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**Glycosylation-mediated targeting of carriers**

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Abstract
For safe and effective therapy, drugs should be delivered selectively to their target tissues or cells at an optimal rate. Drug delivery system technology maximizes the therapeutic efficacy and minimizes unfavorable drug actions by controlling their distribution profiles. Ligand-receptor binding is a typical example of specific recognition mechanisms in the body; therefore, ligand-modified drug carriers have been developed for active targeting based on receptor-mediated endocytosis. Among the various ligands reported thus far, sugar recognition is a promising approach for active targeting because of their high affinity and expression. Glycosylation has been applied for both macromolecular and liposomal carriers for cell-selective drug targeting. Recently, the combination of ultrasound exposure and glycosylated bubble liposomes has been developed. In this review, recent advances of glycosylation-mediated targeted drug delivery systems are discussed.

Keywords: macromolecular carriers, liposomes, targeting, glycosylation, drug delivery
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1. Introduction

After the administration of drugs to an individual, they are distributed according to their physicochemical and/or biological properties. Subsequently, they are eliminated from the body. For safe and effective drug therapy, they should be selectively delivered to their target tissues or cells at an optimal rate. Drug delivery systems are a technology that maximizes drug therapeutic efficacy and minimizes their unfavorable actions by controlling distribution profiles. Thus, controlled drug disposition is an important factor that determines the therapeutic efficacy of drugs. Drug delivery systems have been used clinically, particularly for anticancer drugs and are essential for the practical application of recombinant protein and nucleic acid medicine.

One major approach of targeting is the use of drug carriers that possess an affinity towards a population of target cells. To date, many macromolecules and lipid dispersion preparations, i.e., liposomes and emulsions, as well as various natural and synthetic macromolecules have been developed as drug carriers. Important characteristics of drug carriers include: i) biocompatibility, ii) lack of toxicity and immunogenicity, iii) biodegradability or minimal accumulation in tissues or organs, iv) adequate functional group for chemical modification of ligands and/or drugs, and v) productivity [1,2].

Ligand-receptor binding is a typical example of a specific recognition mechanism. Therefore, ligand-modified drug carriers have been developed for active cell-selective drug delivery by receptor-mediated endocytosis. So far, galactose, mannose, fucose, mannose-6-phosphate, sialyl Lewis x, peptides, and proteins (i.e., transferrin, epidermal growth factor, and anti-human epidermal growth factor receptor 2 antibodies) have been used as ligands [3-10]. Among the various ligands reported, sugar recognition mechanisms are a promising approach for active targeting because of their high affinity and expression.

Recently, various nucleic acids have been developed for medication including pDNA, antisense DNA, small interfering RNA (siRNA), and microRNA [11-15]. The receptor-mediated targeting by glycosylation has been used for therapy with nucleic acids. In the case of nucleic acid medicine, not only recognition by receptors but also intracellular fate, i.e., endosomal escape, should be considered. To solve this problem, multifunctional glycosylated particulates and a combination method of glycosylated bubble particles with ultrasound exposure have been developed. This review focuses on the recent progress of glycosylation-mediated targeting of low-molecular-weight drugs and nucleic acid drugs by drug carriers using macromolecules and liposomes.
2. Distribution characteristics of macromolecules

The distribution of macromolecular carriers is determined by their physicochemical properties, such as electric charge, molecular size, and hydrophilic/hydrophobic balance. Although ligand modifications with a high affinity to target cells are essential for macromolecular carriers in active targeting, the physicochemical properties of macromolecular carriers have an important function in determining the targeting efficacy of ligand-modified macromolecular carriers. The optimal design of macromolecules that possess suitable physicochemical properties of distribution following their systemic administration should be considered for precise active targeting by glycosylated macromolecular carriers.

2.1. Overcoming the rapid clearance of macromolecular carriers

Prolonged retention of drug-ligand-modified macromolecular carrier conjugates in the blood promotes their distribution to target cells. The controlled physicochemical properties of macromolecular carriers are an important factor for their prolonged retention and are based on the biological characteristics present in tissues and organs. These distribution characteristics and pharmacokinetic analysis of macromolecules have been summarized in our previous review [1, 2, 18, 19].

The kidney has an important role in the elimination of drugs from the body. Therefore, the reduction of glomerular filtration in the kidney should be considered. Macromolecules with a molecular weight of < 50,000 (approximately 6 nm in diameter) are suitable for glomerular filtration. In addition, the surface charge of macromolecules affects the reabsorption process in the kidney. For example, positively charged dextran (70 kDa) has higher glomerular permeation via endocytosis than negatively charged dextran although they have a similar molecular weight [20].

The liver also has an important role in the fate of drugs in the body, and thus the reduction of liver uptake should be considered. Our group previously showed that positively charged macromolecules (bovine serum albumin (BSA) and dextran) were distributed to the liver according to their contact area in the parenchymal and non-parenchymal cells via electrostatic interactions [21, 22]. In contrast, negatively charged BSA and dextran were distributed to the liver non-parenchymal cells via scavenger receptor-mediated endocytosis [23-25].

It is necessary to consider the general fate of macromolecules from the viewpoint of their physicochemical and biological characteristics when rationally designing ligand-modified macromolecular carriers for active targeting.
3. Glycosylated macromolecules for cell-selective targeting

Table 1 summarizes an example of glycosylation-mediated targeting of macromolecular carriers. Glycosylation-mediated targeting is a promising approach for cell-selective targeting because it has specificity for target cells and productivity for standardization. In this case, the controlled distribution of non-modified macromolecular carriers determined by physicochemical properties is important for cell-selective targeting. In addition, the density of ligands in macromolecular carriers might affect recognition by the receptor. Fig. 1 summarizes design of glycosylated macromolecular carriers.

### 3.1. Galactose modification

Asialoglycoprotein receptors are exclusively expressed on hepatocytes and have a high affinity and rapid internalization rate. Therefore, galactosylated macromolecules...
have been studied for hepatocyte-selective drug targeting. Discontinuous capillaries (sinusoidal capillaries) are commonly observed in the liver [26], and contain large interendothelial junctions with a mean diameter of 150 nm [26, 27]. Intravenously injected galactosylated macromolecules must pass through the sinusoidal capillaries to reach the hepatocytes expressing asialoglycoprotein receptors for recognition. To date, dextran [28], poly-L-lysine (PLL) [29-33], poly-glutamic acid (PLGA) [34-36], polyethlenimine (PEI) [37-42], chitosan[43-47], dendrimer/α-cyclodextrin conjugate [48], and their derivatives [49, 50] have been studied for galactosylated macromolecular carriers for hepatocyte-selective targeting.

The design of carrier types based on the physicochemical properties and galactose density of the carriers are of significance for targeting efficacy by galactosylated macromolecular carriers. Our group previously compared the targeting efficiency between positively charged galactosylated PLL and negatively charged galactosylated PLGA in mice [51]. After intravenous administration, galactosylated PLGA was selectively taken up by liver parenchymal cells and was significantly inhibited by the co-administration of excess galactosylated BSA, suggesting the asialoglycoprotein receptors mediated uptake of PLGA. Galactosylated PLL showed a higher accumulation in the liver compared with unmodified PLL. However, the effect of galactosylation on liver parenchymal cells-selective targeting was less pronounced than for galactosylated PLGA. These results suggest that the selection of macromolecular carriers is important to achieve efficient cell-selective targeting.

The galactose density of macromolecular carriers might affect targeting efficacy. Our group studied the effect of galactose moiety surface density on galactosylated proteins such as superoxide dismutase (SOD) and BSA. After intravenous administration in mice, galactosylated proteins were recovered in the liver and were shown to be highly dependent upon the degree of galactose modification. Thus, targeting efficacy can be controlled by the degree of macromolecular carrier galactosylation.

Hepatocytes are notable targets for gene and nucleic acid therapy, because many refractory metabolic diseases are caused by hepatocyte-derived proteins. In addition, hepatocytes are considered targets for the production of therapeutic proteins to secrete into the blood because the liver possesses a rich blood flow. The asialoglycoprotein receptor-mediated targeting of pDNA and/or nucleic acids to hepatocytes using galactosylated PLL, galactosylated chitosan, galactosylated PEI, or galactosylated dendrimer/α-cyclodextrin conjugate was previously investigated. To achieve high gene expression by galactosylated polycations/pDNA complexes, a rationally designed delivery system is needed for optimal pharmacokinetics including: i) condensation of
pDNA for the penetration of fenestrae, ii) pharmacokinetic processes of pDNA to hepatocytes by asialoglycoprotein receptor recognition, and iii) release of pDNA from endosomes/lysosomes into cytoplasm after internalization. Of these, the release of pDNA from endosomes/lysosomes into the cytoplasm after internalization is a major obstacle for efficient gene expression in hepatocytes. Therefore, manipulation of this process by functional peptides or the use of PEI with pH buffering functions in lysosomes [53] by galactosylated macromolecules might allow high transfection rates in vivo [40, 49, 54-56].

3.2. Mannose modification

Macrophages have an important role as effector cells in inflammation and antigen presentation. Mannose receptors are expressed on Kupffer cells, alveolar, peritoneal, and splenic macrophages, dendritic cells, and a subset of vascular endothelial cells [57-61]. Therefore, mannosylated macromolecules have been studied for mannose receptor-expressing cell-selective targeting. Similar to galactosylated macromolecular carriers described above, dextran [28], PLL [62-64], PLGA [65], PEI [66-70], chitosan [71-73], and dendrimer/α-cyclodextrin conjugate [74, 75] have been studied for mannosylated macromolecular carriers to selectively target mannose receptor expressing cells. The design of carrier types based on the physicochemical properties and mannose density of the carriers is of significance for targeting efficacy by mannosylated macromolecular carriers.

Our group developed a mannosylated macromolecular carrier system for cell-selective targeting based on dextran, which has high solubility, an abundance of hydroxyl groups for chemical modification, low immunogenicity, and is used in the clinic as a plasma expander [2]. Our group studied carboxymethyl dextran to minimize the interaction with endogenous components and then coupled them with mannose moieties as a ligand [28]. After intravenous injection into mice, mannosylated carboxymethyl dextran was rapidly eliminated from the plasma and accumulated in liver non-parenchymal cells including Kupffer cells and liver endothelial cells that expressed mannose receptors. When we compared the targeting efficiency between positively charged mannosylated PLL and negatively charged mannosylated PLGA in mice [51], the liver non-parenchymal cell selectivity of mannosylated PLL was lower than for mannosylated PLGA. These observations correspond with results observed for galactosylated macromolecular carriers. Thus, the selection of macromolecular carriers based on their physicochemical properties is important to achieve efficient cell-selective targeting.
Similar to galactosylated macromolecular carriers, the mannose density of macromolecular carriers can affect the targeting efficacy. We previously reported the effect of the surface density of mannose moieties in mannosylated proteins such as SOD, BSA, and IgG [76]. After intravenous administration in mice, mannosylated proteins were recovered in the liver and were highly dependent on the degree of mannose modification. These results suggest that targeting efficacy can be controlled by the degree of mannosylation of macromolecular carriers.

Mannose-expressing cells are notable targets for gene and nucleic acid therapy for refractory diseases such as chronic inflammation, cancer, and virus infection, amongst others. Mannose receptor-mediated targeting of pDNA and/or nucleic acids to macrophages was previously reported using mannosylated PLL, and mannosylated chitosan. When considering mannosylation, the condensation of pDNA is not critical because it is not required to pass through fenestrae to reach the target cells unlike galactosylated macromolecular carriers. However, the release of pDNA from endosomes/lysosomes into the cytoplasm after internalization is a major obstacle for efficient gene expression in mannose receptor-expressing cells. To solve this problem, mannosylated PEI, or mannosylated dendrimer/α-cyclodextrin conjugate that can accelerated the cytoplasmic release of pDNA have been investigated.

3.3. Mannose-6-phosphate (M6P) modification

Two M6P receptors, cation-dependent M6P receptors and cation-independent M6P/insulin-like growth factor (M6P/IGF-II), belong to the family of p-type lectins and are involved in the transport of cellular proteins from the cellular surface or trans-golgi network to lysosomes [77-79]. M6P/IGF-II receptors are highly expressed on activated hepatic stellate cells (HSCs) [80-82]. Liver fibrosis is the excessive accumulation of extracellular matrix proteins including collagen. Excessive collagen is considered responsible for activated HSCs, which constitute 5–8% of liver cells [83].

M6P modification of macromolecular carriers has been studied for cell-selective targeting to HSCs or cancer cells that highly express M6P/IGF-II receptors. M6P modified human serum albumin (HSA) was accumulated in the liver by an M6P-modification-dependent mechanism [84]. After intravenous administration, M6P$_{28}$-HSA (M6P:HSA=28:1) preferentially accumulated in HSCs of fibrotic rats and liver endothelial cells in normal rats. In contrast, M6P$_{28}$-HSA predominantly accumulated in liver endothelial cells in normal rats, suggesting a degree of the fibrosis is required for the M6P modification targeting system. These results demonstrate the importance of targeting efficacy to HSCs determined by the degree of M6P expression
of macromolecular carriers in fibrotic rats. This observation corresponds with galactosylated and mannosylated macromolecular carriers as previously described. In addition, the uptake of M6P-HSA in slices of human liver was reported [85].

HSC-selective targeting by macromolecular carriers has been studied using various drugs conjugated to M6P-HSA. Greupink et al. developed a doxorubicin (DOX)-conjugated M6P-HSA (M6P-HSA-DOX) for HSC-selective targeting of bile duct-ligated rats [86]. They showed that M6P-HSA-DOX was distributed in the liver and immunohistochemical double staining revealed accumulation in HSCs. In addition, M6P-HSA-DOX reduced HSC proliferation by 82% in vitro. These results indicate that inhibition of activated HSC proliferation by cytostatic drugs can prevent the fibrotic process. Similarly, Hagens et al. developed glitoxin (GTX), an apoptosis-inducing agent, conjugated with M6P-HSA (M6P-HSA-GTX), which attenuated liver fibrosis in bile duct-ligated rats [87]. Although GTX induced adverse effects on hepatocytes, M6P-HSA-GTX did not show such effects. To date, various drugs including tyrosine kinase inhibitor, angiotensin type 1 receptor blockers, Rho kinase inhibitor, and conjugated M6P-HSA have been developed to inhibit liver fibrosis [88-93]. Prakash et al. reported tumor-selective targeting by M6P-HSA-DOX through M6P/IGF-II receptors in tumor-bearing mice [94].

Regarding oligonucleotide delivery, Ye et al. developed M6P-BSA conjugated with oligonucleotides via a disulfide bond [95]. A triplex-forming oligonucleotide (TFO) was used to inhibit transcription via interactions with genomic DNA. After intravenous injection, M6P20-BSA-[33P]TFO was rapidly eliminated from the blood and a significant increase in uptake by the liver was observed. Study of intrahepatic distribution revealed that M6P20-BSA-[33P]TFO was preferentially taken up by HSCs compared with hepatocytes, Kupffer cells and/or endothelial cells. In addition, pre-injection of excess M6P20-BSA significantly decreased the liver uptake in normal and fibrotic rats, suggesting M6P/IGF-II receptors mediated the uptake of M6P20-BSA-TFO [96]. Zhu et al. developed siRNA-PEG-M6P conjugates for hepatic stellate cell-selective targeting [97]. Although there have been few reports regarding nucleic acid delivery using M6P modified macromolecules, the release from endosomes/lysosomes into the cytoplasm after internalization is a common obstacle for efficient gene expression in HSCs.

4. Distribution characteristics of liposomes

Liposomes have been used in the clinic as drug delivery carriers [98-100]. Therefore, targeted drug delivery by surface modification of liposome ligands is a promising approach for their wide application and delivery of drugs. In general, the
distribution of liposomes is greatly determined by their surface charge, particle size, lipid composition, and dose used. A major factor that determines their rapid elimination is their uptake by the reticuloendothelial (mononuclear phagocyte) system, predominantly the liver and spleen. Phagocytosis of liposomes is accelerated by association with complement and immunoglobulins in the blood. The optimal design of liposomal dosage is one that possesses suitable physicochemical properties, especially avoiding uptake by the reticuloendothelial system upon systemic administration and this should be considered for precise active targeting using glycosylated liposomes.

4.1. Overcoming the rapid clearance of liposomes

After intravenous administration, liposomes are taken up by the reticuloendothelial system in the liver and spleen [101]. Therefore, the reduction and/or blockage of the reticuloendothelial system uptake are promising approaches to enhance the blood concentration of liposomes.

The lipid composition of liposomes might affect the clearance of liposomes depending on the contents and type of lipids present. Regarding the effect of cholesterol incorporation in liposomes, cholesterol-free liposomes and cholesterol-poor (20 mol%) liposomes were cleared more readily from blood circulation than cholesterol-rich (46.6 mol%) liposomes [102]. The predominant factor of clearance from the blood circulation is uptake by the reticuloendothelial tissues. Semple et al. analyzed the in vivo association of blood proteins with liposomes composed of saturated phosphatidylcholines and cholesterol [103]. When liposomes composed of dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearylphosphatidylcholine (DSPC), and diarachidoylphosphatidylcholine (DAPC) were administered intravenously, liposomes containing DMPC and DOPC, that are liquid crystalline prior to injection, showed relatively long circulation times. In contrast, liposomes composed of DPPC, DSPC, and DAPC that are in a gel state at 39°C, were rapidly cleared from blood circulation and were associated with large quantities of associated blood proteins. The incorporation of cholesterol into DSPC liposomes resulted in their deceased protein binding and enhanced circulation time in blood.

The surface charge of liposomes might also affect the clearance of liposomes. Juliano et al. reported that neutral liposomes containing phosphatidyl choline and cholesterol at a 1:1 ratio and positively charged liposomes containing phosphatidyl choline, stearyl amine, and cholesterol at an 18:2:10 ratio were cleared less rapidly than negatively charged liposomes containing phosphatidyl choline, phosphatidyl serine, and cholesterol at a 1:1:1 ratio [104]. Allen et al. reported that the incorporation of greater
than 2 mol% phosphatidyl serine into liposomes caused their rapid elimination from blood circulation. Daeman et al. reported a different intrahepatic distribution of phosphatidyl glycerol and phosphatidyl serine liposomes [105]. Phosphatidyl serine-containing liposomes were found mainly in the liver (75%), while phosphatidyl glycerol-containing liposomes were found in the liver (40%) and spleen (40%) equally. Regarding intrahepatic distribution, phosphatidyl serine-containing liposomes were distributed between Kupffer cells and hepatocytes equally, while phosphatidyl glycerol-containing liposomes were only taken up by Kupffer cells. Gabizon and Papahadjopoulos reported that increasing the molar ratio of negatively charged phosphatidyl inositol from 9% to 23% did not change the distribution, but an increase from 23% to 41% accelerated the clearance of liposomes [106]. It was reported that cationic liposomes/plasmid DNA (lipoplex) can be selectively transfected to lungs following intravenous administration [107-109]. We previously showed that cationic liposomes containing 1,2-di-O-octadecenyl-3-trimethylammonium propane or N-[1-(2,3-dioleoyloxy)propyl]-N, N, N-trimethylammonium methyl sulfate and cholesterol at a 1:1 ratio that were used for gene transfection were mainly distributed in the lung [110]. This is consistent with the transfection characteristics of lipoplex. Therefore, the selection of lipid composition is important when designing targeted drug delivery systems using glycosylated liposomes.

Surface modification of liposomes by ganglioside GM1 [111] and poly(ethylene glycol) (PEG) [112, 113] could achieve long blood circulation by avoiding reticuloendothelial uptake from the blood. To date, many reports have described the use of PEGylated liposomes for long blood circulation. Woodle et al. reported the effect of various molecular weights (120, 750, 1,900, and 5,000) of PEG conjugated with distearoylphosphatidylethanolamine (DSPE)-modified liposomes on prolonged blood circulation [114]. They reported that longer forms of PEG with molecular weights of 1,900 and 5,000 kDa used to PEGylate liposomes with a size of about 100 nm prolonged their circulation in the blood. Maruyama et al. also reported a similar effect using large unilamellar liposomes with a size about 200 nm [115]. DOX-encapsulated liposomes showed enhanced tumor distribution through enhanced permeability and retention effects [116] and reduced adverse effects of DOX, especially cardiac toxicity [98, 117]. A DOX-encapsulated PEGylated liposome formulation (Doxil®) was approved by the US Food and Drug Administration in 1995. Recently, the development of Doxil® was reviewed by Barenholz et al. [98].

Although PEG modification of liposomes is a promising approach, the immune response against PEGylated liposomes should be considered for their therapeutic use.
Dams et al. reported that repeated injections of PEGylated liposomes significantly altered the pharmacokinetics of subsequently injected PEGylated liposomes [118]. A second dose of injected PEGylated liposomes was rapidly cleared from the blood. They studied the factors affecting the accelerated blood clearance of subsequently injected PEGylated liposomes [118]. Macrophage deletion experiments using clodronate-containing liposomes [120] provided evidence that Kupffer cells and spleen macrophages were involved in the reduced blood circulation. This effect did not depend on liposomal size but rather on lipid dose. In addition, they found that DOX-encapsulated PEGylated liposomes did not induce the enhanced clearance of a second injected “empty” PEGylated liposome. Ishida et al. also systemically investigated the accelerated clearance of subsequent PEGylated liposome administration [121-123] and demonstrated that PEGylated liposomes induced PEG-specific IgM that were responsible for the rapid elimination of second injected PEGylated liposomes [124, 125]. Furthermore, the induction of anti-PEG-specific IgM that recognized DOX-encapsulated PEGylated liposomes was significantly lower than that for “empty” PEGylated liposomes [126]. This observation partly explains the observation by Laveman et al. described above [119]. Such information about the immunological responses induced by PEGylated liposomes is important for the design of glycosylated liposomes.

5. Glycosylated liposomes for cell-selective targeting

Glycosylated liposomes have been studied for cell-selective delivery of drugs. It is important to consider the glycosylation methods used. Thus far, glycosylation of liposomes is achieved using glycoproteins or synthetic glycolipids attached to the liposomal surface. Compared with glycoproteins, it is easy to control synthetic glycolipids for targeting. Because glycosylated liposomes interact with endogenous components in blood, the hydrophobicity of synthetic glycolipids is important for stable incorporation in vivo. In addition, an adequate spacer length in synthetic glycolipids is required to display the ligands for recognition by receptors. Ligand modified PEGylated lipids have been used to display ligands on the surface of PEGylated liposomes because of a steric hindrance effect caused by PEG. However, PEG might be immunogenic. Thus, the design of glycosylated liposomes should be considered in a comprehensive way according to their application to disease. Fig. 2 summarizes the design of glycosylated liposomes by glycosylated lipids.
Recently, there has been increasing attention paid to nucleic acid drugs and their application for refractory diseases. Various glycosylated liposomes have been developed for cell-selective delivery. However, both receptor recognition and release of nucleic acids from endosomes/lysosomes into the cytoplasm after internalization should be considered.

Table 2 summarizes an example of cell-selective targeting with liposomal carrier systems based on glycosylation. In this section, the use of glycosylated liposomes for cell-selective targeting of low-molecular-weight drugs and nucleic acids is reviewed.
5.1. Galactose modification

Similar to galactosylated macromolecular carriers, galactosylated liposomes have been studied as hepatocyte-selective targeting agents via asialoglycoprotein receptor-mediated uptake. To date, glycoproteins or synthetic glycolipids have been used to prepare galactosylated liposomes for targeted delivery.

5.1.1. Galactosylated liposomes

For efficient recognition by hepatocytes via asialoglycoprotein receptors, a rational design of galactosylated liposomes is required. Asialofetuin is a glycoprotein that possesses several tri-antennary galactose-terminated sugar chains, and is taken up by the

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PC: parenchymal cells
NPC: non-parenchymal cells
DC: Dendritic cells
TAM: tumor associated macrophages
CHE: cholesteryl hexadecyl ether
DLPL: dilinoleoylphosphatidylethanolamine
liver after intravenous administration in rats [127]. Tsuchiya et al. developed asialofetuin-modified liposomes for hepatocyte-selective targeting of drugs [128]. Asialofetuin was conjugated with palmitic acid, a hydrophobic anchor that fixes asialofetuin on to the liposomal surface. After intravenous administration, asialofetuin-modified liposomes were rapidly eliminated from the blood, and most were recovered from the liver [129]. Intrahepatic distribution studies revealed that asialofetuin-modified liposomes were selectively found in liver parenchymal cells [130] and Wu et al. reported that asialofetuin-modified liposomes distributed in the liver in combination with intravenously injected vitamin E improved the protective effect against CCl4-induced acute liver injury in mice [130].

Synthetic glycolipids have been designed and studied for cell-selective targeting. Spanjer et al. reported that tri-antennary galactose-terminated cholesterol (tri-gal-chol) modified liposomes were efficiently distributed to the liver [131]. However, an intrahepatic distribution study showed that tri-gal-chol-modified liposomes were taken up by liver non-parenchymal cells (including Kupffer cells) via galactose/fucose recognizing receptors and not by liver parenchymal cells via the asialoglycoprotein receptors in rats. However, a rapid exchange of tri-gal-chol to endogenous components in the blood occurred because of the relatively high hydrophilicity of tri-gal-chol [132]. Sliedregt et al. synthesized a hydrophobic tri-gal-chol by adding one or more fatty acid chains to the glycolipid to increase their stable incorporation in galactosylated liposomes [132]. They found that 5% (mol) hydrophobic tri-gal-chol-modified galactosylated liposomes were taken up by asialoglycoprotein receptors on liver hepatocytes. In contrast, 50% (mol) hydrophobic tri-gal-chol-modified galactosylated liposomes were taken up by the galactose/fucose recognizing receptors on Kupffer cells in the liver. Sasaki and Murahashi et al. synthesized various synthetic galactosyl lipids to prepare galactosylated liposomes [133, 134] and found that galactose density on galactosylated liposomes was a critical factor for hepatocyte-selective targeting rather than the branching structure of the galactosylated lipid [135]. Recently, Garg et al. observed that uptake of galactosylated liposomes with a low galactosyl lipid content by parenchymal cells was significantly higher than for non-parenchymal cells [136]. This corresponded with a report by Sliedregt et al. [132]. These observations demonstrate the importance of the galactose density of galactosylated liposomes as well as the design of synthetic galactosylated lipids for hepatocyte-selective delivery.

For hepatocyte-selective targeting, our group synthesized cholesten-5-yloxy-N-(4-(1-imino-2-D-thiogalactosyl(ethyl)amino)butyl)formamide (Gal-C4-Chol), a galactosylated cholesterol derivative, to prepare galactosylated
liposomes [137]. Introduction of many hydrophilic galactose moieties to a hydrophobic anchor might result in their removal by interaction with endogenous components in the blood. Therefore, one galactose moiety was conjugated to cholesterol via a spacer. After intravenous administration in mice, 5% (mol) Gal-C4-Chol-modified galactosylated liposomes were efficiently distributed to liver parenchymal cells [138]. The high uptake in liver parenchymal cells was observed the 3.5, 5.0, and 7.5% (mol) for Gal-C4-Chol-modified galactosylated liposomes [139]. To determine the effect of lipid composition in targeting, we prepared 5% (mol) Gal-C4-Chol-modified galactosylated liposomes involving different egg phosphatidylcholine or DSPC and cholesterol contents [140, 141]. Liver parenchymal cells selectivity was significantly affected by the lipid composition of galactosylated liposomes, suggesting that the lipid composition of galactosylated liposomes should be considered for cell-selective targeting.

To achieve a targeted and sustained drug delivery by galactosylated liposomes, we prepared polysorbate (Tween) 20 or 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol (PEG(x)-DSPE) where x represents a mean molecular weight of PEG (2,000 or 350 kDa), which was added to galactosylated liposomes [142]. Incorporation of Tween 20 and PEG-DSPE reduced blood elimination and hepatic uptake. Furthermore, the incorporation of PEG350-DSPE into galactosylated liposomes could control delivery to hepatocytes without losing its targeting capabilities. Recently, Wang et al. developed a novel cleavable PEG lipid, PEG2,000-cholesteryl hemisuccinate (PEG2,000-CHEMS), that can be metabolized by esterases in plasma and tissues, for targeted and sustained targeting into hepatocytes [143]. The incorporation of PEG2,000-CHEMS into galactosylated liposomes directed the delivery of encapsulated DOX into hepatocytes without losing its targeting capability. DOX-encapsulated PEGylated and galactosylated liposomes exhibited the highest inhibitory effect in hepatocarcinoma 22-bearing mice. Therefore, a controlled and targeted method is an effective strategy to exert a high pharmacological effect of drugs used for therapy.

Regarding low-molecular-weight drug delivery, studies have reported the delivery of anti-cancer drugs to hepatocellular carcinomas (HCC). Xiao et al. synthesized a tetravalent galactosylated diethylenetriaminepenta-acetic acid-distearoyl phosphatidylethanolamine (4Gal-DTPA-DSPE) to prepare doxorubicin encapsulated galactosylated liposomes [144]. Either 5 or 10% (mol) of 4Gal-DTPA-DSPE was added to lipids to prepare galactosylated liposomes. Galactosylated liposomes had a prolonged blood retention and high liver accumulation after intravenous administration. Study of intrahepatic distribution demonstrated galactosylated liposomes selectively accumulated
in hepatocytes. As an example of applications for drug delivery, vitamin E [130], probucol [140], prostaglandin E\textsubscript{1} [145], DOX [146], retinoic acids [147], ara-C [148], N4-Octadecyl-1-\(\beta\)-D-arabinofuranosylcytosine [148], and stabudine [136, 149] have been used for hepatocyte-selective targeting. Because asialoglycoprotein receptors are expressed on normal hepatocytes, tumor selectivity is required for a rational cancer therapy. Our group developed a novel drug delivery system using enzymatic activity expressed specifically in tumors. It was reported that matrix metalloprotease-2 (MMP-2) plays a critical role in tumor progression, angiogenesis, and metastasis, and are overexpressed in HCC [150-153]. Using the enzymatic activity of MMP-2 could provide HCC-selective properties to galactosylated liposomes. Thus, our group developed a novel functional lipid, cleaved by the protease activity of MMP-2. The amino group of dioleoylphosphatidylethanolamine (DOPE) was conjugated [148] with PEGylated MMP-2 substrate peptide (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln), and MMP-2-cleavable PEG-peptide-DOPE (PEG-PD) was synthesized. PEG-PD incorporated galactosylated liposomes were taken up by human hematoma HepG2 cells in a dose-dependent manner and was dependent upon MMP-2 expression. Hatakeyama et al. also reported that a PEG-PD-modified multifunctional envelope-type nano device for tumor-selective delivery of pDNA and siRNA used the enzymatic degradation of the peptide by MMP-2 [153-155].

5.1.2. Nucleic acid delivery

Our group investigated the hepatocyte-selective delivery of nucleic acids using galactosylated cationic liposomes based on their physicochemical properties. Pharmacokinetic analysis of pDNA complexed with galactosylated PLL demonstrated that physicochemical properties such as charge and size of complexes should be optimized for efficient hepatocyte-selective targeting [32]. Therefore, we investigated the hepatocyte-selective delivery of pDNA using galactosylated cationic liposomes based on their physicochemical properties [156]. When pDNA was complexed with galactosylated cationic liposomes (galactosylated lipoplexes) via electrostatic interactions, a 5% glucose solution enabled the preparation of about 140-nm-sized galactosylated lipoplexes at a relatively high concentration for \textit{in vivo} use. The cationic charge ratio (−:+) of galactosylated lipoplexes was from 1.0:2.3 to 1.0:3.1 and showed high gene expression in the liver. Furthermore, hepatocyte-selective gene expression was observed when lipoplexes were prepared at a charge ratio (−:+) of 1.0:2.3. In addition, galactosylation of the lipoplexes increased the tissue binding and internalization rate via asialoglycoprotein receptor-mediated endocytosis on hepatocytes.
Letrou-Bonneval et al. reported that galactosylated multi-modular lipoplexes prepared at a charge ratio (−:+) of 1.0:2.0 showed asialoglycoprotein receptor-mediated transfection in primary cultured rat hepatocytes [158]. Penetration across the fenestrated sinusoidal endothelium by galactosylated lipoplexes might be limited. In addition, lipoplexes easily aggregate in higher salt concentrations [159]. Therefore, a reduction in size or improved stability of galactosylated lipoplexes in higher salt conditions would be a promising approach to enhance the transfection activity in hepatocytes. To solve this problem, we developed stabilized galactosylated lipoplexes by adding moderate amounts of sodium chloride during lipoplex formation based on our surface charge regulation (SCR) theory [160]. Galactosylated SCR lipoplexes had a mean diameter of 120 nm and had high stability in a high ionic solution (150 mM NaCl). The hepatic transfection activity of galactosylated SCR lipoplexes was about 10- to 20-fold higher than that of galactosylated lipoplexes. The pre-administration of excess galactosylated BSA inhibited hepatic gene expression in galactosylated SCR lipoplexes, suggesting the involvement of asialoglycoprotein receptor-mediated uptake on hepatocytes. Thus, controlling the physicochemical properties of galactosylated lipoplexes is important for the efficient hepatocyte-selective targeting of DNA.

Cationic-charged galactosylated lipoplexes interact with non-target cells or erythrocytes [161] via electrostatic interaction, therefore the use of PEGylated lipids would be a promising strategy to reduce such non-specific interactions. Perouzel et al. synthesized various glycosylated cholesterol derivatives to prepare glycosylated cationic liposomes, adenovirus core peptides, and pDNA ternary complexes [162]. Then, PEG2,000-DSPE were incorporated into glycosylated ternary complexes by pre- or post-modification methods. Although a stabilizing effect was observed by the incorporation of PEG2,000-DSPE (5-10% (mol)) in high salt conditions, transfection into HeLa cells was abolished using the same preparation conditions. Therefore, galactose might be required to conjugate the terminal of PEG2,000 displayed on the liposomal surface for both efficient recognition by asialoglycoprotein receptors and the prevention of complex aggregation in higher salt conditions. As an alternate strategy, the use of degradable PEGylated lipids by either acid [163] or enzyme [148, 153] would be a promising approach. Frisch et al. synthesized a novel galactosylated lipid. To display galactose moieties, a tri-antennary galactose moiety was attached to the distal end of the PEGylated lipids. The positive charge of the galactosylated-PEGylated lipoplex was shielded by the PEG effect to some extent. Chloroquine inhibits endosomal maturation and can enhance the transfection activity of lipoplexes [164]. In the presence of chloroquine, targeted gene transfection was observed in human hepatoma HepG2 cells,
suggesting asialoglycoprotein receptor-mediated uptake. This result is in accord with those reported by our group using galactosylated lipoplexes [165]. These results also suggest that the pharmacokinetic process of endosomal escape is a rate-limiting process for efficient gene expression.

Considering these findings, both biodistribution and intracellular distribution should be controlled for efficient gene expression in hepatocytes. Therefore, a multifunctional carrier system by galactosylation should be developed. The use of a pH buffering effect could be an effective strategy to enhance transfection efficiency by promoting the release of drugs from the endosome to cytoplasm. Our group focused on histidine (His), an amino group with a pKa of 6.0. We synthesized a novel pH-sensitive histidine-modified galactosylated cholesterol derivative (Gal-His-C4-Chol), for efficient transfection to hepatocytes [166]. Galactosylated lipoplexes containing Gal-His-C4-Chol showed much greater transfection activity in HepG2 cells than conventional galactosylated lipoplexes and was dependent upon an asialoglycoprotein receptor-mediated mechanism. Recently, our group also demonstrated that lysine-histidine dendron-modified chitosan could improve the transfection efficiency of chitosan based on pH buffering capacity in HEK293 cells [167]. Recently, Hu et al. developed multifunctional galactosylated and PEGylated lipoplexes for the efficient nuclear delivery of pDNA in mice. In this multifunctional system, galactose, PEG, calcium phosphate, and monocyclic octa-arginine were introduced to control both biodistribution and intracellular distribution processes [168]. A high gene expression was observed in mouse hepatocytes. Therefore, both biodistribution and intracellular distribution should be considered as efficient strategies to obtain high gene expression in hepatocytes.

RNA interference (RNAi) can be induced by double-stranded siRNA, consisting of 21–25 nucleotides, which is incorporated into the RNAi-induced silencing complex and is a guide for cleavage of complementary target messenger RNA (mRNA) in the cytoplasm [169, 170]. An effective delivery system is essential for the application of siRNA as clinical treatment. Our previous study demonstrated that a siRNA (Ubc-13)/galactosylated cationic liposomes complex was efficiently delivered to hepatocytes [171]. Sonoke et al. also reported the hepatocyte-selective delivery of siRNA (firefly luciferase) using siRNA/galactosylated cationic liposomes complexes [172]. Regarding the use of these complexes for clinical use, Jiang et al. prevented liver ischemia reperfusion or concanavalin A-induced hepatitis via the hepatocyte-selective delivery of siRNA using siRNA/galactosylated cationic liposomes complexes [173, 174]. Thus, galactosylated cationic liposomes can deliver siRNA into hepatocytes.
5.2. Mannose modification

Similar to mannosylated macromolecular carriers, mannosylated liposomes have been studied for macrophage- and/or dendritic cell-selective targeting via mannose receptor-mediated uptake in various tissues. To date, mannosylated lipids have been used to prepare mannosylated liposomes for targeted delivery.

5.2.1. Development of mannosylated liposomes

Mannose-containing phospholipids from the cell wall of mycobacteria [175] and synthetic mannosylated lipids [176-191] have been used to prepare mannosylated liposomes for drug delivery or imaging of macrophages and/or dendritic cells. Mannosylated liposomes have been used for the treatment of diseases such as infections, inflammation, and cancer, etc..

For macrophage- and/or dendritic cell-selective targeting, our group synthesized cholesten-5-yloxy-N-(4-((1-imino-2-D-thiomannosylethyl)amino)butyl) formamide (Man-C4-Chol), a galactosylated cholesterol derivative, to prepare mannosylated liposomes [179]. To deliver drugs into macrophage- and/or dendritic cell in various tissues, we investigated various administration routes including intravenous [179, 192-197], intraperitoneal [198-201], and intratracheal [202, 203] administration. In addition, vaccination of peptides by subcutaneous [178] and intraperitoneal [204, 205] routes have been studied. Mannosylated liposomes were efficiently taken up by macrophages and/or dendritic cells in various tissues according to each administration route.

To obtain efficient recognition of complexes via mannose receptors expressed by macrophages, the rational design of mannosylated liposomes is required. Engel et al. synthesized alkylmannoside derivatives that possessed a hydrophobic anchor and hydrophilic head group containing a PEG spacer with increasing length between the hydrophilic head group and hydrophobic anchor moieties [182]. The effect of PEG as a spacer on macrophage uptake was investigated using mannosylated liposomes with alkylmannoside derivatives. Cellular uptake was increased by increasing PEG numbers of alkylmannoside derivatives. Thus, the spacer length of synthetic mannosylated lipids in mannosylated liposomes is important for recognition by mannose receptors on macrophages. Mannose density of mannosylated liposomes is also an important factor in the recognition of mannose receptors. Espuelas et al. synthesized multi-branched mannosylated lipids [186]. The dimannosylated ligands were recognized as efficiently as tetramannosylated lipids by mannose receptors expressed on immature human
dendritic cells. Man-C4-Chol contents in mannosylated liposomes enhanced the uptake of mannosylated liposomes from 2.5 to 7.5% (mol) by mannose receptor-mediated endocytosis in cultured rat alveolar macrophages [202]. This finding is in line with our previous report using mannosylated emulsion in mouse peritoneal macrophages [206]. Chono et al. showed that the uptake of bare liposomes by rat alveolar macrophages was dependent on particle size (100, 200, 400, 1,000, and 2,000 nm) and that mannosylation of liposomes with 4-aminophenyl-α-D-mannopyranoside enhanced uptake [185]. These results suggest the structure of mannosylated lipids, mannose density, and size of mannosylated liposomes should be optimized for efficient recognition by mannose receptors on macrophages and/or dendritic cells.

Examples of drug delivery or imaging applications by encapsulation of muramyl dipeptide [193], stavudine [184], dexamethasone palmitate [202], cytidine 5’ diphosphocholine [187], amphotericin B [189], 64Cu [191], and indocyanine green [206] have been reported. Similarly, encapsulation of various peptides to induce cytotoxic T lymphocytes for vaccination have been investigated [178, 204, 205].

5.2.2. Nucleic acid delivery
pDNA, NF-κB decoy, mRNA, and siRNA have been used for delivery to macrophages and/or dendritic cells. We investigated macrophage- and/or dendritic cell-selective delivery of nucleic acids using mannosylated cationic liposomes based on their physicochemical properties. Because it is not necessary to pass through fenestrae in the liver to reach Kupffer cells and liver endothelial cells, we prepared 200-nm-sized pDNA/mannosylated lipoplexes [192]. After intravenous administration in mice, the highest gene expression was observed in liver non-parenchymal cells including Kupffer cells and liver endothelial cells. A cationic charge ratio (−:+) of mannosylated lipoplexes from 1.0:2.3 to 1.0:3.1 demonstrated high gene expression in the liver [208]. When complexes were formed with higher charge ratios, transfection efficiency in the lung was highest, indicating non-specific interactions. These findings correspond with our previous reports of galactosylated lipoplexes in vivo and in situ [156, 157].

Consideration of the administration route is also important for pDNA delivery. After intravenous administration, many mannosylated lipoplexes were rapidly taken up by the liver [194]. Therefore, the intraperitoneal administration route was selected to avoid rapid uptake by Kupffer cells and achieve sustained gene expression in macrophages and dendritic cells, but not Kupffer cells [198]. Following intraperitoneal administration of mannosylated lipoplexes into mice, high gene expression was observed in macrophages and dendritic cells from peritoneally exuded cells and the spleen. This
supports a report by Ikehara et al. using ovalbumin-encapsulated oligomannose-coated liposomes via intraperitoneal administration [205]. Recently, Li et al. reported that mannosylated liposome/protamine/pDNA complexes exhibited a high transfection activity in bone marrow-derived dendritic cells [209] supporting the view that lipoplex mannosylation is an efficient strategy to obtain high transfection activity in macrophages and/or dendritic cells.

To control endosomal escape, we synthesized a histidine-modified mannosylated cholesterol derivative (Man-His-C4-Chol), for efficient transfection to macrophages [210]. Mannosylated lipoplexes containing Man-His-C4-Chol functioned similarly to galactosylated lipoplexes containing Gal-His-C4-Chol, and showed a higher transfection activity than conventional mannosylated lipoplexes, via a mannose receptor-mediated mechanism both in vivo and in vitro. These results are consistent with those recently reported by Perche et al. [188]. Therefore, both biodistribution and intracellular distribution could be useful strategies to obtain high gene expression in macrophages and/or dendritic cells.

The use of nucleic acids, DNA or mRNA vaccination for cytotoxic T lymphocyte induction [188, 199, 200, 211], CpG DND for cancer immunotherapy [196, 201], and NF-κB decoy delivery for anti-inflammatory therapy [H195, 203] has been reported.

### 5.2.3. Combination of bubble formulation with ultrasound exposure

Fig. 3 summarizes the combination method of glycosylated liposomes with physical stimuli for targeted drug delivery. Microbubbles can be destroyed by ultrasound exposure to generate microstreams or microjets. Consequently, transient holes are generated in cellular membranes. Therefore, the combined use of ultrasound exposure and microbubble formulation used as ultrasound contrast agents can enhance the transfection efficacy of naked pDNA by facilitating pDNA entry into the cells [212]. Suzuki et al. prepared ultrasound imaging gas-encapsulated liposomes (bubble liposomes) for naked pDNA mediated transfection in mice [213]. The bubble liposomes had a small size (about 446 nm) compared with conventional microbubble formulation [212]. We developed mannosylated bubble liposome/pDNA complexes (mannosylated bubble lipoplexes) for efficient cell-selective gene transfection with ultrasound exposure [214-217]. This system could be used for delivery of oligonucleotides such as siRNA or NF-κB decoy delivery into liver NPC [218] or tumor-associated macrophages [219]. Because cationic-charged bubble lipoplexes interact with erythrocytes, we developed ultrasound-responsive anionic-charged bubble lipopolyplexes as a platform of
glycosylation for safe transfection [220]. Therefore, optimization of physicochemical properties of mannosylated bubble formulations might be used for safe and efficient gene transfection into macrophages and/or dendritic cells. Another glycosylated bubble formulation, sialyl LewisX-modified microbubbles were previously developed for molecular imaging applications [221, 222].

5.3. Fucose modification

Fucosylated liposomes have been studied as carriers for Kupffer cell or pancreatic cancer cell-selective delivery. Fucose rectin is found exclusively in hepatic Kupffer cells [223]. We synthesized cholesten-5-yloxy-N-(4-((1-imino-2-D-thiofucosylethyl)amino)butyl)formamide, a fucosylated cholesterol derivative, to prepare the fucosylated liposomes [179]. Intravenously injected fucosylated liposomes were rapidly taken up by the liver and were recovered from liver NPC. An in vitro uptake study using primary cultured rat sinusoidal endothelial cells and Kupffer cells demonstrated that fucosylated BSA was taken up by both sinusoidal cells and Kupffer cells [224]. The uptake of fucosylated BSA was significantly inhibited in the presence of excess mannosylated BSA and fucosylated BSA. In addition, fucosylated BSA was more Kupffer cell selective than mannosylated BSA. Thus, fucosylated liposomes might be more selective as Kupffer cell-targeting carriers.

Based on these findings, we investigated the use of fucosylated liposomes for NF-κB decoy delivery into Kupffer cells for the treatment of acute hepatitis [225]. NF-κB decoy was mainly recovered from liver NPC following the intravenous administration of fucosylated cationic liposomes/NF-κB decoy complexes (fucosylated complexes). In an acute hepatitis model, proinflammatory cytokines, alanine transaminase, and aspartate transaminase serum levels in LPS-infected mice were significantly attenuated by treatment with fucosylated complexes.
Recently, Yoshida et al. reported the pancreatic cancer cell-selective delivery of cisplatin using fucosylated liposomes by simultaneous injection of excess D-mannose (5 mg) to inhibit the hepatic uptake in tumor-bearing mice [226]. Under these experimental conditions, fucosylated liposomes selectively accumulated in pancreatic cancer cells and efficiently inhibited tumor growth as well as prolonging survival of tumor-bearing mice.

5.4. M6P modification

M6P/IGF II receptors are expressed on hepatic stellate cells [80-82] and M6P-modified liposomes have been studied as carriers for hepatic stellate cell-selective delivery. Kamps et al. conjugated M6P-HSA to the surface of liposomes for hepatic stellate cell-selective targeting [227-229]. In bile duct-ligated rats, M6P-HSA-modified liposomes selectively accumulated in hepatic stellate cells. Greupink et al. reported that M6P-HSA-DOX-conjugated modified liposomes inhibited hepatic stellate cell proliferation in bile duct-ligated rats. Similarly, Adrian et al. reported the use of M6P-HSA-modified liposomes or hemagglutinating virus of Japan liposomes for hepatic stellate cell-selective targeting [230, 231]. Therefore, M6P-modified liposomes might be an efficient carrier for hepatic stellate cell-selective delivery of drugs.
6. Future Perspectives

Although this review focused on glycosylated macromolecules and liposomes, glycosylated polymeric micelles would be a promising approach for targeted drug and gene delivery [232-235]. Kataoka et al. have reviewed the recent progress of the functional polymeric micelles for drug and gene delivery [236, 237].

For targeted drug delivery by glycosylation, both the glycosylation method and physicochemical properties should be optimized to achieve efficient cell-selective targeting by macromolecular and liposomal carriers. For nucleic acid delivery, the control of intracellular delivery is also required because of nucleic acid degradation by lysosomal enzymes. Therefore, introduction of functional groups that facilitate endosomal/lysosomal escape might be an effective strategy to improve transfection activity. Hossain and Akaike et al. reported that carbonate apatite/siRNA complex can facilitate the intracellular delivery of siRNA [238]; therefore the development of such a new carrier for glycosylation is important. To exploit the shielding effects of PEG, the use of biodegradable PEG cleaved by enzymes or low pH might provide a new targeting system. Moreover, the combined use of physical stimuli such as ultrasound exposure could enable enhanced targeting efficacy by glycosylated liposomes [239]. Because the use of ultrasound exposure is a promising approach, further optimization of the physicochemical properties and/or development of glycosylated lipids for glycosylated bubble formulations are required for more efficient and safe drug delivery systems.
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