Development of functional liposomes by modification of stimuli-responsive materials and their biomedical applications

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Abstract

Liposome is a promising nanocarrier for drug delivery because of its biocompatibility and the encapsulation capacity of drugs. Liposomes can be functionalized easily by introduction of functional materials such as stimulus-responsive materials. Temperature-responsive liposomes and pH-responsive liposomes are representative stimulus-responsive liposomes that can deliver drugs to locally heated target tissues and intracellular organelles. Here, temperature-responsive liposomes for the selective release of cargo and pH-responsive liposomes for the induction of antigen-specific immunity are overviewed. Temperature-responsive polymer-modified liposomes immediately released

drugs in response to heating, which achieved selective drug release at a tumour after topical heating of tumour-bearing mice. Introduction of MR-detectable molecules enabled the tracing of liposome accumulation into target sites to optimize the heating timing. These liposomes can also be combined with magnetic nanoparticles or carbon nanomaterials to attain magnetic field-responsive, electric field-responsive and light-responsive properties to support on-demand drug release or control of biological reactions using these external stimuli. pH-Responsive liposomes were produced by modification of poly(carboxylic acid) derivatives or by pH-responsive amphiphiles. These liposomes delivered antigenic proteins into the cytosol of antigen presenting cells, which induced cross-presentation and antigen-specific cellular immunity. Adjuvant molecules or bioactive polysaccharide-based pH-responsive polymers improved their immunity-inducing effect further, leading to tumour regression in tumour-bearing mice. Precise design and control of structures of stimulus-responsive materials and combination with functional materials are expected to create novel methodologies to control biological functions and to produce highly potent liposomal drugs that can achieve selective release of bioactive molecules.

1. Introduction

Recent progress in biotechnology and nanotechnology has fostered novel methodologies to treat cancer and intractable diseases. Protein therapeutics are representative examples of such a drastic change in medical fields in recent decades[1]. Especially, antibody medicine has emerged for treatment of cancer and autoimmune diseases by controlling human immune systems, which further opens up another treatment options designated as "immunotherapy"[2-6]. Another achievement of remarkable progress in biotechnology and nanotechnology fields is the development of nanocarriers and their application in biomedical fields[7-9]. Nanocarriers can prolong circulation time in the bloodstream, can deliver their cargo into target site or cells and can protect their cargo from degradation by enzymes in body fluids. These features of nanocarriers are important to improve the bioavailability of drugs and decrease adverse events by controlling the drug partition in the body.

To date, nanocarriers of various types such as polymeric particles, polymeric micelles, polymersomes, liposomes, nanogels, and organic–inorganic nanohybrid particles have been studied[7-9]. Among them, liposomes, lipid-based nanovesicles, are classical but promising carriers for use in drug delivery fields. Since the discovery of liposomes by Prof. Bangham, numerous studies have been aimed at their clinical application[10-12]. Already, more than 18 liposomal drug products have been approved for clinical treatment of cancer, infectious diseases and age-related macular degeneration[12]. A representative liposomal drug Doxil, which is doxorubicin (Dox)-loaded liposome modified with poly(ethylene glycol) (PEG), shows accumulation to tumour sites *via* enhanced permeation and retention (EPR) effects and reduces adverse events induced by Dox partition into normal tissues such as heart[13]. However, a recent report demonstrates that

overall therapeutic effects of Doxil are almost identical to parent low molecular weight anticancer drug formulation[14]. This equivalence might be explained by the lack of selectivity to target cells and the lack of drug release properties because of quite high stability of the Doxil lipid membrane. Therefore, further improvements, especially in drug release behaviours, would be necessary to produce more efficient liposomal drugs. To control drug release profiles precisely, stimulus-responsive liposomes have been developed and studied intensively (Figure 1) [15–18]. This review is the first to describe the design of stimulus-responsive liposomes to improve drug delivery function of liposomes. Subsequently, examples of temperature-responsive liposomes for cancer chemotherapy and pH-responsive liposomes for cancer immunotherapy are described.

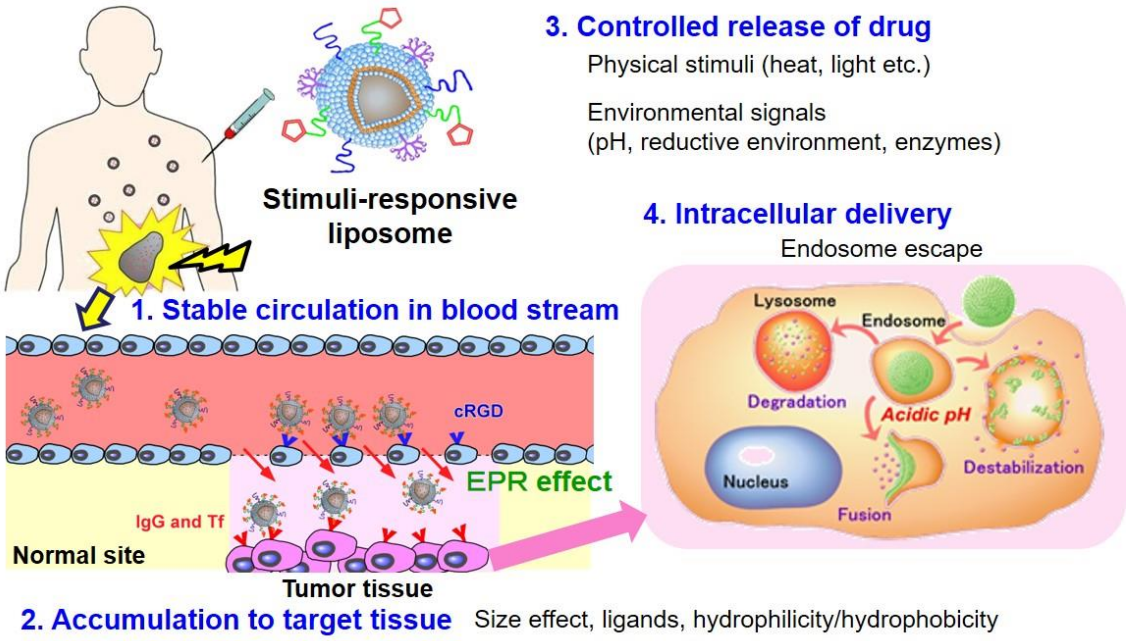

Figure 1. Design of stimuli-responsive liposomes to overcome various barriers in the body.

2. Design of stimulus-responsive liposomes

Combination of external or internal stimuli with stimulus-responsive liposomes is a promising approach to improve the therapeutic efficacy of liposomal DDS and to decrease adverse effects. There are two mainly used strategies to prepare stimulus-responsive liposomes: the use of phase transition of lipid membrane in response to stimuli and the modification of stimulus-responsive molecules onto the liposomes (Figure 2) [16].

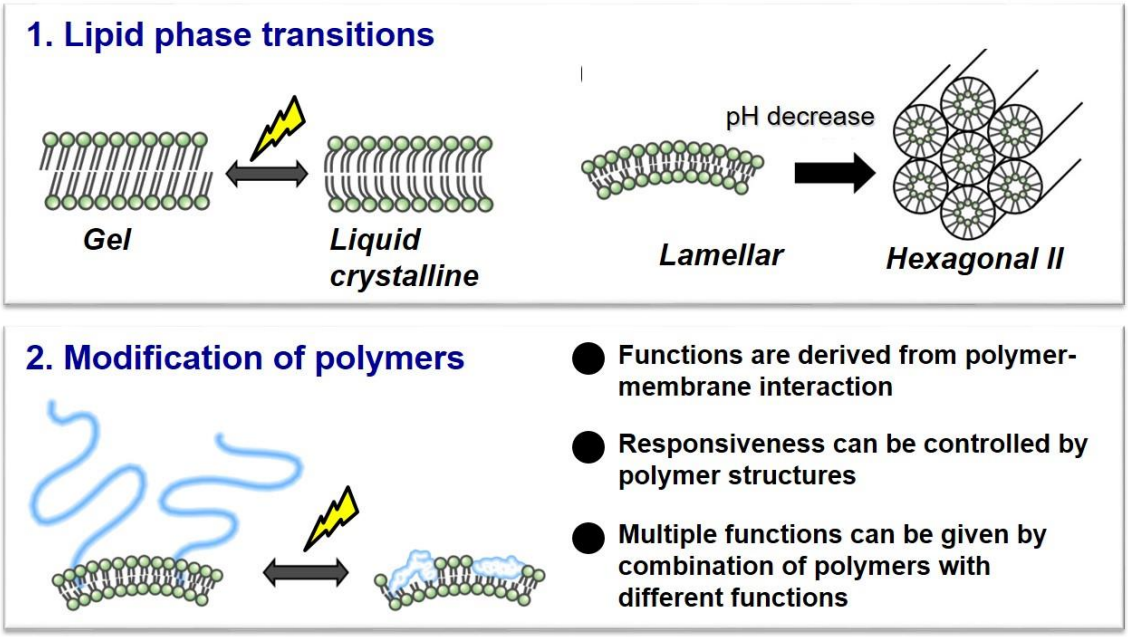

Figure 2. Strategies to prepare stimuli-responsive liposomes.

For production of liposomes with response to temperature, which is representative external stimulus, gel-to-liquid crystalline transition of lipid bilayer has been used. Dipalmitoylphosphatidylcholine (DPPC)-based liposome has a transition temperature of 41 °C, at which the lipid membrane permeability is enhanced and is used to prepare temperature-responsive liposomes[19]. Inclusion of a lysolipid into DPPC liposomes can further increase its temperature-responsive drug release property from the liposomes[20]. One promising temperature-responsive liposomal formulation (ThermoDox™) is

currently undergoing phase III clinical trials as a treatment for hepatocellular carcinoma[21]. For preparation of liposomes with responsiveness to pH, which is a representative internal stimulus, mixtures of dioleoylphosphatidylethanolamine (DOPE) and carboxy-group-possessing amphiphiles such as oleic acid and cholesteryl hemisuccinate have been well-studied[22,23]. Such a mixture forms a bilayer at neutral pH, at which times carboxy group is deprotonated. After protonation of carboxy groups at acidic pH, the bilayer structure changes to hexagonal II packing, an intrinsic property of DOPE, which induces rapid drug release or membrane fusion with other membranes[24]. When using a stimulus-responsive lipid-based approach, the liposome responsiveness is defined precisely by lipid chemical structures and characteristics that are beneficial for the precise production of drug formulation. By contrast, the selection of stimulus-responsive lipids that show the suitable responsiveness at a desired temperature, pH, or other parameter is restricted.

Another strategy to prepare stimulus-responsive liposomes is the modification of stimulus-responsive polymers onto a liposomal membrane. By this approach, the responsiveness of the liposomes can be controlled by polymer structures because drug release from the liposomes is induced by the interaction of the polymers with a lipid membrane. Furthermore, multiple functions can be introduced into a single polymer, which provides multiple stimuli-responsive liposomes.

Subsequent sections present findings obtained for various polymer-based or lipid-based stimulus-responsive liposomes and their biofunctions in biomedical fields.

3. Temperature-responsive liposomes

3.1. Poly(*N*-isopropylacrylamide)

Poly(*N*-isopropylacrylamide) (pNIPAM, Figure 3a) is a well-studied

thermoresponsive polymer that changes its water solubility in response to temperature[25]. At temperatures higher than 32 °C, pNIPAM becomes water-insoluble. The specific temperature is designated as a lower critical solution temperature (LCST). Polymers showing LCST behaviour have been used for various bio-related materials including drug delivery systems[26–28]. Actually, modification of pNIPAM or its copolymer onto the liposomes creates a temperature-responsive liposome[29]. Liposomes that are modified by pNIPAM or its derivative showed content release at temperatures higher than LCST because pNIPAM becomes hydrophobic and leads to membrane destabilization *via* hydrophobic interaction with lipid membrane[29-32]. The temperature at which content release is induced can be controlled by copolymerization of hydrophilic/hydrophobic monomers with NIPAM, which changes the LCST of obtained copolymers[31]. In addition, copolymers with high transition enthalpy showed a sharp temperature responsiveness compared to copolymers with low enthalpy, even though these copolymers have almost identical LSCT[32]. Methods of modifying copolymers onto liposomes are also important for temperature-responsive behaviours of liposomes. pNIPAM copolymers with anchoring groups in the polymer chain end induced sharper temperature-responsiveness than copolymers with anchoring groups at random sites through polymer chains[32]. These reports suggest that the temperature-sensitivity of polymer-modified liposomes can be controlled in terms of copolymerization, polymer mobility, and transition behaviours through precise control of polymer structures.

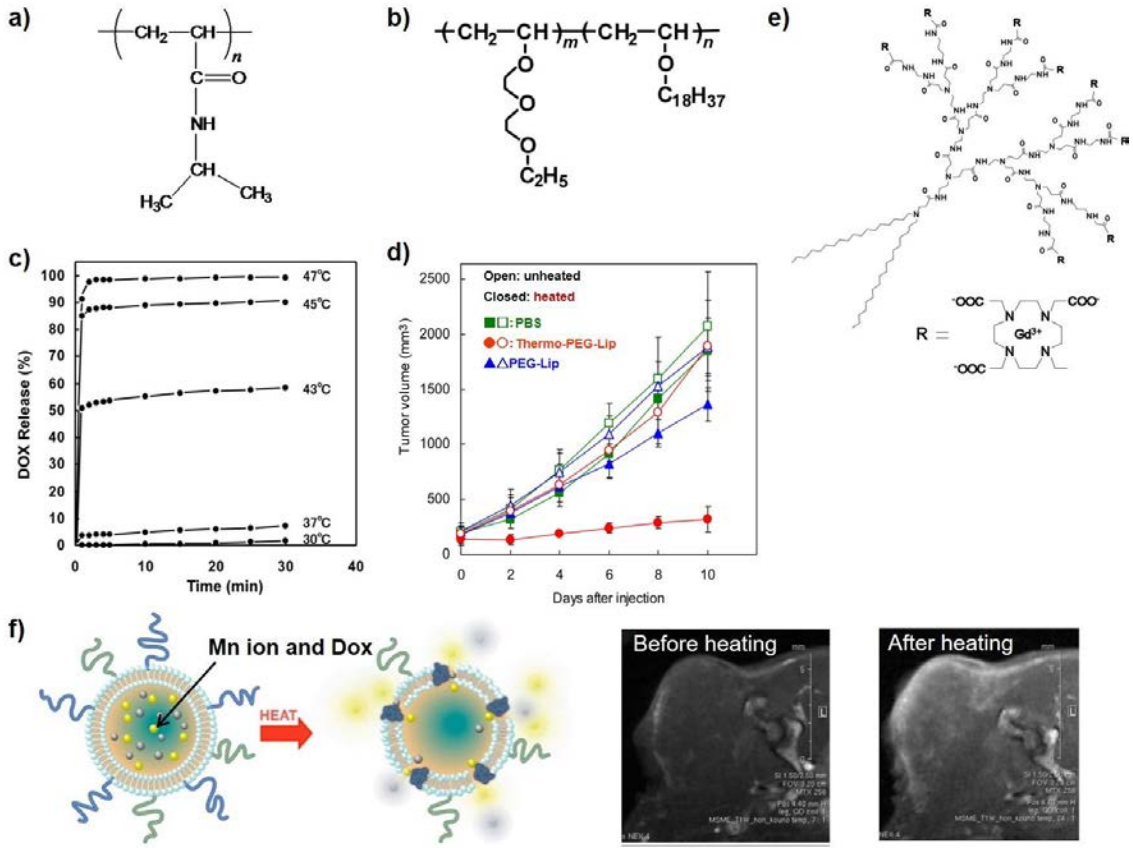

Figure 3. Chemical structures of pNIPAM (a) and pEOEOVE with ODVE anchor block. (c) Temperature-responsive drug release profiles of liposomes modified with pEOEOVE. Partially reproduced from Ref. 35 with permission from Elsevier. (d) Tumour growth suppression by intravenous injection of Dox-loaded liposomes modified with pEOEOVE with subsequent local heating of tumour site of colon26 tumour-bearing mice. Partially reproduced from Ref. 35 with permission from Elsevier. (e) Chemical structure of MR-detectable gadolinium chelate-having dendron lipid. (f) Manganese ion- and Dox-loaded thermoresponsive liposomes and representative MR images before and after heating of tumour site after intravenous injection of the liposomes. Partially reproduced from Ref. 40 with permission from Elsevier.

3.2. Poly[(2-ethoxy)ethoxyethyl vinylether]

For biomedical applications, temperature-responsive liposomes ideally should show negligible content release at temperature under physiological temperature for reduction of adverse effects. Simultaneously, temperature-responsive liposomes must induce rapid drug release under heating procedures to avoid damage to the body by the heating itself. Typically, the temperature range for clinical hyperthermia is 40–45 °C. Therefore, temperature-responsive liposomes that can show sharp responsiveness at this temperature range is promising in a viewpoint of clinical application. Poly[(2-ethoxy)ethoxyethyl vinylether] (pEOEOVE, Figure 3b) has LCST at around 41 °C and its side chain structure resembles that of PEG, a representative biocompatible polymer[33]. pEOEOVE formed a highly hydrophobic domain at temperatures higher than its LSCT. It also exhibited definite temperature-responsiveness[34]. Block copolymers of pEOEOVE with octadecyl vinylether block as an anchoring group were designed for temperature-sensitization of liposomes. These copolymer-modified PEG-liposomes retained the encapsulated anticancer drug (Dox) under body temperature, but Dox molecules were released immediately within a few minutes at 45 °C (Figure 3c) [35]. Combination of intravenous administration of the liposome to tumour-bearing mice and subsequent local heating of tumour site at 45 °C using a radio wave applicator significantly suppressed tumour growth compared with this liposome without local heating (Figure 3d) [35].

3.3. Multifunctional liposomes

Imaging function is important to monitor liposome accumulation to tumour sites or drug release behaviour from liposomes at tumour sites, which provides useful information to elucidate the DDS function of the liposomes for additional optimization of liposomal DDS[36]. Typical modalities used for *in vivo* imaging of DDS are fluorescence imaging,

nuclear magnetic resonance imaging (MRI), X-ray CT, positron emission tomography (PET) and ultrasonic imaging. Actually, MRI is a widely used imaging modality in clinical settings because MRI provides high-resolution images without exposure to radiation and because MR signals are unaffected by the half-life of imaging reagents[37]. MR-detectable functions were introduced to temperature-responsive liposomes to produce multifunctional liposomes. Multiple gadolinium chelate-grafted dendron-bearing lipids (Figure 3e) were introduced to temperature-responsive polymer-modified liposomes[38]. After intravenous injection to tumour-bearing mice, MR images were taken to reveal the biodistribution of the liposomes. Reportedly, MR signal derived from gadolinium chelates at the tumour site increased with time and reached a plateau at 8 h after liposome injection[38]. The MR signal intensity at tumour site varied with liposome size: liposomes with 110 nm size showed higher accumulation into tumour site than liposomes with 48 nm size. The accumulation behaviour of liposomes also affected the tumour size. Furthermore, liposomes exhibited heterogeneous distribution in the tumour: both high MR signal intensity areas and low signal intensity areas were excited in the tumour. Considering that liposomes accumulate to the tumour *via* EPR effects, these data suggest that the distribution and permeability of tumour blood vessels are heterogeneous and that some parts of tumours might be stroma-rich, which suppress the permeation of liposomal DDS. MRI is applicable not only to the detection of liposomal DDS distribution in the body but also the visualization of drug-release behaviour *in vivo*. Both Dox and manganese ion were encapsulated into the liposomes (Figure 3f) [39,40]. Manganese ion shows negligible MR signal during complexation with Dox inside of liposomes, but the MR signal is recovered when Dox molecules are released from the liposomes under heating. Eight hours after injection of the liposomes, MR signals at tumour site were low.

After local heating at the tumour site, an intense MR signal was observed (Figure 3f), indicating the release of manganese ion and Dox molecules from the liposomes under heat application[40].

The introduction of targeting ligands onto the surface of nanocarriers can improve the DDS function further by enhanced accumulation into target sites and target cells[41,42]. Transferrin or Herceptin was introduced to temperature-responsive polymer-modified liposomes[43]. These targeting liposomes actually improved the cellular uptake by tumour cells and the accumulation to tumour site after intravenous injection, leading to strong anti-tumour effects[43]. Therefore, temperature-responsive polymer-modified liposomes are promising nano-DDS to produce personalized cancer treatments that can optimize anticancer drug delivery processes by real time monitoring of liposomes and drug release behaviours and which can maximize anticancer therapeutic effects through selective drug release in tumour lesions under heating at optimum timing.

3.4. Thermoresponsive dendron-bearing lipids

Thermoresponsive behaviours of thermoresponsive polymers are controlled by hydrophobic interaction between side chain units and hydrogen bond-formation between water molecules and polar groups in the side chain unit. This finding spurred us to investigate another strategy to temperature-sensitizing of functional molecules. Actually, the introduction of alkyl amide groups, which are identical structures to those of side chains of typical thermoresponsive polymer, into temperature-insensitive dendritic molecules such as polyamidoamine (PAMAM) dendrimers, polypropyleneimine dendrimers and hyperbranched polyglycidols provided thermoresponsive properties to these molecules[44–48]. These properties might derive from interactions of alkyl amide

groups on the periphery of dendritic molecules. We further applied this technique to thermoresponsive molecular assemblies. Isobutyramide (IBAM) groups were introduced to the terminal amino groups of PAMAM dendron-bearing lipids (DLs) (Figure 4) [49]. An aqueous solution of IBAM-introduced DLs exhibited turbidity changes with the increase of temperature: IBAM-DL solution changed from transparent to turbid at a specific temperature designated as the cloud point. Cloud points of IBAM-DLs solution varied with the generation of PAMAM dendron and solution pH. Analysis using small-angle X-ray scattering (SAXS) revealed that IBAM-DLs forms vesicles at low temperature, whereas the assembling structure changes immediately to a fibre-like structure composed of hexagonal II phase at high temperature (Figure 4), which results from change in the molecular shape from a cylinder to a truncated cone *via* dehydration of polar head group. In addition, cloud points can be controlled precisely by the density of IBAM groups on the surface of lipid assemblies by mixing temperature-insensitive DLs. These thermoresponsive self-assemblies are applicable to various DDS that can control interaction with cells and drug release behaviour using heating.

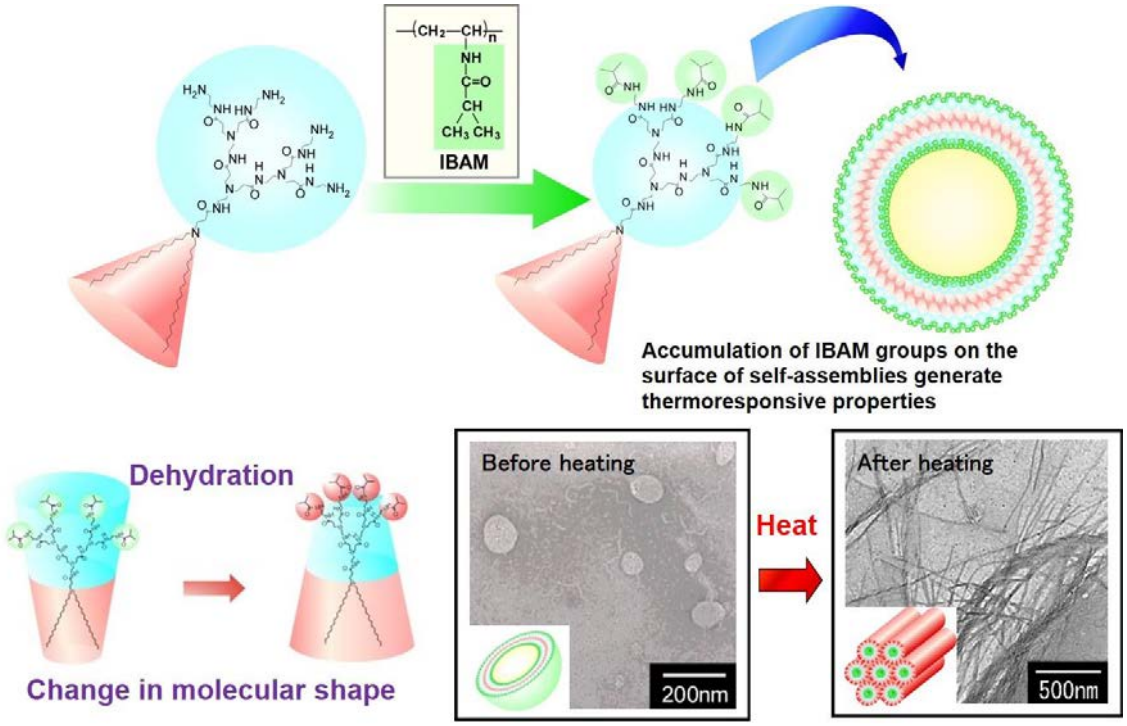

Figure 4. Thermoreponsive molecular assemblies prepared from isobutyramide(IBAM)-modified dendron lipids. IBAM groups showed dehydration after heating, which induces the change in molecular shape and assembling structures confirmed by transmission electron microscopy. Partially reproduced from Ref. 49 with permission from Wiley.

3.5. Combination of thermoresponsive liposomes with functional nanomaterials

Combination of thermoresponsive liposomes with various nanomaterials further provides advanced drug delivery systems with multiple modality-responsive nanocarriers. Incorporation of gold nanoparticles and growth of gold nanoshells on the surface of the thermoresponsive liposomes have been reported[50-52]. Gold nanoparticles and gold nanoshells generate heat under light irradiation. Therefore, such nanohybrids of liposomes and gold nanostructures can achieve light-triggered drug release from the liposomes. In addition, gold nanostructures are detectable using X-ray CT, which also provides an imaging modality. Magnetic nanoparticles modified with oleic acids can be

incorporated into the lipid bilayer of thermoresponsive liposomes *via* hydrophobic interaction[53-55]. These liposomes can induce drug release only during application of alternating magnetic field, which generates heat inside of the lipid bilayer, and induces the transition of thermoresponsive polymer (pEOEOVE) leading to destabilization of lipid membrane and drug release from the liposomes[55]. These hybrid liposome properties are useful for on-demand drug release application at disease sites using external magnetic fields.

Nanohybrids of thermoresponsive liposomes with carbon nanotubes were also designed as electric field/light-responsive nanomaterials[56,57]. The movement of these nanohybrids on the chip can be controlled by an electric field. Furthermore, laser irradiation to these nanohybrids generates heat that triggered the content release from thermoresponsive liposomes. Such a property of nanohybrids is useful for molecular transport *via* electric fields and light-controlled monitoring of biological reactions. Actually, these nanohybrids delivered substrates of β-galactosidase (fluorescein digalactoside, FDG) at a desired site where β-galactosidase exists. They released FDG after laser irradiation, which caused the enzyme reaction. Furthermore, these nanohybrids were applied the delivery of amiloride, which is an inhibitor of sodium ion channel to control the biological reaction in living worms[57]. After injection of nanohybrids into the body cavity of the *Caenorhabditis elegans*, mechanosensory neuron functions of the worms were successfully inhibited *via* amiloride release by topical laser irradiation.

Magnetic nanoparticle-embedded carbon nanohorns were incorporated into the thermoresponsive liposomes to prepare the nanomaterials responding to magnetic field, light and temperature. FDG-loaded liposome-based nanohybrids were injected intravenously into β-galactosidase-overexpressing transgenic mice[58]. Accumulation of

nanohybrids in mice bodies can be controlled by a magnet. In addition, fluorescence derived from enzymatically cleaved FDG was detected only at laser irradiated areas, indicating that enzyme reaction took place *in vivo* by FDG release from nanohybrids after laser irradiation. These nanohybrids represent functional nanomachines that achieve spatiotemporal control of biological reaction *in vivo* through external stimuli.

4. pH-Responsive liposomes

4.1. Poly(carboxylic acid)s

Poly(carboxylic acid)s have been well-studied as polyelectrolytes to pH-sensitize the lipid membrane because poly(carboxylic acid)s show a coil-to-globule transition in response to pH change[59,60]. Poly(2-ethylacrylic acid) (Figure 5a) induces lipid membrane lysis *via* mixed micelle formation with lipid components by hydrophobic interaction after protonation of carboxy groups at acidic pH 5 [61]. Poly(2-propylacrylic acid) (Figure 5b) showed membrane lysis at a higher pH region than poly(2-ethylacrylic acid) did because the pKa of poly(2-propylacrylic acid) is higher than that of poly(2-ethylacrylic acid) [62]. Hydrophobicity near the carboxy group strongly affects its pKa. A series of poly(carboxylic acid)s was synthesized *via* the reaction of polyallylamine with dicarboxylic acid anhydrides (Figure 5c) [63]. With increase of the carbon number of spacer structures between the carboxy group and amide bond, pKa of carboxy group increased. Also, the membrane destabilization property was improved. These reports suggest that the design of hydrophobicity next to carboxylate is important to obtain poly(carboxylic acid)s that induce strong interaction with lipid membranes in response to decreased pH.

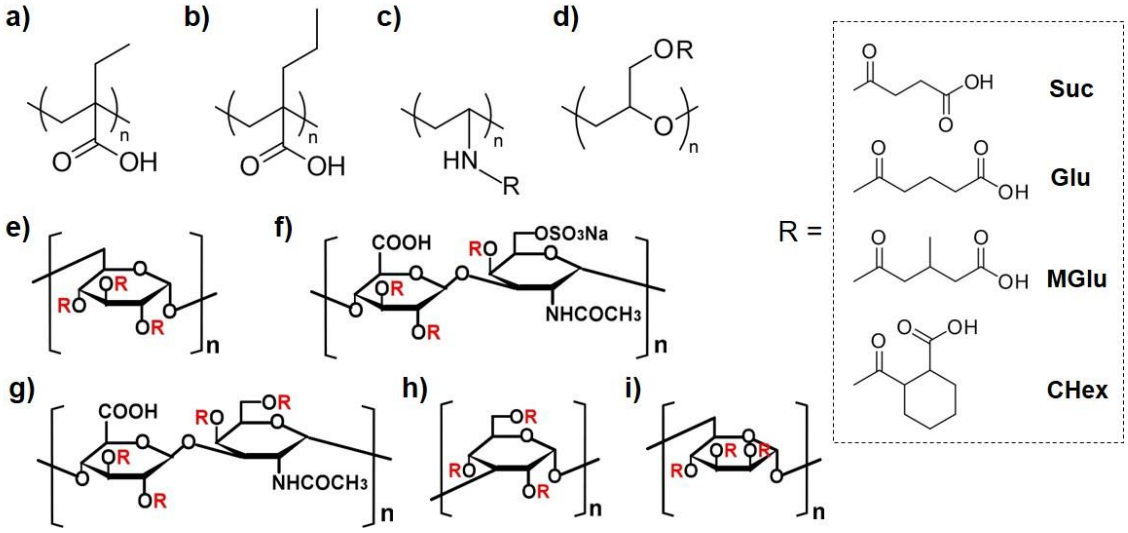

Figure 5. Chemical structures of (a) poly(2-ethylacrylic acid), (b) poly(2-propylacrylic acid), (c) carboxylated polyallylamines, (d) carboxylated polyglycidols, (e) carboxylated dextrans, (f) chondroitin sulfate derivatives, (g) hyaluronic acid derivatives, (h) carboxylated curdlan and (i) carboxylated mannan. Carboxylated groups with various spacer units are also shown.

4.2. Carboxylated polyglycidols

Polyglycidol has a PEG-like backbone and hydroxy groups in its side chain. Hydroxy group can be functionalized *via* esterification to obtain polyglycidol-based poly(carboxylic acid)s (Figure 5d). Succinylated polyglycidol (SucPG), first established polyglycidol-based pH-responsive polymer, induced pH-responsive content release from egg yolk phosphatidylcholine liposomes after modification of SucPG *via* anchoring (decylamide) group[64,65]. In addition, fluorescence resonance energy transfer (FRET) technique reveals that SucPG-modified liposomes promote membrane fusion with other lipid membranes at acidic pH [65]. Vinyl backbone-based pH-responsive polymers, as described in the last section, induce membrane lysis *via* mixed micelle formation because

polymer chains can be incorporated completely into lipid membranes *via* strong hydrophobic interaction. By contrast, PEG-like hydrophilic backbone of polyglycidol would interfere the complete embedding of polymer chains into the deep hydrophobic site of the lipid membrane. This embedding induces defects on the lipid bilayer and promotes membrane fusion with other membranes to compensate these membrane defects (Figure 6). A series of polyglycidol derivatives was also synthesized by reaction with various dicarboxylic acid anhydrides[66]. Similarly to polyallylamine-based polymers, polyglycidol derivatives with more hydrophobic spacer groups between the carboxy group and ester bond had higher pKa and exhibited stronger membrane fusion activities. Considering the pH region in intracellular organelle such as endosomes or lysosomes, 3-methylglutarylated polyglycidol (MGluPG) (pKa: 6.3) was selected as a suitable pH-responsive polymer towards its application to intracellular delivery systems. MGluPG-modified liposomes delivered fluorescence dye (calcein) molecules into cytosol of HeLa cells with earlier timing than SucPG-modified liposomes did.

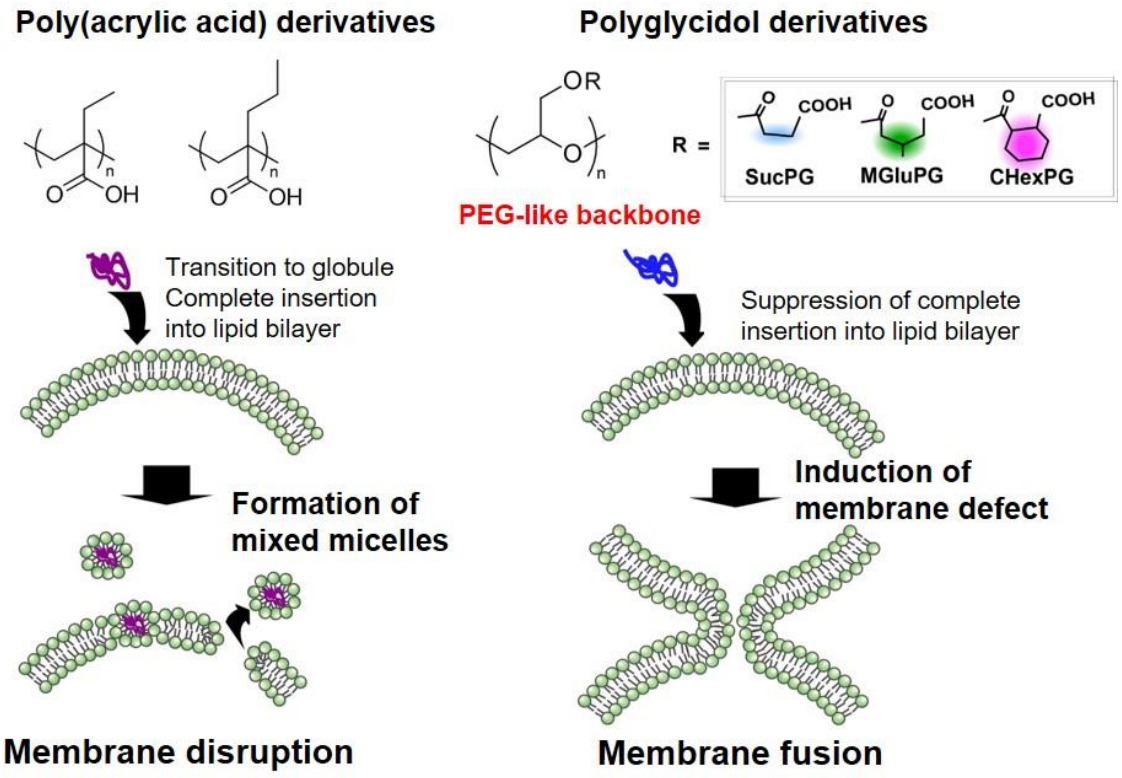

Figure 6. Plausible interaction modes of poly(carboxylic acid)s derivatives with lipid membrane.

Considering that membrane fusion is initiated from membrane defects, bulkier MGluPG-derivatives were designed using hyperbranched polyglycidol (HPG) backbone instead of linear polyglycidol[67]. The steric structure of HPG backbone and the peripheral pH-responsive groups (MGlu groups) promoted interaction with the lipid membrane compared with linear MGluPG. In addition, cellular association of liposomes modified with MGluHPG to the murine dendritic cell line (DC2.4 cell) was higher than that of linear MGluPG-modified liposomes. Dendritic cells have scavenger receptors that recognize anionic molecules and anionic phosphatidyl serine-exposing apoptotic cells[68,69]. Carboxy groups on the surface of MGluHPG-modified liposomes might interact with these scavenger receptors *via* multivalent interactions, leading to higher cellular

association than that of linear MGluPG-modified liposomes.

4.3. Application of pH-responsive liposomes to cancer immunotherapy

Antigen presenting cells (APCs) are target cells for antigen delivery carriers because APCs such as dendritic cell or macrophage can induce antigen-specific acquired immune responses[70,71]. In addition, uptake modes by APCs or intracellular fate of antigen inside of APCs affect the induced immune responses (Figure 7). When an antigen is taken up *via* endocytosis and degraded in endo/lysosomes, the degraded antigen peptides are carried onto major histocompatibility complex (MHC) II molecules and are presented to CD4-positive naïve T lymphocytes. This process is designated as antigen presentation. In this case, CD4-positive naïve T lymphocytes differentiate to antigen-specific helper T (Th) cells to activate humoral immunity. In contrast, when antigen is taken up *via* specific receptor-mediated endocytosis or is carried into cytosol of APCs, degraded antigen peptide fragments are bound onto MHC I molecules to be presented to CD8-positive naïve T lymphocytes. This specialized antigen presentation process of endogenous antigen *via* MHC I molecules is designated as cross-presentation, which induces the differentiation of CD8-positive naïve T lymphocytes into antigen-specific cytotoxic T lymphocyte (CTL) to activate cell-based immunity (cellular immunity) [72,73]. Cellular immunity plays a crucially important role in infectious diseases and cancer immunity to eliminate the virus-infected cells and to attack antigen-expressing tumour cells directly. However, the efficiency of cross-presentation is generally low when an antigen is taken up directly by APCs. Therefore, efficient antigen carriers that can induce cross-presentation are necessary to induce antigen-specific CTLs for the treatment of a viral infection or cancer.

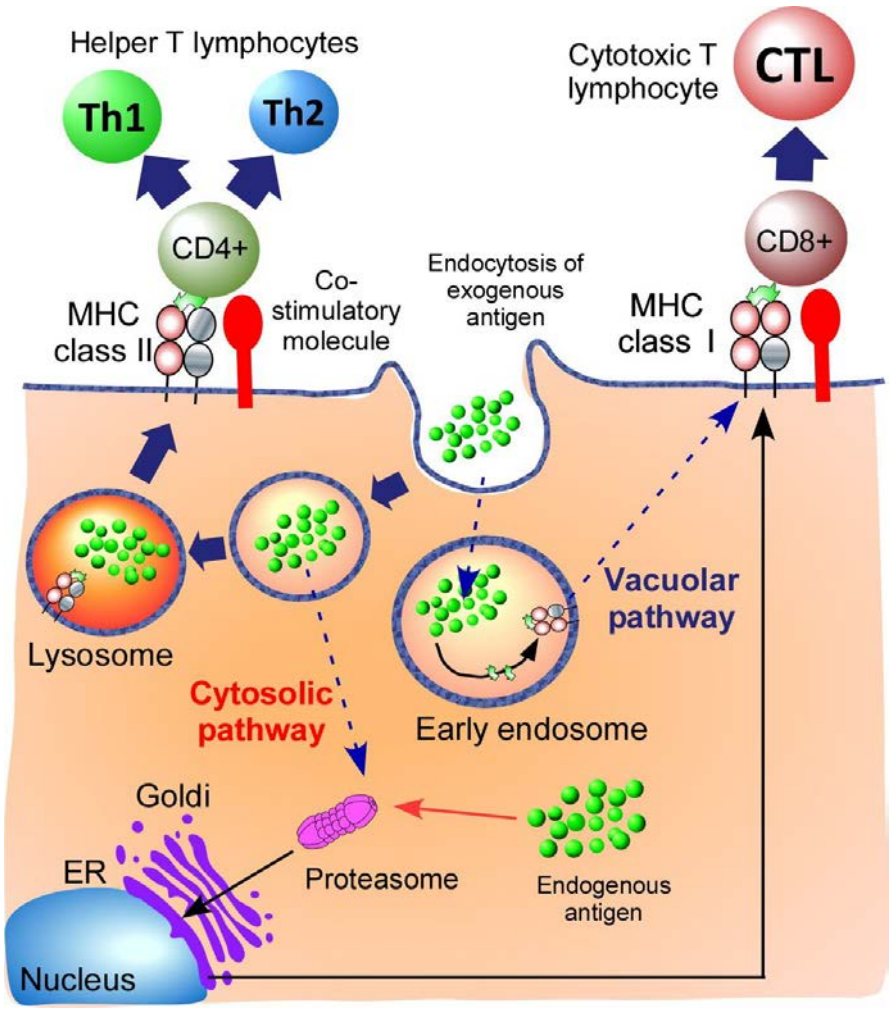

Figure 7. Antigen presenting pathway in antigen presenting cell. Most of exogenous antigens taken up *via* endocytosis are degraded in lysosomes and presented onto MHC II molecules, which induces activation of helper T lymphocytes. A part of exogenous antigens are transferred into cytosol and presented onto MHC I molecules as well as endogenous antigens to induce cytotoxic T lymphocytes (Cross-presentation by cytosolic pathway). Antigens transferred into low acidic compartments are also presented onto MHC I molecules (Cross-presentation by vacuolar pathway).

A main pathway to induce cross-presentation is the delivery of antigens into the cytosol of APCs (Figure 7) [72,73]. Because most nanocarriers are taken up by APCs *via*

endocytosis, the promotion of endosomal escape of antigen-loaded carriers is a crucially important step for the induction of cross-presentation[74]. Considering the intracellular delivery performance of pH-responsive polymer-modified liposomes *via* membrane fusion, these liposomes were applied to antigen delivery carriers into immune cells. As a model antigen, ovalbumin (OVA) is loaded into liposomes modified with MGluPG or MGluHPG. Such MGluPG-modified and MGluHPG-modified liposomes achieved the delivery of OVA into the cytosol of dendritic cell lines and bone marrow-derived dendritic cells (Figure 8a) [75,76]. Subsequently, FRET analysis revealed that this cytoplasmic delivery process of antigen was induced *via* intracellular fusion of liposomes with endosomal membranes (Figure 8b) [67]. Cytoplasmic delivery of antigen, as expected, promoted cross-presentation of OVA: MHC I-mediated antigen presentation of OVA peptide was detected when MGluPG-modified or MGluHPG-modified liposomes were applied to dendritic cells (Figure 8c) [76]. It is noteworthy that not only MHC I mediated antigen presentation, but also MHC II mediated antigen presentation was promoted by pH-responsive polymer-modified liposomes (Figure 8c), which reflects the enhanced antigen uptake by these liposomes compared to that of free OVA or OVA-loaded polymer unmodified liposomes. After subcutaneous, intranasal or intraperitoneal injection of MGluPG-modified or MGluHPG-modified liposomes to mice, OVA-specific antibody responses in serum and OVA-specific CTLs in spleen were detected, which reflects efficient antigen delivery into APCs in the body and induction of both MHC I mediated and MHC II mediated antigen presentations *in vivo*[75–78].

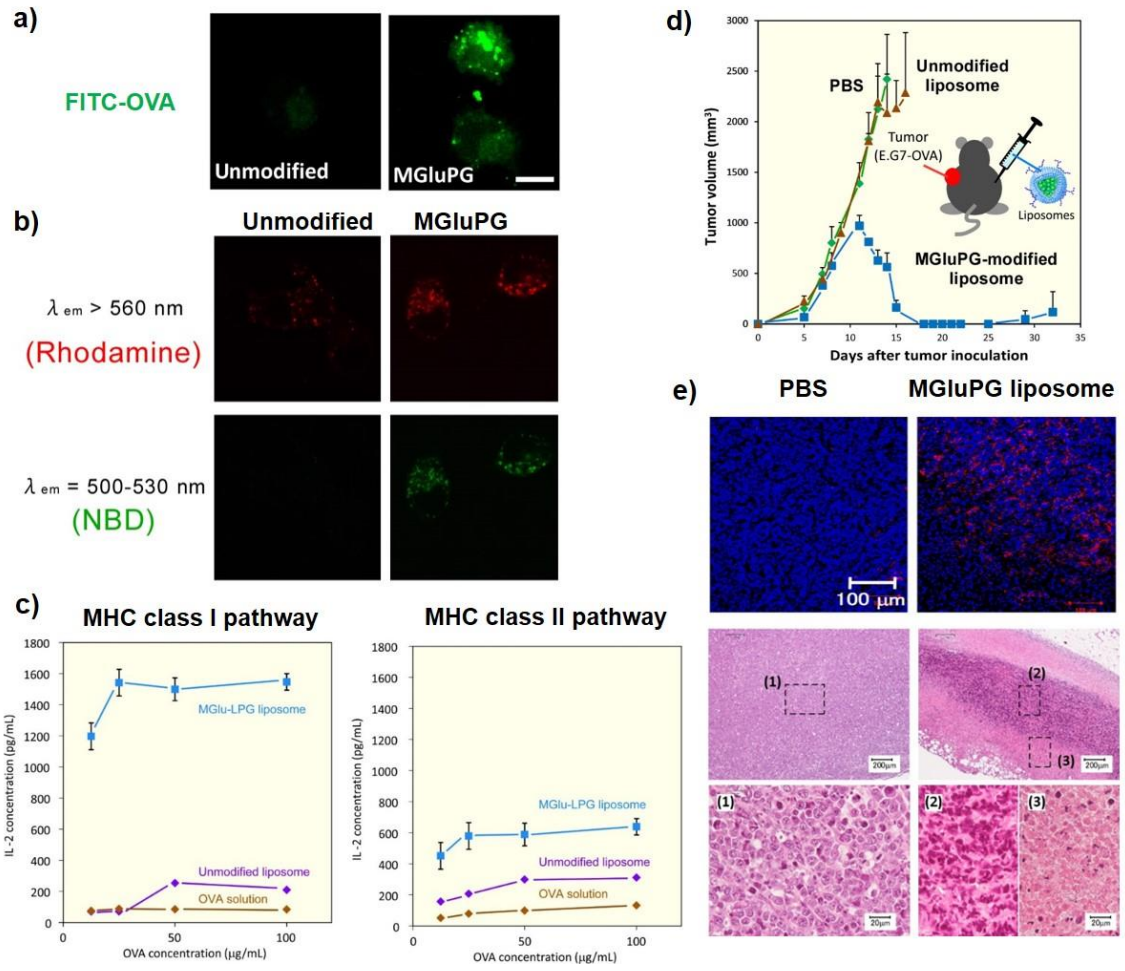

Figure 8. (a) Cytosolic delivery of FITC-OVA in bone marrow-derived dendritic cells induced by intracellular fusion of MGluPG-modified liposomes with endosomes detected by FRET (b). Cytosolic delivery of OVA also promoted cross-presentation of OVA (c). Subcutaneous injection OVA-loaded liposomes modified with MGluPG exhibited tumour regression in E.G7-OVA tumour-bearing mice (d), which was caused by enhanced infiltration of CD8-positive cells (shown as red in upper panel of e)) and induction of necrosis and apoptosis in tumour tissues (e). Partially reproduced from Ref. 67, 76 and 83 with permission from Elsevier.

4.4. Inclusion of adjuvant molecules to promote immune responses

During antigen presenting processes, not only antigen presentation *via* MHC molecules (first signal) but also stimulation *via* co-stimulatory molecules (second signal) and cytokine production (third signal) are required for the completion of activation of naïve T lymphocytes into helper T cells or CTLs. To induce upregulation of co-stimulatory molecules and cytokine production from APCs, adjuvant molecules that are recognized by pattern-recognition receptors on APCs are well used[79,80]. Therefore, antigen carriers require not only intracellular antigen delivery performance but also incorporation of adjuvant molecules. Lipid-based adjuvants are used often to provide the adjuvant function into liposomes because these lipid-like molecules are readily incorporated *via* hydrophobic interaction. Monophosphoryl lipid A (MPLA), a clinically approved adjuvant, is recognized by Toll like receptor 4 (TLR4) on APCs, which leads to strong activation of APCs[81]. Actually, incorporation of MPLA into MGluPG-modified or MGluHPG-modified liposomes promotes OVA-specific immune responses compared with results obtained in the absence of MPLA[82]. Pre-immunization of these liposomes completely rejected the growth of OVA-expressing tumour cells (E.G7-OVA cells) [76]. Furthermore, subcutaneous injection of these liposomes induced tumour regression in established E.G7-OVA tumours (Figure 8d) [76]. However, the same liposomes showed no tumour growth suppressive effects on OVA-non-expressing EL4 tumour-bearing mice. Numerous CD8-positive cells were detected in the tumour section of mice treated with MGluPG-modified liposomes 6 days after liposome injection (Figure 8e) [83]. At the same timing, many damaged cells were observed in E.G7-OVA tumour section from H&E staining results (Figure 8e). These data indicate clearly that OVA-specific immune responses recognize the OVA-expression on the tumour cells and OVA-specific CTLs migrates from spleen into tumour tissue to induce E.G7-OVA tumour cell killing. As

another lipid adjuvant, α-galactosylceramide (α-GalCer)-incorporated MGluPG-modified liposomes also induced strong cellular immune responses after subcutaneous injection[84]. Chemically synthesized cationic lipids have been used also as a lipid-type adjuvant[85-88]. Incorporation of cationic lipid 3,5-didodecyloxybenzamidine (TRX) into MGluHPG-modified liposomes increased the cellular association of the liposomes to DC2.4 cells[89]. Furthermore, cytokine production from liposome-treated cells and expression of MHC molecules/co-stimulatory molecules were promoted compared with MGluHPG-modified liposomes without TRX. Moreover, TRX-incorporation affected the intracellular distribution of antigens. After incorporation of TRX, some OVA were delivered into cytosol but most OVA were entrapped inside of endosomes because of the suppression of polymer interaction with endosomal membrane caused by MGluHPG chains restriction on the surface of the parent liposomes. Such a difference in the intracellular fate of antigenic proteins also changed the *in vivo* immune responses. TRX-incorporated MGluHPG-modified liposomes promoted both CTL induction and Th1 activation, whereas MGluHPG-modified liposomes without TRX mainly induced CTL responses. Th1 cells can activate CTLs *via* secretion of cytokine such as IL-12. Actually, therapeutic effects on tumour-bearing mice were enhanced by TRX incorporation into MGluHPG-modified liposomes.

The presence of cationic lipids on the liposomal membrane enables further incorporation of anionic adjuvant molecules *via* electrostatic interaction. Bacteria-derived CpG-motif and virus-derived double strand RNA are recognized respectively *via* TLR9 and TLR3 in APC endosomes to induce bacteria-specific or virus-specific immune responses[80]. Therefore, CpG-oligonucleotide (CpG-ODN) or synthetic RNA such as poly(I:C) have been used as adjuvant molecules. CpG-ODN was incorporated

additionally into TRX-incorporated MGluHPG-modified liposomes[82]. CpG-ODN molecules were entrapped to the TRX-incorporated MGluHPG-modified liposomes 10 times more efficiently than that of liposome without TRX because of electrostatic interaction with TRX on the liposome surface. In addition, CpG-ODN-loaded liposomes delivered CpG-ODN into endosomes where TLR9 (the receptor for CpG-ODN) exists, whereas most of the free CpG-ODN absorbed onto the cell surface. After subcutaneous administration into mice, CpG-ODN-loaded liposomes induced OVA-specific cellular immune responses more efficiently than that of conventional MGluHPG-modified liposomes, resulting in tumour regression in tumour-bearing mice.

4.5. Carboxylated dextrans

pH-Responsive polymers having a biodegradable backbone were designed using naturally occurring polysaccharides. Polysaccharides also have a hydrophilic backbone and many hydroxy groups to be modified with pH-responsive groups. In addition, the bioactivity of polysaccharides and targeting properties to polysaccharide-specific receptors such as lectins is expected to provide multifunctional pH-responsive polymers. As a first example of pH-responsive polysaccharides, dextran was used as a base polymer[90]. Similarly to polyglycidol derivatives, pH-responsive group-introduced dextrans were synthesized *via* reaction of hydroxy groups of dextran with dicarboxylic acid anhydrides. Carboxylated dextrans (MGlu-Dex, Figure 5e) showed lipid membrane destabilization activity in acidic pH. MGlu-Dex-modified liposomes exhibited content release under weakly acidic pH and were taken up efficiently by DC2.4 cells. In addition, MGlu-Dex-modified liposomes achieved cytoplasmic delivery of OVA, leading to MHC I-mediated antigen presentation. However, therapeutic effects on E.G7-OVA tumour-

bearing mice were insufficient to regress the tumour burden completely after subcutaneous injection of MGlu-Dex-modified liposomes.

As a measure for improving dextran-based pH-responsive polymers, the hydrophobicity of spacer units in pH-responsive groups was increased[91]. Dextran was reacted with 1,2-cyclohexanedicarboxylic acid anhydrides to obtain CHex-Dex with cyclohexyl spacer unit in pH-responsive groups. CHex-Dex formed hydrophobic domains at pH less than 7 and induced membrane destabilization activities at very weakly acidic pH. Reflecting its hydrophobic character, CHex-Dex-modified liposomes showed higher cellular association than that of MGlu-Dex-modified liposomes. In addition, quite high IL-12 production from DC2.4 cells treated with CHex-Dex was observed compared with MGlu-Dex. These data suggest that the hydrophobicity in the spacer unit of pH-responsive group is important to promote Th1 cytokine production from APCs. However, CHex-Dex-modified liposomes showed no anti-tumour effects on tumour-bearing mice after subcutaneous injection[92]. These unexpected results might be attributable to non-specific interaction of the liposomes after subcutaneous injection because of the overly hydrophobic character of CHex-Dex.

4.6. Chondroitin sulfate derivatives and hyaluronic acid derivatives

Introduction of CHex groups into dextran is effective to improve cellular association and cytokine production from dendritic cells *via* hydrophobic interactions. However, *in vivo* results suggest that non-specific cellular uptake of hydrophobic CHex groups also interferes with the cellular association into APCs *in vivo*. To modulate the hydrophobicity of CHex group, charged group-having polysaccharides were used as a backbone instead of dextran. Here, chondroitin sulfate (CS) that has carboxy groups and sulfo groups was

selected as the backbone[92]. CHex group-introduced CS (CHex-CS, (Figure 5f)) induced the content release from liposomes at acidic pH. Furthermore, CHex-CS-modified liposomes exhibited high cellular association to DC2.4 cells, but the same liposomes showed negligible cellular association to the fibroblasts. By contrast, CHex-Dex-modified liposomes exhibited high uptake by DC2.4 cells and fibroblasts, which supports the *in vivo* non-specific uptake of CHex-Dex-modified liposomes. CHex-CS-modified liposomes also induced significantly high cytokine production from DC2.4 cells compared with CHex-Dex-modified liposomes, resulting in tumour growth suppression in tumour-bearing mice. Hyaluronic acid was also used as a backbone having a charged group. The modification of CHex group-introduced hyaluronic acids (CHex-HA, Figure 5g) onto liposomes also achieved highly cellular association with dendritic cells and macrophages and negligible uptake by the fibroblasts[93]. Furthermore, knockdown of CD44 (HA receptor) using CD44-specific siRNA significantly reduced the cellular association of CHex-HA-modified liposomes (Figure 9a). These findings indicate that HA receptors can recognize CHex-HA even after chemical modification of the HA backbone by CHex groups, which provides CD44-specific delivery carriers with intracellular delivery performance. Actually, CHex-HA-modified liposomes were applied not only to antigen delivery but also to anticancer drug delivery to CD44-expressing cancer cells[94].

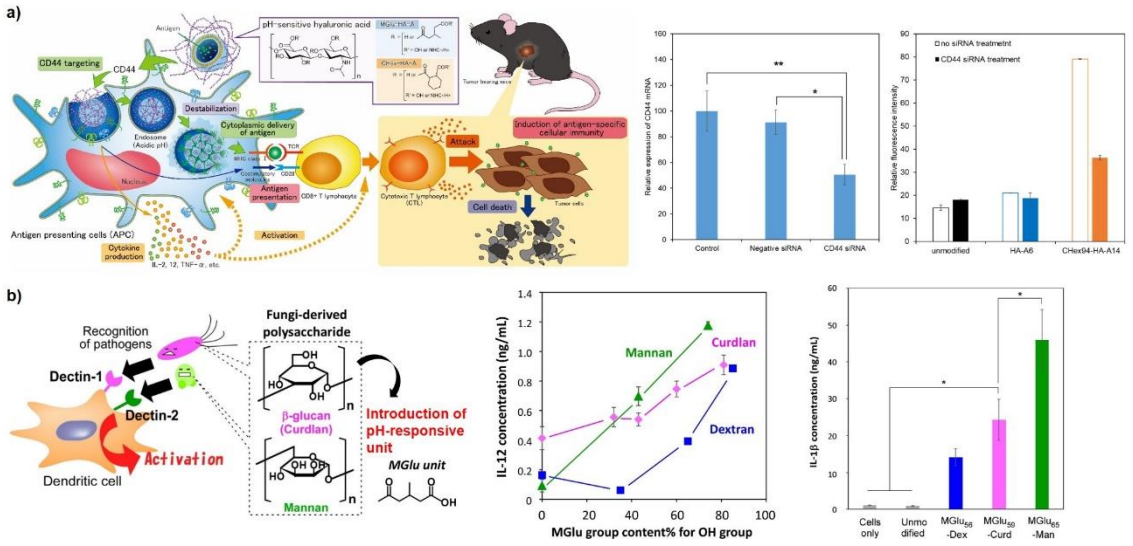

Figure 9. (a) Hyaluronic acid derivative-based CD44-specific antigen delivery system. After knockdown of CD44 on dendritic cell line (left panel), cellular association of CHex-HA-modified liposomes was significantly decreased (right panel). Partially reproduced from Ref. 93 with permission from ACS publications. (b) Modification of bacteria-derived polysaccharides promoted the cytokine production from dendritic cell lines by polysaccharides themselves (left panel) and polysaccharide-modified liposomes (right panel). Partially reproduced from Ref. 98 with permission from Elsevier.

4.7. Carboxylated curdlan and mannan

Some polysaccharides possess specific bioactivity including adjuvant effects. The use of bioactive polysaccharides as a backbone of pH-responsive polymer would also provide multifunctional polysaccharides. Curdlan, typical linear β-1,3-glucan, and mannan were selected as the backbone because curdlan and mannan are recognized respectively by surface receptors on APCs, Dectin-1 and Dectin-2[95-97]. Recognition of curdlan or mannan by these receptors leads to APC maturation. MGlu groups were introduced to curdlan and mannan to obtain MGlu-Curd and MGlu-Man (Figures 5h and

5i)[98]. Both curdlan derivatives and mannan derivatives having medium amounts of MGlu groups induced IL-12 production from DC2.4 cells more efficiently than parent curdlan, mannan or MGlu-Dex did, indicating that MGlu group introduction improved the adjuvant property compared with parental polysaccharides and indicating that the backbone structure actually affected the adjuvant property of carboxylated polysaccharides. More interestingly, MGlu-Curd, MGlu-Man and MGlu-Dex with high MGlu groups showed almost identical cytokine production (Figure 9b), suggesting that both the backbone structure and MGlu group contents are important to prepare highly effective adjuvant polysaccharides. Among these polysaccharides, MGlu-Curd-modified liposomes showed superior antigen delivery performance and anti-tumour effects in tumour-bearing mice. Especially, MGlu-Curd-modified liposomes induced almost complete regression of tumours, even in the absence of conventional lipid adjuvant (MPLA). Therefore, backbone structure selection and optimization of the pH-responsive group contents and spacer structures are expected to produce further effective multifunctional polysaccharides for cancer immunotherapy.

4.8. Dendron-bearing lipid as pH-responsive lipid nanocarriers

PAMAM dendron-bearing lipids (DLs) can also be used for pH-responsive amphiphiles to obtain pH-responsive molecular assemblies[99]. PAMAM dendron has several primary and tertiary amines to be protonated in response to decreased pH [100]. Protonation in the dendron part would change the hydrophilic–hydrophobic balance of DL at a molecular level, which changes its self-assembly structures. Actually, the DL suspension formed vesicles above pH 6.4, whereas micelle-like structures were observed pH under 6.3 (Figure 10), which pH corresponds to the starting point of the protonation

of tertiary amine in the PAMAM dendron. Such a drastic change in the self-assembling structure is applicable to intracellular protein delivery. DL-based self-assemblies can encapsulate OVA molecules at neutral pH, but OVA molecules were released immediately at pH 6.0. In addition, OVA molecules were delivered into cytosol of dendritic cell lines after 4 h-incubation with OVA-loaded DL self-assemblies (Figure 10). After internalization to the cells *via* endocytosis, DL self-assemblies changed their assembly structure into micelles in response to weakly acidic pH in the endosomes, resulting in destabilization of endosomal membrane and release of OVA.

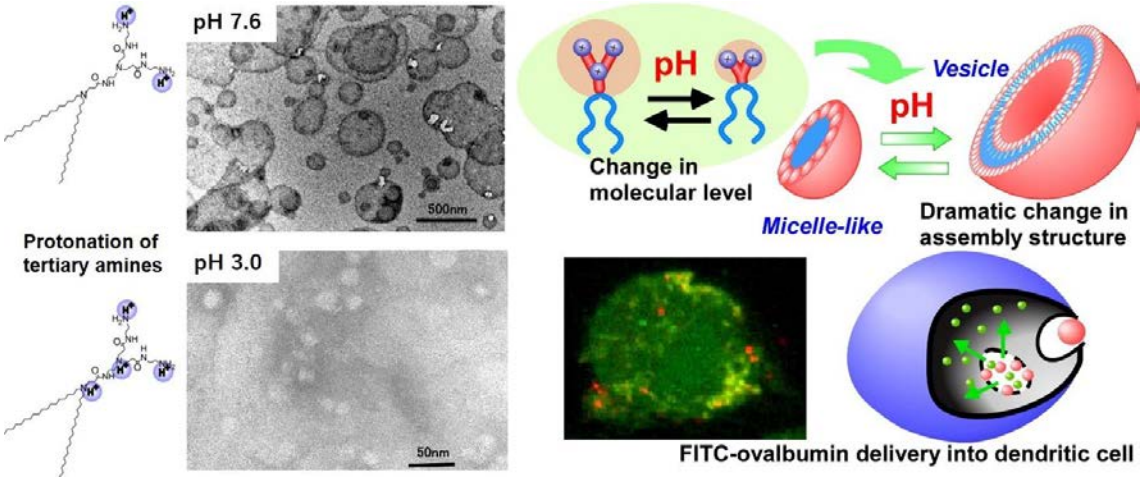

Figure 10. Polyamideamine dendron-bearing lipid changed its self-assembling structure from vesicle to micelles after protonation of tertiary amines at acidic pH, which can be applied to cytosolic delivery of proteins into immune cells. Partially reproduced from Ref. 99 with permission from ACS publications.

5. Concluding remarks and perspectives

This review summarized strategies to prepare highly effective stimulus-responsive liposomes modified with various functional materials. For the preparation of

thermoresponsive liposomes, thermoresponsive polymers were incorporated into anticancer drug-loaded liposomes. These liposomes were able to induce the selective release of anticancer drugs at the disease site *in vivo* in response to local heating. Furthermore, the accumulation of liposomes and drug release processes was monitored using MRI, which provides further optimization of liposomal DDS for each cancer patient by adjusting the heating timing and DDS characteristics. In addition, these liposomes can be combined with conventional radiotherapy such as heavy ion radiotherapy to improve cancer treatment efficacy[101]. Thermoresponsive liposomes can be combined with various functional nanomaterials such as inorganic nanoparticles and carbon nanomaterials. These nanohybrids are expected to lead to novel methodologies for controlling biological reactions inside of a living body or in a cell level artificially through precise control of biodistribution of nanohybrids and external stimuli, which might provide new biological information supporting future biology and medicine. Thermoresponsive lipid-based molecular assemblies were also developed, which further opens novel chemical design of thermoresponsive nanocarriers using a drastic transition of self-assembling structures to control drug release behaviours and interaction with the cell or tissues by external heating.

For the preparation of pH-responsive liposomes, various poly(carboxylic acid)s were modified on the liposome surface. Especially, polyglycidol-based poly(carboxylic acid)s are useful to induce pH-sensitive membrane fusion towards intracellular drug delivery including antigenic proteins for induction of antigen-specific immune responses. Already, these liposomes have been applied to the delivery of antigenic peptides identified from various human cancers[102-104]. Cross-presentation of these antigenic peptides by pH-responsive polymer-modified liposomes was confirmed using human-derived dendritic cells, which indicates the practical usefulness of these pH-responsive polymer-modified

liposomes towards antigen carriers for cancer immunotherapy. Polysaccharide-based poly(carboxylic acid)s were also prepared as multifunctional polymers for the induction of antigen-specific immunity. Targeting properties, adjuvant properties and immunity-inducing functions depend on their backbone structures and spacer structures in pH-responsive groups. These suggest precise control of immune responses by changing the molecular structures of polysaccharide derivatives and selecting suitable polymer backbones. Through further investigation of correlation of polymer structure and interaction with surface receptors on antigen presenting cells, novel chemical designs to produce highly effective adjuvant molecules will be fabricated.

Recent progress in antibody medicine, especially in the area of cancer immunotherapy, has revealed the importance of cancelling of immunosuppression in a tumour microenvironment such as immune checkpoints, immunosuppressive cells, and cytokines[105,106]. We have also investigated that the combination of pH-responsive polymer-modified liposomes with inhibitors of immunosuppressive cytokine signals or Th1 cytokine-encoding plasmid DNA delivery systems markedly improved the anti-tumour effects by promotion of CTL infiltration into tumour tissues[83,107]. Not only has the molecular design of stimulus-responsive polymers improved antigen delivery performance: a combination of modulation systems of immunity or tumour microenvironments can be a promising approach to construct highly potent therapeutic system from a practical viewpoint. Deep understanding of the material chemistry side will be important to prepare potent delivery carriers, as will knowledge of the biology side in areas such as tumour immunology and cell biology. Such interdisciplinary studies will create potent next-generation medicines and personalized medicines.

6. Conflicts of interest

There is no conflict to declare

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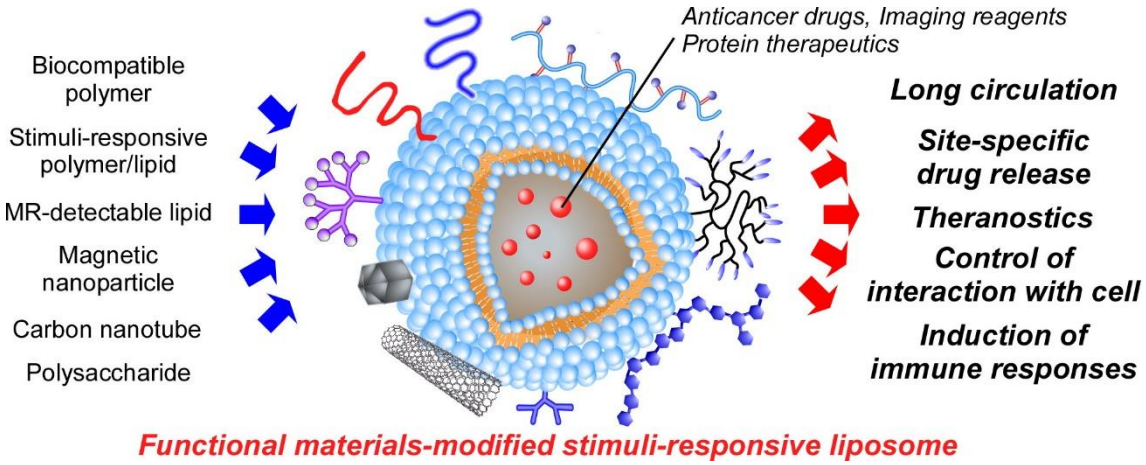

The fabrication strategies and biomedical applications of stimuli-responsive materials-modified liposomes are summarized and reviewed.

Biography

Dr. Eiji Yuba received his BS degree from Department of Applied Materials Science, Osaka Prefecture University, Japan in 2006. He received MS and Ph.D. degrees from Department of Applied Chemistry, Osaka Prefecture University, Japan in 2008 and 2010, respectively. He worked as an Assistant Professor of Osaka Prefecture University, Japan, from 2010 to 2017 and as an Associate Professor from 2017 to date in Department of Applied Chemistry, Osaka Prefecture University. Also, he stayed in the University of Chicago (Jeffrey Hubbell laboratory) as a visiting scholar in 2019. His research interests are functional polymer-based nanomaterials for biomedical applications including drug delivery, gene vectors, diagnosis and immunotherapy.