles because these particles can achieve introduction of antigenic proteins into cytosol of DC using biological processes such as membrane fusion. For example, liposomes modified with viral fusion proteins exhibit capabilities to fuse with or to disrupt endosomal and/or lysosomal membranes and introduce encapsulated antigenic OVA into DC cytosol, thereby inducing OVA-specific cellular immunity [12–14]. However, such liposomes might induce unexpected immune responses originating from viral proteins. Consequently, synthetic molecules with fusogenic or membrane disruptive activity might be strongly desired for the construction of liposomes with biological functions for efficient antigen delivery into cytosol of DCs.

To date, synthetic molecules of various types have been used to provide membrane fusion and/or membrane disruptive abilities to liposomes including synthetic polymers and fusion peptides [15–19]. Especially, pH-sensitive molecules which destabilize lipid membranes at weakly acidic pH are used for production of functional liposomes for cytoplasmic delivery. When taken up by cells through endocytosis, liposomes modified with these pH-sensitive molecules can deliver contents through fusion or disruption of endosome and lysosome, which have mildly acidic interiors. Considering their use as antigen delivery systems, it is highly desired that, after being taken up by DCs, they introduce contents into cytosol promptly and efficiently for the effective induction of cellular immunity. To accomplish that, highly pH-sensitive property and strong membrane-destabilizing character of polymers are necessary for construction of liposome-based antigen delivery carriers with polymers, which achieve efficient induction of cellular immunity.

We have developed pH-sensitive fusogenic polymers, which generate fusion ability under weakly acidic conditions, by carboxylation of poly(glycidol)s, which are known to be highly biocompatible [20], with acid anhydrides of various kinds [18,19,21]. These pH-sensitive poly(glycidol) derivatives were obtained by reacting various anhydrides, such as succinyl anhydride, to the polymer hydroxyl groups. We demonstrated that surface modification with these pH-sensitive poly(glycidol) derivatives can provide pH-sensitive destabilizing property to stable egg yolk phosphatidylcholine (EYPC) liposomes. The resultant liposomes achieved delivery of contents into cytosol of cells of various types [18,19]. Especially, 3-methylglutarylated poly(glycidol) (MGlu-PG) (Fig. 1), which has carboxyl groups with hydrophobic spacer moiety, produced pH-sensitive fusogenic liposomes with excellent performance as cytosolic delivery carriers through their strong fusion ability [19]. Additionally, we synthesized MGlu-PGs of two types using poly(glycidol)s with different backbone structure, which are hyperbranch poly(glycidol) and linear poly(glycidol). Results suggest that the backbone structure might affect the performance of polymer-modified liposomes in terms of cellular association and intracellular behavior [21]. Considering the high performance of these pH-sensitive fusogenic MGlu-PG-modified liposomes as cytosolic delivery vehicles, in this study, we examined their ability to deliver antigenic proteins into cytosol of DCs and to activate cellular immune response, and investigated their feasibility as antigen delivery carriers for use in cancer immunotherapy.

## 2. Materials and methods

### 2.1. Materials

EYPC and L-dioleoyl phosphatidylethanolamine (DOPE) were kindly donated by NOF Co. (Tokyo, Japan). Lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). OVA, monophosphoryl lipid A (MPLA) and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO.). Pyranine 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). 3-Methylglutarylated hyperbranched poly(glycidol) with polymerization degree of 60 (MGlu-HPG) and 3-methylglutarylated linear poly(glycidol) with polymerization degree of 76 (MGlu-LPG) were prepared as previously reported [19,21]. The ratios of MGlu units to decyl amide units for MGlu-LPG and MGlu-HPG were respectively 81/10 and 85/10, as estimated using $^1$H NMR (Fig. 1) [19,21]. 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 [21].

### 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 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]. CD8-OVA1.3 cells, a T–T hybridoma against $OVA_{257-264}$/H-2K$^b$ complex, were kindly provided by Dr. C.V. Harding [24], and were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 μM 2-ME, and antibiotics. OT4H.1D5 cells, a T–T hybridoma against $OVA_{265-277}$/I-A$^b$ complex, were kindly provided by Dr. J.A. Kapp [25], and were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, and antibiotics.

### 2.3. Generation of murine bone marrow-derived DCs

Bone marrow-derived dendritic cells (BMDCs) were prepared according to the method of Lutz et al. with slight modification [26]. Briefly, bone marrow cells flushed from the femurs and tibias of C57BL/6 mice were seeded at $5 \times 10^6$ cells per sterile 100-mm bacterial grade culture dish in 10 mL of RPMI 1640 containing 10% FBS, 10 ng/mL recombinant murine granulocyte/macrophage colony-stimulating factor (GM-SCF, PeproTech EC Ltd.), 50 μM 2-ME, and antibiotics. On day 5, another 10 mL of culture medium was added to the dish for medium replenishment. Nonadherent cells were harvested on days 6–8 as immature BMDCs.

### 2.4. Animals

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

### 2.5. Preparation of liposomes

To a dry, thin membrane of EYPC (5.0 mg) and DOPE (4.7 mg) was added 1.0 mL of OVA/PBS solution (pH 7.4, 4 mg/mL), and the mixture was vortexed at 4 °C. The liposome suspension was further hydrated by freezing and thawing, and was extruded through a polycarbonate membrane with a pore size of 100 nm. The liposome suspension was applied to a sepharose 4B column to remove free OVA from the OVA-loaded liposomes. Polymer-modified liposomes were also prepared according to the above procedure using dry membrane of a lipid mixture with polymers (lipids/polymer = 7/3, w/w). For induction of immune responses, MPLA (4 g/mol lipids) was introduced into liposomal membrane.

### 2.6. Dynamic light scattering and zeta potential

Diameters and zeta potentials of the liposomes were measured using a Nicomp 380 ZLS dynamic light scattering instrument (Particle Sizing Systems, Santa Barbara, CA) equipped with a 35 mW laser (632.8 nm wavelength). Zeta potentials were measured by equipped an Avalanche photodiode detector, and were detected at an 18.9 angle treated with 9.75 mV. Data was obtained as an average of more than three measurements on different samples.

### 2.7. Release of pyranine from liposome

Pyranine-loaded liposomes were prepared as described above except that mixtures of polymers and EYPC/DOPE were dispersed in aqueous 35 mM pyranine, 50 mM DPX, and 25 mM phosphate solution (pH 7.4). Release of pyranine from liposome was measured as previously reported [19,21,27]. 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

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