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The Insertion Timing of PEGylated Lipids to Galactosylated Lipoplexes Is Important for Liver-Selective Transfection in Mice

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Abstract: In the present study, we demonstrated the importance of PEGylation timing of galactosylated liposome/plasmid DNA (pDNA) complexes (lipoplexes) for liver-selective transfection in mice. Because a fenestrated endothelium can be a barrier for penetration of lipoplexes though sinusoids, the particle size of lipoplexes is one of the determining factors for *in vivo* liver parenchymal cell (hepatocyte, PC)-selective transfection. Here, we found that syn-insertion, as a novel PEGylation timing, was useful to control the particle size of galactosylated lipoplexes. Syn-insertion of PEGylated lipids was performed by simple mixing of pDNA solution containing PEGylated lipids and dispersion of the cationic liposomes. Both syn- and pre-insertion of PEGylated lipids decreased the particle size of lipoplexes, whereas post-insertion did not. Moreover, syn-insertion of PEGylated lipids to galactosylated lipoplexes improved liver selectivity and the PC/non-parenchymal cell ratio of transgene expression after intravenous injection in mice. Hence, these data will be valuable for the design and preparation of PEGylated lipoplexes for gene targeting.

Keywords: Gene Therapy, PEGylation, Galactosylated Liposomes, Targeting, Hepatocyte

1. Introduction

Gene delivery to the liver has a great therapeutic potential against intractable and refractory diseases such as hepatocellular carcinoma and familial hypercholesterolemia [1, 2]. Both viral and non-viral vectors targeted to the liver have been developed worldwide [3-9]. The transduction efficiency of viral vectors is generally high. However, the toxicities of viral vectors are problematic [10, 11]. In contrast, non-viral vectors have several advantages including low immunogenicity, easy handling, and low cost compared with viral vectors. Thus, the development of effective and safe non-viral vectors is promising for clinical use.

Liver parenchymal cells (hepatocytes, PCs) are a major target, because PCs perform various biological functions such as secretion of proteins (e.g., albumin.), metabolism, and excretion. To deliver foreign genes to liver PCs, several factors must be considered, including the administration route, blood circulation, stability of the vectors in blood, penetration though a fenestrated endothelium, selection of ligands, and uptake by hepatocytes [12, 13]. In terms of the delivery routes for liver-targeted gene transfer, topical application has been reported, such as direct injection to the liver [14] and injection via the portal vein [6, 7]. However, topical administration to the liver requires surgery. Alternatively, an intravenous route is attractive. Naked

plasmid DNA (pDNA), a simple and safe non-viral vector, is generally inactive because of degradation by nucleases and rapid elimination by the reticuloendothelial system [15]. Hydrodynamic injection, rapid injection of a large volume of pDNA solution, is a highly effective method for liver-directed gene transfer [16, 17]. However, in hydrodynamic injection, the volume of injection is similar to the blood volume. Consequently, it is hardly applicable to humans without devisal such as topical application. Carrier systems are also required for gene transfer targeted to liver PCs. In particular, sugar modification of carriers is an effective approach for targeting [12, 13]. Unfortunately, the transfection efficiency after intravenous injection of galactosylated lipoplexes is inferior to that after intraportal injection [6]. This property might be mainly due to trapping of lipoplexes by the lung. In such a case, polyethylene glycol modification (PEGylation) is useful to prevent lung accumulation and improve retention in systemic circulation. In general, however, PEGylation can also decrease uptake by target cells. This phenomenon is known as the 'PEG dilemma' [18].

To overcome the PEG dilemma, the use of pH-responsive PEGylated lipids [19], enzyme-cleavable PEGylated lipids [20], and PEGylated lipids with short acyl chains [21] have been reported. As modification methods for PEGylation of lipoplexes, pre-insertion and post-insertion of PEGylated lipids have been reported [22, 23]. In pre-insertion, PEGylated lipids are inserted into the lipid bilayer of liposomes prior to lipoplex formation, whereas post-insertion incorporates PEGylated lipids into already-formed lipoplexes. Thus, post-insertion involves two steps to prepare lipoplexes, i.e. lipoplex formation and insertion of PEGylated lipids. In contrast, post-insertion has an advantage in transfection efficiency compared with pre-insertion [21, 24].

In this study, to achieve hepatocyte-selective gene transfer via an intravenous route, we selected PEGylated lipids with a short acyl chain for PEGylation of galactosylated lipoplexes. Importantly, we demonstrated that the PEGylation timing plays a crucial role in determining liver selectivity of the transgene expression of PEGylated galactosylated lipoplexes. To improve both the formulation step and transfection efficiency, we developed a novel insertion timing, 'syn-insertion', in which PEGylated lipids were inserted into lipoplexes during lipoplex formation.

2. Materials and Methods

2.1. Materials

Sodium pentobarbital was purchased from Kyoritsuseiyaku Corporation (Ibaraki, Japan). 1,2-Dioleoyl-3-trimethylammonium-propane methyl sulfate salt (DOTAP), N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (mPEG-Cer8), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), chloroform, and cholesterol (Chol) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All chemicals were of the highest purity available.

2.2. Experimental Animals

Male ddY mice (5 weeks old, 24.7–30.4 g) were purchased from Kyudo Co., Ltd. (Kumamoto, Japan). They were housed in a cage in an air-conditioned room and maintained on a standard laboratory diet (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

2.3. Preparation of pDNA

The pCMV-luciferase vector encoding firefly luciferase under the control of the cytomegalovirus promoter was constructed previously [25]. pDNA was amplified in Escherichia coli strain DH5α, isolated, and purified using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA dissolved in a 5% glucose solution was stored at –20°C prior to experiments. Fluorescein (FL) or tetramethyl-rhodamine (TMR) labeling of pDNA was performed using a Label IT Nucleic Acid Labeling Kit (Mirus Co., Madison, WI, USA).

2.4. Synthesis of the Galactosylated Cholesterol Derivative

The galactosylated Chol derivative (Gal-C4-Chol) was synthesized as reported previously [6, 7]. Briefly, cholesteryl chloroformate and N-(4-aminobutyl)carbamic acid tert-butyl ester were reacted in chloroform for 24 h at room temperature. A solution of trifluoroacetic acid and chloroform was added dropwise, and then the mixture was stirred for 4 h at 4°C. The solvent was evaporated to obtain N-(4-aminobutyl)-(cholesten-5-yloxyl)formamide that was then combined with 2-imino-2-methoxyethyl-1-thiogalactoside [26], followed by stirring for 24 h at 37°C. After evaporation, the resultant material was suspended in water, dialyzed against distilled water for 48 h (12 kDa cut-off dialysis tubing), and then lyophilized.

2.5. Preparation of Cationic Liposomes

Cationic liposomes were prepared as reported previously [6, 7]. For DOTAP/Chol liposomes, DOTAP and Chol were dissolved in chloroform at a molar ratio of 1:1, vacuum desiccated, and then resuspended in a sterile 5% glucose solution at 8 mg/ml to form liposomes. For galactosylated liposomes, the molar ratio of DOTAP, Chol and Gal-C4-Chol was 2:1:1. To form small unilamellar vesicles, liposomes were extruded through polycarbonate membrane filters (100- and 50-nm pore sizes, 11 times for each pore sizes) using a commercially available instrument (Mini-Extruder, Avanti

Polar Lipids, Inc.).

2.6. Preparation of PEGylated Lipoplexes

To form lipoplexes without PEGylation, 150 μl of 600 μg/ml pDNA in a 5% glucose solution was mixed with an equal volume of cationic liposomes and then incubated for 30 min at 60°C, except in experiments measuring temperature dependency. The charge ratio, which is the molar ratio of cationic lipids to pDNA phosphate residues, was 2.3. The charge ratio of unity was 3.52 and 2.70 μg of total lipid/μg of pDNA for DOTAP/Chol and DOTAP/Chol/Gal-C4-Chol liposomes, respectively. For pre-insertion of PEGylated lipids, the liposomes were incubated with an appropriate concentration of mPEG-Cer8 for 30 min prior to lipoplex formation. For syn-insertion, mPEG-Cer8 was added to the pDNA solution prior to forming lipoplexes. For post-insertion, lipoplexes without PEGylated lipids were incubated with mPEG-Cer8 for 30 min. The particle size and ζ-potential of lipoplexes at 25°C were measured using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK).

2.7. In Vivo Gene Transfer

Lipoplexes (90 μg pDNA in 300 μl) were injected via the tail vein into mice. Six hours after administration, the mice were sacrificed under anesthesia (sodium pentobarbital, 40–60 mg/kg, intraperitoneal injection). Subsequently, the liver and lungs were removed with surgical scissors. The tissues were washed twice with saline and homogenized in lysis buffer (0.1 M Tris/HCl, pH 7.8, 0.05% Triton X-100, and 2 mM EDTA) [6, 7, 25]. The volume of the lysis buffer added was 4 μl/mg of tissue. The homogenates were centrifuged at $15{,}000 \times g$ for 5 min. Each supernatant was then assayed for luciferase activity. To evaluate intrahepatic localization of transgene expression, the luciferase activities in PCs and non-parenchymal cells (NPCs) were independently determined after centrifugal separation of PCs and NPCs in collagenase-digested liver as described previously [6].

2.8. Luciferase Assay

Twenty microliters of tissue homogenate supernatant was mixed with 100 μl luciferase assay substrates (PicaGene®, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan), The light produced was immediately measured using a luminometer (Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany). Luciferase activity is indicated as relative light units (RLU)/g of tissue.

2.9. Evaluation of Hemagglutination

Lipoplexes were incubated with a murine erythrocyte suspension in serum (mimic of blood, erythrocytes:serum = 2:3) for 5 min at 37°C, and then observed under a phase-contrast microscope (Leica DM IL LED, Leica Microsystems GmbH, Wetzlar, Germany). The camera for image acquisition was an EC3 (Leica Microsystems GmbH). The acquisition software was Leica Application Suite EZ

(Leica Microsystems GmbH).

2.10. Fluorescent Resonance Energy Transfer (FRET) Analysis

We have demonstrated that FRET analysis is useful to evaluate the mechanism of lipoplex formation [7]. To evaluate membrane fusion during lipoplex formation, liposomes were double labeled with two types of fluorescent lipids, NBD-DOPE (1 mol%) and Rh-DOPE (1 mol%). To evaluate potential incorporation of several molecules of pDNA into one lipoplex particle, FL-pDNA and TMR-pDNA were mixed at a ratio of 1:4 prior to lipoplex formation. The fluorescent intensity spectra were measured using a spectrofluorophotometer (RF5300pc; Shimadzu Co., Kyoto, Japan). The excitation wavelengths for NBD and FL were set at 460 and 480 nm, respectively. Fusion of the lipid membrane in lipoplexes was measured by FRET reduction caused by lipid mixing between NBD-DOPE/Rh-DOPE double-labeled liposomes and unlabeled liposomes during lipoplex formation. Incorporation of several molecules of pDNA into one lipoplex particle was measured by FRET induction from FL-pDNA to TMR-pDNA incorporated in the same lipoplex particle.

2.11. Statistical Analysis

Statistical comparisons were performed by Student's t test for two groups or by Dunnett's test for multiple comparisons with a control group.

3. Results

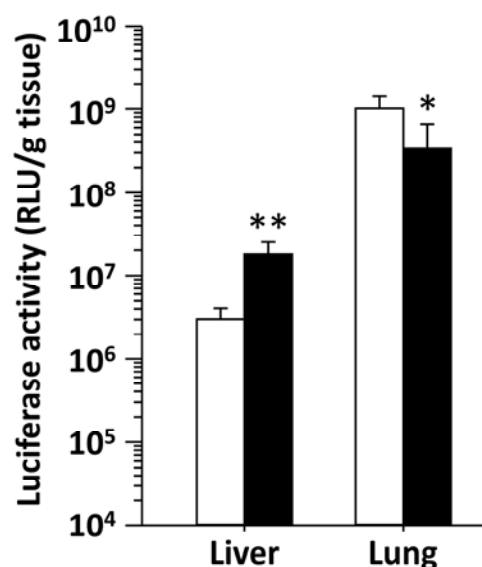## 3.1. Liver Selectivity of Lipoplexes Without PEGylation

Fig. 1. *Effect of galactosylation of lipoplexes without PEGylation on transgene expression in the liver and lung after intravenous injection in mice. Open bars: DOTAP/Chol liposomes; closed bars: DOTAP/Chol/Gal-C4-Chol liposomes. Each bar represents the mean + S.D. of four experiments. * P < 0.05, ** P < 0.01 vs. DOTAP/Chol group.*

To confirm superior liver selectivity of galactosylated

lipoplexes, we compared transgene expression of DOTAP/Chol and DOTAP/Chol/Gal-C4-Chol lipoplexes (Fig. 1). Transgene expression in the lung was much higher than that in the liver for both lipoplexes, especially the DOTAP/Chol lipoplex. Galactosylation of lipoplexes significantly increased transgene expression in the liver, but decreased that in the lung. The ratio of transgene expression in the liver to that in the lung (liver/lung ratio) of DOTAP/Chol/Gal-C4-Chol lipoplexes was 0.110 ± 0.096, which was 37-fold higher than that of DOTAP/Chol lipoplexes (0.003 ± 0.001). Thus, DOTAP/Chol/Gal-C4-Chol lipoplexes were selected for the following experiments to evaluate the effects of insertion timing of PEGylated lipids to lipoplexes.

3.2. Effect of PEGylation Timing on Physicochemical Characteristics of Lipoplexes

Figure 2 shows the effects of insertion timing of PEGylated lipids on the particle size and ζ-potential of lipoplexes. Pre-insertion and syn-insertion of mPEG-Cer8 to lipoplexes significantly decreased the particle size, whereas post-insertion did not. The ζ-potential of lipoplexes was decreased by PEGylation, indicating that PEG moieties had sufficiently covered the surface of lipoplexes.

To elucidate the mechanism of the reduction in particle size by PEGylation of lipoplexes, we assessed lipid membrane fusion during lipoplex formation (Fig. 3a and b) and potential incorporation of several molecules of pDNA into one lipoplex particle by FRET analysis (Fig. 3c and d). For membrane fusion, FRET reduction was analyzed by the increase in the ratio of NBD-DOPE fluorescence to Rh-DOPE fluorescence. The ratio of NBD-DOPE fluorescence to Rh-DOPE fluorescence in free liposomes was 0.139 ± 0.0002 (n =3). Extensive FRET reductions were detected in all lipoplexes (Fig. 3b). PEGylation inhibited FRET reduction, especially in syn-insertion, indicating that the PEG moieties had prevented membrane fusion. Next,

incorporation of several molecules of pDNA into one lipoplex particle was assessed by FRET induction from FL-pDNA to TMR-pDNA (Fig. 3c and d). The ratio of TMR-pDNA fluorescence to FL-pDNA fluorescence in lipoplexes (>0.53) was slightly higher than that in free pDNA (0.467 ± 0.0005, n = 3) (Fig. 3d). This result indicated that several molecules of pDNA had occasionally incorporated into the same lipoplex particle. The ratios in pre- and syn-insertion lipoplexes were significantly lower than that in lipoplexes without PEGylation, whereas post-insertion did not change the ratio. Thus, the presence of PEGylated lipids during lipoplex formation suppressed the incorporation of several molecules of pDNA into one lipoplex particle.

The absolute values of the ζ-potential of PEGylated lipoplexes were still high. Therefore, we evaluated the potential hemagglutination caused by positively charged lipoplexes (Fig. 4). PEGylation of lipoplexes prevented hemagglutination. This result also suggests that PEGylated lipids were successfully inserted into lipoplexes. From the viewpoints of physicochemical characteristics, pre- and syn-insertion of PEGylated lipids are preferable for hepatocyte-selective gene transfer.

3.3. Liver-Selective Transgene Expression of Syn-insertion Lipoplexes

Figure 5 shows transgene expression in the liver and lung after intravenous injection of lipoplexes in mice. Using pre-insertion, transgene expression in the liver and lung was extremely low compared with lipoplexes without PEGylation (Fig. 5a). Syn- and post-insertion of PEGylated lipids maintained transgene expression in the liver, while decreasing that in the lung compared with lipoplexes without PEGylation. The liver/lung ratio of the syn-insertion group was more than the unity, which was 20-fold higher than that of lipoplexes without PEGylation (Fig. 5b). The liver/lung ratio of post-insertion was less than the unity. Thus, syn-insertion was the best timing in terms of liver-selective gene transfer.
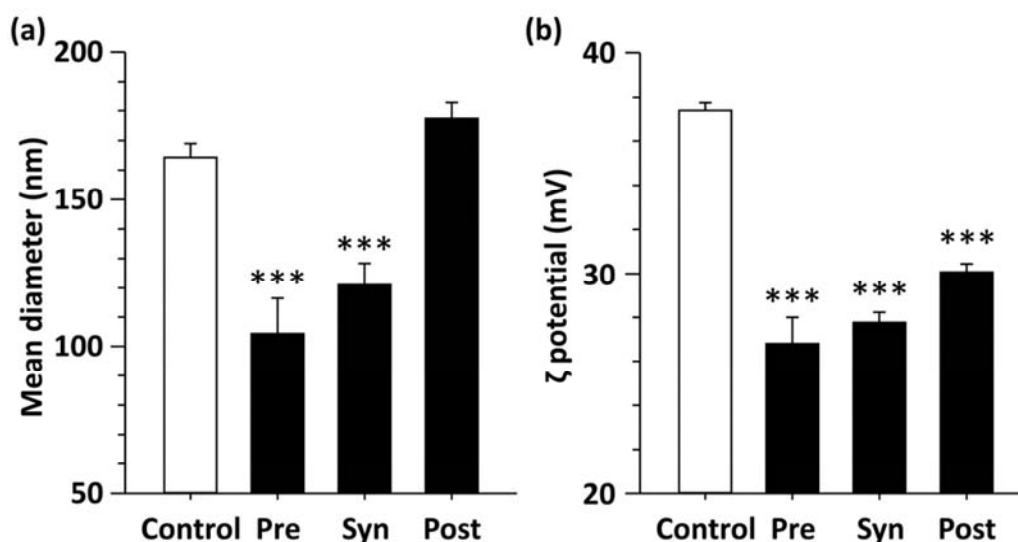

*Fig. 2. Effect of insertion timing of PEGylated lipids on the physicochemical characteristics of lipoplexes. (a) Particle size. (b) ζ-potential. The concentration of PEGylated lipids was set to 20 mol%. Each value represents the mean + S.D. of three experiments. *** $P < 0.001$ vs. control group (without PEGylation).*
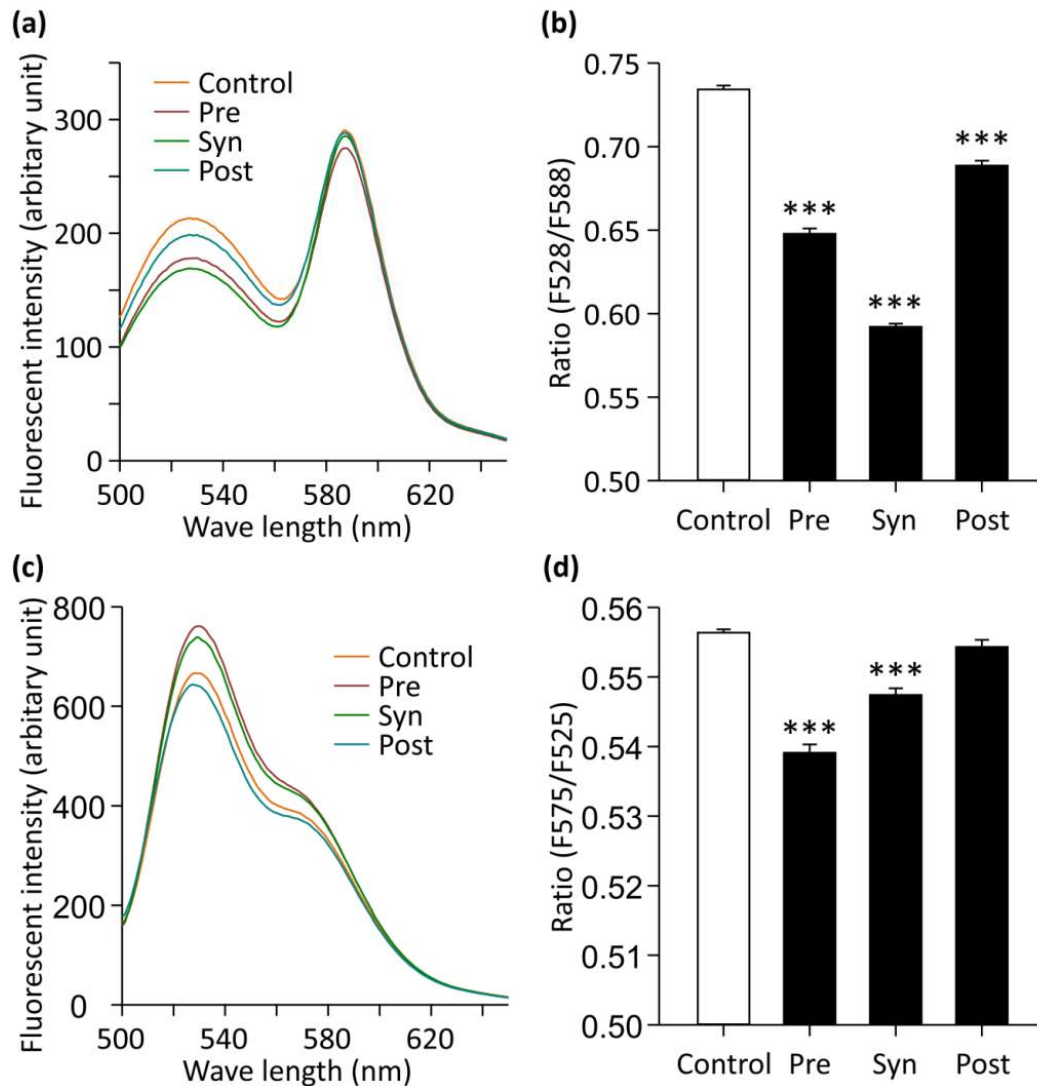
Fig. 3. *FRET analysis of membrane fusion among liposomes during lipoplex formation (a, b) and incorporation of several molecules of pDNA into one lipoplex particle (c, d). (a, c) Mean emission spectra. Fluorescent intensity spectra were measured at excitation wavelengths of 460 nm (a) and 480 nm (c). (b) Ratio of the fluorescent intensity of NBD (F528) to Rh (F588), indicating membrane fusion. NBD and Rh double-labeled liposomes were mixed with unlabeled liposomes at a ratio of 1:4 prior to lipoplex formation. (d) Ratio of the fluorescent intensity of TMR (F575) to FL (F525), indicating the presence of several molecules of pDNA in one lipoplex particle. FL-pDNA and TMR-pDNA were mixed at a ratio of 1:4 prior to lipoplex formation. The concentration of PEGylated lipids was set to 20 mol%. Each bar represents the mean + S.D. of three experiments. *** P < 0.001 vs. control group (without PEGylation).*
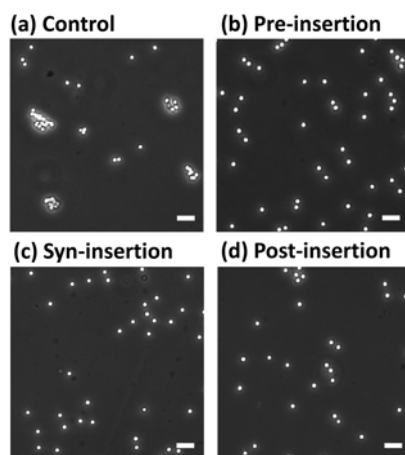

Fig. 4. *Assessment of hemagglutination induced by lipoplexes. The concentration of PEGylated lipids was set to 20 mol%. Scale bar: 20 μm.*

3.4. Effect of the Concentration of PEGylated Lipids in Syn-insertion

To decrease the particle size of lipoplexes, 5 mol% of PEGylated lipids was sufficient for syn-insertion (Fig. 6a). Increasing the concentration of PEGylated lipids in syn-insertion to more than 5 mol% did not affect the particle size. To decrease the ζ-potential of lipoplexes, PEGylated lipids of more than 10 mol% were required (Fig. 6b). PEGylation of lipoplexes tended to decrease transgene expression in the lung (Fig. 7a). In contrast, the decrease of transgene expression in the liver was slight. As a result, increasing the concentration of PEGylated lipids in syn-insertion improved the liver/lung ratio of transgene expression (Fig. 7b).

3.5. Tissue Distribution of Transgene Expression

To compare the profiles of tissue distribution, we examined transgene expression in other tissues (Fig. 8). Without PEGylation, transgene expression in the spleen and heart was comparable to that in the liver (Fig. 8a). In contrast, syn-insertion improved liver selectivity not only against the lung but also the spleen and heart (Fig 8b). Transgene expression in the kidney was low using both types of lipoplexes.

3.6. Effect of Temperature During PEGylated Lipoplex Formation

Figures 9 and 10 show the effects of temperature during PEGylated lipoplex formation on the physicochemical characteristics and transgene expression. To reduce the particle size, 37°C or higher during lipoplex formation was necessary (Fig. 9a). In contrast, PEGylation of lipoplexes reduced the ζ-potential regardless of the temperature during lipoplex formation (Fig.9b). Decreasing the temperature significantly affected transgene expression in the lung, while that in the liver did not change (Fig. 10).
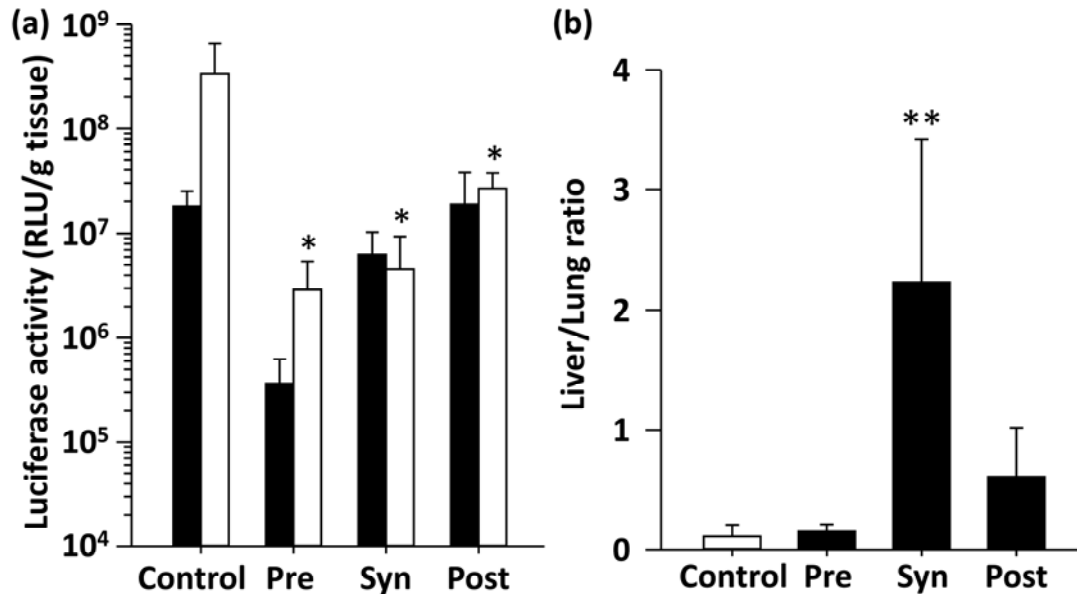

Fig. 5. *Effect of insertion timing of PEGylated lipids on transgene expression in the liver and lung after intravenous injection in mice. The concentration of PEGylated lipids was set to 20 mol%. (a) Transgene expression in the liver (closed bars) and lung (open bars). (b) Ratio of transgene expression in the liver to that in the lung after intravenous injection of lipoplexes. Each bar represents the mean + S.D. of four experiments. * P < 0.05, ** P < 0.01 vs. control group (without PEGylation).*
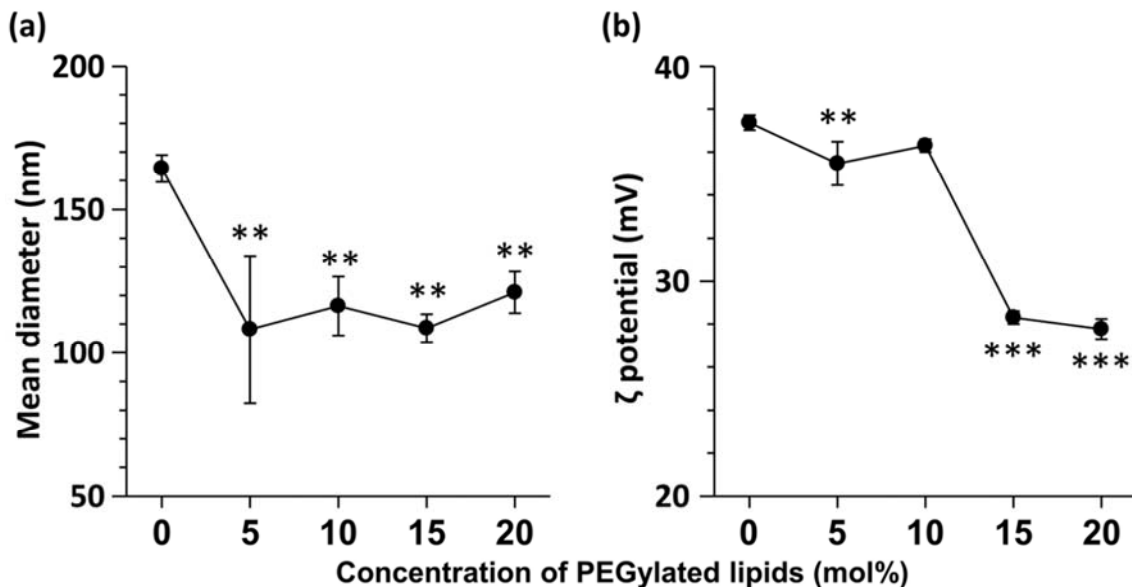

Fig. 6. *Effect of the concentration of PEGylated lipids on the physicochemical characteristics of syn-insertion lipoplexes. Each value represents the mean ± S.D. of three experiments. ** P < 0.01, *** P < 0.001 vs. 0 mol% group.*
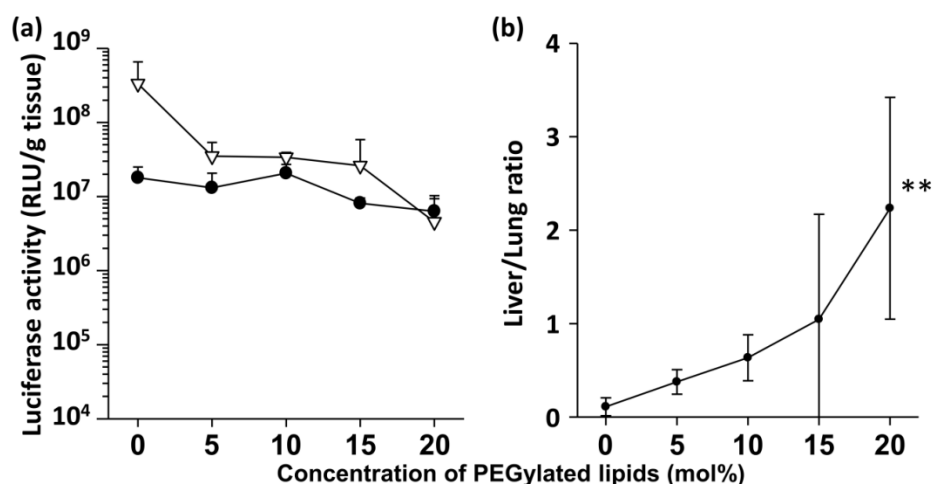
Fig. 7. *Effect of the concentration of PEGylated lipids on transgene expression after intravenous injection of syn-insertion lipoplexes in mice. (a) Transgene expression in the liver (closed circles) and lung (open inverted triangles). Each value represents the mean + S.D. of four experiments. There were no significant differences between transgene expression in the liver and lung. (b) Ratio of transgene expression in the liver to that in the lung. Each value represents the mean ± S.D. of four experiments. ** P < 0.01 vs. 0 mol% group.*
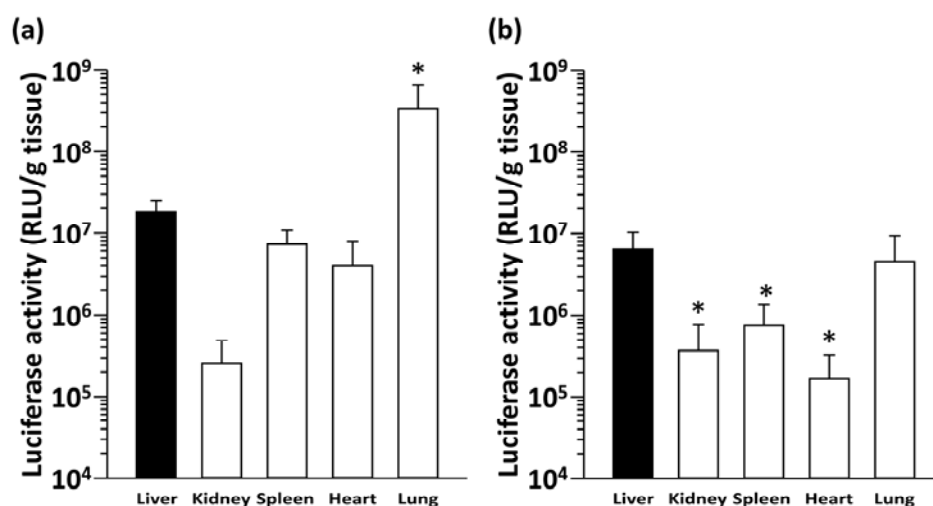

Fig. 8. *Tissue distribution of transgene expression. (a) Without PEGylation. (b) Syn-insertion (20 mol% PEGylated lipids). Each bar represents the mean + S.D. of four experiments. * P < 0.05 vs. liver.*
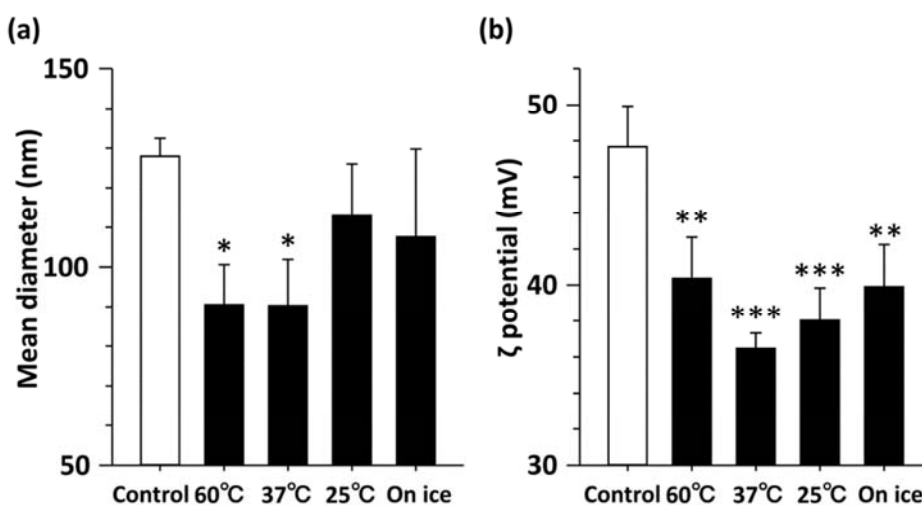

Fig. 9. *Effect of temperature during syn-insertion lipoplex formation on the physicochemical characteristics. (a) Particle size. (b) ζ-potential. The concentration of PEGylated lipids was set to 20 mol%. Each value represents the mean ± S.D. of three experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control group (without PEGylation).*
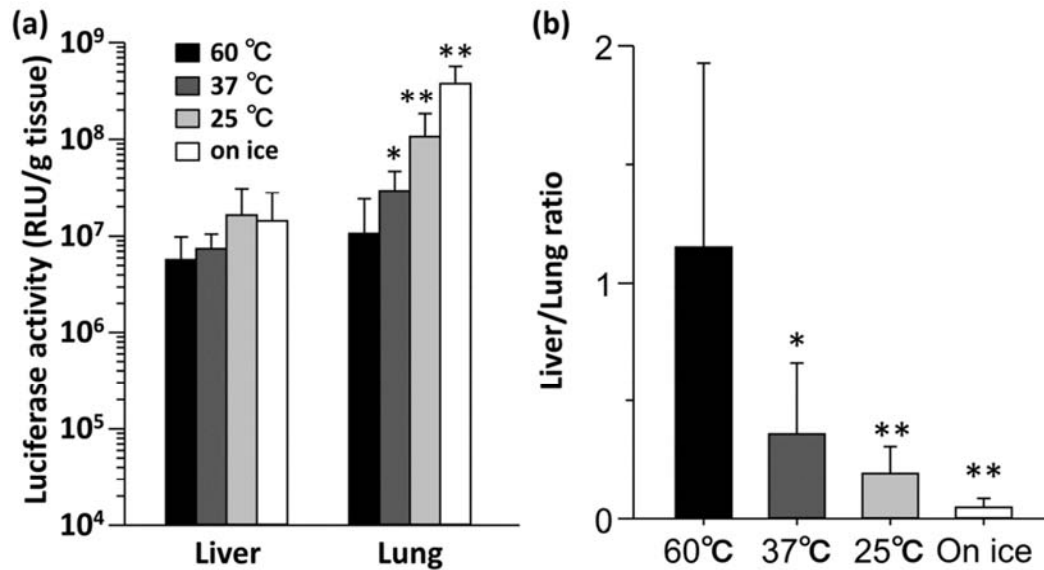
Fig. 10. *Effect of temperature during syn-insertion lipoplex formation on transgene expression in the liver and lung of mice. (a) Transgene expression in the liver and lung. (b) Ratio of transgene expression in the liver to that in the lung. The concentration of PEGylated lipids was set to 20 mol%. Each bar represents the mean + S.D. of five experiments. * P < 0.05, ** P < 0.01 vs. 60°C group.*
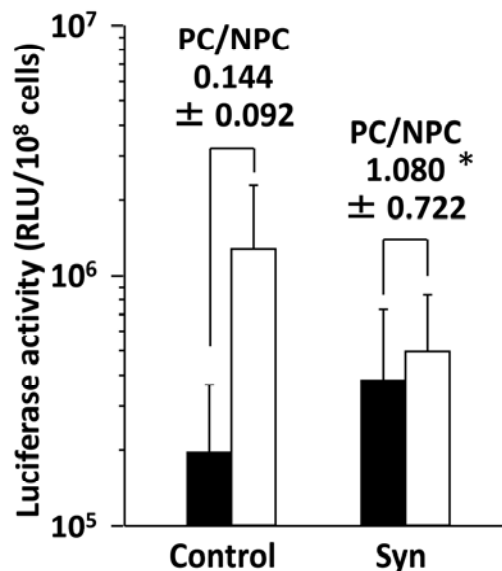

Fig. 11. *Intrahepatic distribution of transgene expression after intravenous injection of syn-insertion lipoplexes in mice. Closed bars: PCs (liver parenchymal cells), open bars: NPCs (liver non-parenchymal cells). The concentration of PEGylated lipids was set to 20 mol%. Each bar represents the mean + S.D. of five experiments. * P < 0.05 vs control group (without PEGylation).*

3.7. PC Selectivity of Transgene Expression

The purpose of the present study was to improve hepatocyte-selective gene transfer. Therefore, we assessed transgene expression in PCs and NPCs (Fig. 11). Transgene expression of syn-insertion lipoplexes in PCs was slightly higher than that of lipoplexes without PEGylation, while transgene expression in NPCs was decreased slightly. In addition, the PC/NPC ratio of syn-insertion lipoplexes was significantly higher than that of lipoplexes without PEGylation.

4. Discussion

In vivo transfection efficiency of non-viral vectors is often inferior to the *in vitro* transfection efficiency. Specific *in vivo* factors such as interactions with blood components and biodistribution restrict *in vivo* transfection. Thus, to deliver foreign genes to liver PCs, rational design is required for the development of non-viral vectors. Rational design of lipoplexes includes prevention of hemagglutination, escape from the lung, the particle size, and targeting ligands. In the present study, we designed small PEGylated galactosylated lipoplexes for liver PC targeting. The most important aspect was the insertion timing of PEGylated lipids to lipoplexes. We developed syn-insertion as a novel timing. The particle sizes of syn- and pre-insertion lipoplexes were small (Fig. 2). Transfection efficiency of syn-insertion lipoplexes was similar to that of post-insertion lipoplexes (Fig. 5a). Syn-insertion also improved liver selectivity of transgene expression (Fig. 5b). Furthermore, syn-insertion improved liver selectivity against other tissues and PC selectivity of transgene expression (Figs. 8 and 11). Thus, syn-insertion may have a good balance of physicochemical characteristics and *in vivo* transfection efficiency.

PEGylation of lipoplexes is a useful strategy to inhibit interactions with blood components. The first event after intravenous injection of lipoplexes is interaction with blood components, followed by trapping in the lung. Both blood cells and plasma components such as lipoproteins are thought to be limiting factors of *in vivo* transfection [27-29]. In particular, the cationic nature of lipoplexes induces hemagglutination [28]. Such large aggregates are easily trapped in lung capillaries. Our previous study indicates that the presence of large amounts of proteins such as albumin prevents hemagglutination [30]. The surface charge of

lipoplexes is inverted from positive to negative by interacting with serum. However, negatively charged lipoplexes covered with serum proteins can still be trapped by lung capillaries, because fibronectin in the blood can mediate interactions between cell surface integrins and lipoplexes [30]. Thus, inhibiting interactions with blood components is necessary to prevent pulmonary transfection. In this study, we selected mPEG-cer8 with a short acyl chain as a detachable PEGylated lipid [21, 24, 31]. Detachable PEGylated lipids can overcome the PEG dilemma in which PEG moieties on the surface of lipoplexes inhibit all interactions including those with target cells. Because a PEG moiety prevents endosomal escape [32] and cellular uptake [33], detachable PEGylated lipids are a rational choice. We showed that mPEG-cer8 inhibited hemagglutination (Fig. 4), which is similar to mPEG-DOPE [28]. This property of mPEG-cer8 is important to prevent the first-pass effect through lung capillaries. Hepatic transgene expression was maintained by syn-insertion of mPEG-cer8 to lipoplexes, while pulmonary transgene expression was decreased (Fig. 5), supporting the hypothesis that mPEG-cer8 detaches from lipoplexes after the first-pass through lung capillaries. Detachment of mPEG-DOPE from lipoplexes had hardly occurred because transgene expression in the liver and lung were negligible when using syn-insertion lipoplexes containing mPEG-DOPE (data not shown). Taking these data into consideration, not only insertion timing, but also selection of PEGylated lipids is important to achieve targeted gene delivery.

In terms of the physicochemical characteristics of lipoplexes, it has been reported that increasing the surface charge and particle size increases uptake by phagocytes including liver Kupffer cells [34-36]. Synergistic effects of steric hindrance by PEGylation [37], a size decrease, and surface charge mask result in escape from the reticuloendothelial system. Syn-insertion of PEGylated lipids decreased the particle size and masked the surface charge of lipoplexes (Fig. 2), suggesting escape from uptake by Kupffer cells. Because the size of fenestrae ranges from 100 to 200 nm [38-40], fenestrae can restrict penetration of lipoplexes. We previously demonstrated restricted penetration of lipoplexes without PEGylation through the fenestrated endothelium of a perfused rat liver [41]. In contrast, polyethylenimine/pDNA complexes, which have a smaller particle size as lipoplexes, easily penetrate through a fenestrated endothelium [42]. Thus, the small particle size of syn-insertion lipoplexes was also preferable for penetration through sinusoids in the liver. In fact, the PC/NPC ratio of syn-insertion was superior to lipoplexes without PEGylation (Fig. 11). Therefore, physicochemical characteristics may largely affect the biodistribution of lipoplexes.

The concentration of mPEG-cer8 was an important factor to determine both the physicochemical characteristics of lipoplexes and their *in vivo* transection efficiency. A high concentration of mPEG-cer8 was required to decrease the ζ-potential (Fig. 6b) and increase liver selectivity of transgene expression (Fig. 7b), whereas a low concentration of mPEG-cer8 was sufficient to decrease the particle size (Fig

6a). The decrease in the ζ-potential was probably due to sufficient covering of PEG moieties on the surface of lipoplexes, which was important to prevent pulmonary transgene expression.

A high temperature during lipoplex formation was also required for high liver selectivity of transgene expression (Fig. 10b). The particle size of lipoplexes was dependent on temperature during lipoplex formation, while the ζ-potential was not (Fig. 9). Thus, the temperature during lipoplex formation mainly affected the particle size of syn-insertion lipoplexes, thereby inhibiting pulmonary transfection.

Pre- and syn-insertion of PEGylated lipids decreased the particle size, whereas post-insertion did not (Fig. 2). Therefore, the presence of PEGylated lipids during lipoplex formation is important to reduce the particle size. Steric hindrance by PEG moieties during lipoplex formation might stabilize a lipoplex intermediate and prevent interactions among lipoplex intermediates and subsequent formation of large aggregates. Accordingly, membrane fusion and incorporation of several molecules of pDNA into one lipoplex particle were inhibited (Fig. 3), which would be the reason for the small particle size of pre- and syn-insertion lipoplexes. To obtain a small particle size of syn-insertion lipoplexes, it was necessary to warm the mixture during lipoplex formation (Fig. 9a). It is well known that increasing the temperature increases the ζ-potential of a particle, which might be important to maintain repulsion among lipoplex intermediates and prevent large aggregate formation. Both the presence of PEGylated lipids and a high temperature during lipoplex formation are important to obtain small particle sizes.

In terms of targeting ligands, we selected galactose for hepatocyte-selective gene transfer. Galactosylation of lipoplexes improved liver selectivity of transgene expression (Fig. 1). For liver targeting, the use of asialofetuin [43] and transferrin [44] has been reported. Because asialofetuin and transferrin are proteins, galactosylation has several advantages such as easy handling and pharmaceutical stability. In contrast, neutralized lipid nanoparticles without any ligands can be delivered to the liver via an interaction with apolipoprotein E [45]. Because the nanoparticle does not contain pharmaceutically unstable components, such a strategy may be useful to deliver foreign genes to the liver.

5. Conclusion

We demonstrated that the physicochemical characteristics and *in vivo* transfection efficiency of lipoplexes were highly dependent on the insertion timing of PEGylated lipids to lipoplexes. The syn-insertion of PEGylated lipids to galactosylated lipoplexes simultaneously decreased the particle size and increased liver selectivity of transgene expression. These properties were superior to those of pre- and post-insertion. Furthermore, we improved liver PC selectivity by syn-insertion of PEGylated lipids to galactosylated lipoplexes. These results suggest that the combination of PEGylated lipids with syn-insertion to galactosylated lipoplexes improves liver selectivity of transgene expression

after intravenous injection in mice. Hence, these data will be valuable for the design and preparation of PEGylated lipoplexes for gene targeting.

Acknowledgements

References

[1] K. Domvri, P. Zarogoulidis, K. Porpodis, M. Koffa, M. Lambropoulou, S. Kakolyris, G. Kolios, K. Zarogoulidis, and E. Chatzaki, "Gene therapy in liver diseases: state-of-the-art and future perspectives," Curr. Gene Ther., vol. 12, pp. 463-483, December 2012.

[2] E. van Craeyveld, F. Jacobs, S. C. Gordts, and B. de Geest, "Gene therapy for familial hypercholesterolemia," Curr. Pharm. Des., vol. 17, pp. 2575-2591, 2011.

[3] G. P. Gao, M. R. Alvira, L. Wang, R. Calcedo, J. Johnston, and J. M. Wilson, "Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy," Proc. Natl. Acad. Sci. USA, vol. 99, pp. 11854-11859, September 2002.

[4] M. Johnson, S. Huyn, J. Burton, M. Sato, and L. Wu, "Differential biodistribution of adenoviral vector in vivo as monitored by bioluminescence imaging and quantitative polymerase chain reaction," Hum. Gene Ther., vol. 17, pp. 1262-1269, December 2006.

[5] F. Park, K. Ohashi, and M. A. Kay, "Therapeutic levels of human factor VIII and IX using HIV-1-based lentiviral vectors in mouse liver," Blood, vol. 96, pp. 1173-1176, August 2000.

[6] S. Kawakami, S. Fumoto, M. Nishikawa, F. Yamashita, and M. Hashida, "In vivo gene delivery to the liver using novel galactosylated cationic liposomes," Pharm. Res., vol. 17, pp. 306-313, March 2000.

[7] S. Fumoto, S. Kawakami, Y. Ito, K. Shigeta, F. Yamashita, and M. Hashida, "Enhanced hepatocyte-selective in vivo gene expression by stabilized galactosylated liposome/plasmid DNA complex using sodium chloride for complex formation," Mol. Ther., vo. 10, pp. 719-729, October 2004.

[8] K. Morimoto, M. Nishikawa, S. Kawakami, T. Nakano, Y. Hattori, S. Fumoto, F. Yamashita, and M. Hashida, "Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver," Mol. Ther., vol. 7, pp. 254-261, February 2003.

[9] Y. Hu, M. T. Haynes, Y. Wang, F. Liu, and L. Huang, "A highly efficient synthetic vector: nonhydrodynamic delivery of DNA to hepatocyte nuclei in vivo," ACS Nano, vol. 7, pp. 5376-5384, June 2013.

[10] S. E. Raper, N. Chirmule, F. S. Lee, N. A. Wivel, A. Bagg, G. P. Gao, J. M. Wilson, and M. L. Batshaw, "Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer," Mol. Genet. Metab., vol. 80, pp. 148-158, September-October 2003.

[11] J. C. Nault, S. Datta, S. Imbeaud, A. Franconi, M. Mallet, G. Couchy, E. Letouzé, C. Pilati, B. Verret, J. F. Blanc, C. Balabaud, J. Calderaro, A. Laurent, M. Letexier, P. Bioulac-Sage, F. Calvo, and J. Zucman-Rossi, "Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas," Nat. Genet., vol. 47, pp. 1187-1193, October 2015.

[12] S. Fumoto, S. Kawakami, M. Hashida, and K. Nishida, "Section 1 Approached to Gene Therapy, Chapter 1 Targeted Gene Delivery: Importance of Administration Routes," INTECH "Novel Gene Therapy Approaches" Editor, Ming Wei; Co-editor, David Good, pp. 3-31, February 2013.

[13] S. Kawakami, and M. Hashida, "Glycosylation-mediated targeting of carriers," J. Control. Release, vol. 190, pp. 542-555, September 2014.

[14] M. A. Hickman, R. W. Malone, K. Lehmann-Bruinsma, T. R. Sih, D. Knoell, F. C. Szoka, R. Walzem, D. M. Carlson, and J. S. Powell, "Gene expression following direct injection of DNA into liver," Hum. Gene Ther., vol. 5, pp. 1477-1483, December 1994.

[15] K. Kawabata, Y. Takakura, and M. Hashida, "The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake," Pharm. Res., vol. 12, pp. 825-30, June 1995.

[16] F. Liu, Y. Song, and D. Liu, "Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA," Gene Ther., vol. 6, pp. 1258-1266, July 1999.

[17] G. Zhang, V. Budker, and J. A. Wolff, "High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA," Hum. Gene Ther., vol. 10, pp. 1735-1737, July 1999.

[18] H. Hatakeyama, H. Akita, and H. Harashima, "The polyethyleneglycol dilemma: advantage and disadvantage of PEGylation of liposomes for systemic genes and nucleic acids delivery to tumors," Biol. Pharm. Bull., vol. 36, pp. 892-899, June 2013.

[19] J. Shin, P. Shum, and D. H. Thompson, "Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids," J. Control. Release, vol. 91, pp. 187-200, August 2003.

[20] H. Hatakeyama, H. Akita, K. Kogure, M. Oishi, Y. Nagasaki, Y. Kihira, M. Ueno, H. Kobayashi, H. Kikuchi, and H. Harashima, "Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid," Gene Ther., vol. 14, pp. 68-77, January 2006.

[21] F. Shi, L. Wasungu, A. Nomden, M. C. Stuart, E. Polushkin, J. B. Engberts, and D. Hoekstra, "Interference of poly(ethylene glycol)-lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions," Biochem. J., vol. 366, pp. 333-341, August 2002.

[22] Y. P. Zhang, L. Sekirov, E. G. Saravolac, J. J. Wheeler, P. Tardi, K. Clow, E. Leng, R. Sun, P. R. Cullis, and P. Scherrer, "Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties," Gene Ther., vol. 6, pp. 1438-1447, August 1999.

[23] K. W. Mok, A. M. Lam, and P. R. Cullis, "Stabilized plasmid-lipid particles: factors influencing plasmid entrapment and transfection properties," Biochim. Biophys. Acta, vol. 1419, pp. 137-150, July 1999.

[24] L. Peeters, N. N. Sanders, A. Jones, J. Demeester, and S. C. de Smedt, "Post-pegylated lipoplexes are promising vehicles for gene delivery in RPE cells," J. Control. Release, vol. 121, pp. 208-217, August 2007.

[25] S. Kawakami, R. Hirayama, K. Shoji, R. Kawanami, K. Nishida, M. Nakashima, H. Sasaki, T. Sakaeda, and J. Nakamura, "Liver- and lobe-selective gene transfection following the instillation of plasmid DNA to the liver surface in mice," Biochem. Biophys. Res. Commun., vol. 294, pp. 46-50, May 2002.

[26] Y. C. Lee, S. P. Stowell, and M. J. Krantz, "2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins," Biochemistry, vol. 15, pp. 3956-3963, September 1976.

[27] F. Sakurai, T. Nishioka, H. Saito, T. Baba, A. Okuda, O. Matsumoto, T. Taga, F. Yamashita, Y. Takakura, and M. Hashida, "Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid," Gene Ther., vol. 8, pp. 677-686, May 2001.

[28] H. Eliyahu, N. Servel, A. J. Domb, and Y. Barenholz, "Lipoplex-induced hemagglutination: potential involvement in intravenous gene delivery," Gene Ther., vol. 9, pp. 850-858, July 2002.

[29] B. M. Tandia, C. Lonez, M. Vandenbranden, J. M. Ruysschaert, and A. Elouahabi, "Lipid mixing between lipoplexes and plasma lipoproteins is a major barrier for intravenous transfection mediated by cationic lipids," J. Biol. Chem., vol. 280, pp. 12255-12261, April 2005.

[30] N. Yoshikawa, S. Fumoto, M. Nakashima, K. Shimokawa, H. Miyamoto, and K. Nishida. "The role of fibronectin in pulmonary gene transfer following intravenous administration of lipoplex in mice," Biol. Pharm. Bull., vol. 36, pp. 1807-1813, November 2013.

[31] K. Kusumoto, H. Akita, A. El-Sayed, and H. Harashima, "Effect of the anchor in polyethylene glycol-lipids on the transfection activity of PEGylated cationic liposomes encapsulating DNA," Biol. Pharm. Bull., vol. 35, pp. 445-448, April 2012.

[32] K. Remaut, B. Lucas, K. Braeckmans, J. Demeester, S. C. de Smedt, "Pegylation of liposomes favours the endosomal degradation of the delivered phosphodiester oligonucleotides," J. Control. Release, vol. 117, pp. 256-266, February 2007.

[33] M. C. Deshpande, M. C. Davies, M. C. Garnett, P. M. Williams, D. Armitage, L. Bailey, M. Vamvakaki, S. P. Armes, and S. Stolnik, "The effect of poly(ethylene glycol) molecular architecture on cellular interaction and uptake of DNA complexes," J. Control. Release, vol. 97, pp. 143-156, May 2004.

[34] S. A. Johnstone, D. Masin, L. Mayer, and M. B. Bally, "Surface-associated serum proteins inhibit the uptake of phosphatidylserine and poly (ethylene glycol) liposomes by mouse macrophages," Biochim. Biophys. Acta, vol. 1513, pp. 25-37, July 2001.

[35] C. R. Miller, B. Bondurant, S. D. McLean, K. A. McGovern, and D. F. O'Brien, "Liposome-cell interactions in vitro: effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes," Biochemistry, vol. 37, pp. 12875-12883, September 1998.

[36] Y. Higuchi, S. Kawakami, S. Fumoto, F. Yamashita, and M. Hashida, "Effect of the particle size of galactosylated lipoplex on hepatocyte-selective gene transfection after intraportal administration," Biol. Pharm. Bull., vol. 29, pp. 1521-1523, July 2006.

[37] G. R. Harper, S. S. Davis, M. C. Davies, M. E. Norman, T. F. Tadros, D. C. Taylor, M. P. Irving, J. A. Waters, and J. F. Watts, "Influence of surface coverage with poly(ethylene oxide) on attachment of sterically stabilized microspheres to rat Kupffer cells in vitro," Biomaterials, vol. 16, pp. 427-439, April 1995.

[38] E. Wisse, "An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids," J. Ultrastruct. Res., vol. 31, pp. 125-150, April 1970.

[39] Z. Gatmaitan, L. Varticovski, L. Ling, R. Mikkelsen, A. M. Steffan, and I. M. Arias, "Studies on fenestral contraction in rat liver endothelial cells in culture," Am. J. Pathol., vol. 148, pp. 2027-2041, June 1996.

[40] E. Wisse, R. B. de Zanger, K. Charels, P. van der Smissen, and R. S. McCuskey, "The liver sieve: considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of Disse," Hepatology, vol. 5, pp. 683-692, July-August 1985.

[41] S. Fumoto, F. Nakadori, S. Kawakami, M. Nishikawa, F. Yamashita, and M. Hashida, "Analysis of hepatic disposition of galactosylated cationic liposome/plasmid DNA complexes in perfused rat liver," Pharm. Res., vol. 20, pp. 1452-1459, September 2003.

[42] S. Fumoto, Kawakami S, M. Ishizuka, M. Nishikawa, F. Yamashita, and M. Hashida, "Analysis of hepatic disposition of native and galactosylated polyethylenimine complexed with plasmid DNA in perfused rat liver," Drug Metab. Pharmacokinet., vol. 18, pp. 230-237, August 2003.

[43] M. A. Arangoa, N. Düzgüneş, C. Tros de Ilarduya, "Increased receptor-mediated gene delivery to the liver by protamine-enhanced-asialofetuin-lipoplexes," Gene Ther., vol. 10, pp. 5-14, January 2003.

[44] J. G. Seol, D. S. Heo, H. K. Kim, J. H. Yoon, B. I. Choi, H. S. Lee, N. K. Kim, and C. Y. Kim, "Selective gene expression in hepatic tumor with trans-arterial delivery of DNA/liposome/transferrin complex," In Vivo, vol. 14, pp. 513-517, July-August 2000.

[45] M. Ukawa, H. Akita, Y. Hayashi, R. Ishiba, K. Tange, M. Arai, K. Kubo, Y. Higuchi, K. Shimizu, S. Konishi, M. Hashida, and H. Harashima, "Neutralized nanoparticle composed of SS-cleavable and pH-activated lipid-like material as a long-lasting and liver-specific gene delivery system," Adv. Healthc. Mater. vol. 3, pp. 1222-1229, August 2014.