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BRIEF ARTICLE

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Improving *in Vivo* Hepatic Transfection Activity by Controlling Intracellular Trafficking: The Function of GALA and Maltotriose

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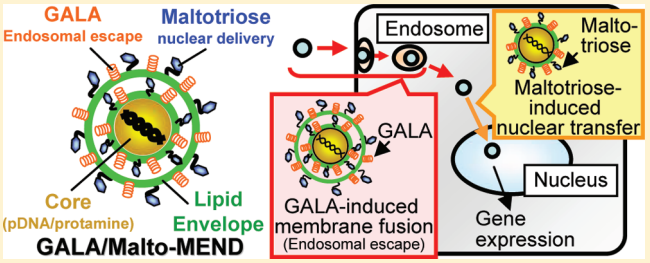
ABSTRACT: The successful control of intracellular trafficking (i.e., endosomal escape and nuclear delivery) is prerequisite for the development of a gene delivery system. In the present study, we developed an *in vivo* hepatic gene delivery system using a plasmid DNA (pDNA)-encapsulating lipid envelope-type nanoparticle, to which we refer as a multifunctional envelope-type nanodevice (MEND). The critical structural elements of the MEND are a DNA/protamine condensed core coated with lipid bilayers including serum-resistant cationic lipids. Intravenous administration of bare MEND represents minimal transfection activity. For the surface modification of functional devices, hydrophobic moieties were chemically attached, which are shed in the spontaneous orientation outward from the MEND surface by anchoring to the lipid bilayers. Modification of the pH-dependent fusogenic peptide GALA as an endosome escape induced transfection activity by 1 and 2 orders of magnitude. In an attempt to induce the nuclear delivery of pDNA, maltotriose, a recently characterized nuclear localization signal, was additionally modified. As a result, transfection activity further enhanced by 1 order of magnitude, and it reached to the higher level obtained for a conventional lipoplex and an *in vivo* jetPEI-Gal, with less hepatic toxicity. The data show that the combination of GALA and maltotriose results in a highly potent functional device that shows an enhanced endosomal escape and nuclear delivery *in vivo*.



KEYWORDS: liver, sugar, nuclear delivery, gene delivery, plasmid DNA

INTRODUCTION

Since the liver plays an important role in homeostasis of the body and detoxification, the loss of function results in the development of cirrhosis and hepatitis. Gene therapy promises to be a promising strategy for curing these intractable diseases.[1] For efficient gene delivery, vectors must be designed to satisfy intracellular pharmacokinetics (iPK), as well as conventional pharmacokinetics in the systemic circulation (PK).[2] We recently reported on the rational design of a multifunctional envelope-type nanodevice (MEND), which consists of a DNA/polycation-condensed core encapsulated in a lipid envelope equipped with functional devices.[3]

Regarding intracellular trafficking, transfection activity is hampered by the presence of a variety of biomembrane barriers (i.e., the plasma/endosome membrane and the nuclear membrane). The influenza virus, an envelope-type RNA virus, releases its genomic DNA into the cytosol by membrane fusion with the aid of hemagglutinin (HA), which undergoes a conformational change in response to the acidic environment.[4,5] Inspired by this, the peptide derived from the N-terminus of hemagglutinin subunit HA-2 (i.e., INF-7),[6−8] or artificial peptides (GALA)[9,10] were developed to induce endosomal escape. Lipid conjugation to these peptides permits them to be displayed on the surface of the lipid envelope of the MEND. The incorporation of cholesterol derivatives of GALA (Chol-GALA) by the liposome stimulates the release of the encapsulated molecules into the cytoplasm.[11,12] Furthermore, modification of the lipid envelope with stearylated INF7 (STR-INF7) resulted in an enhanced hepatic gene expression via iv administration.[13]

Thereafter, the nuclear membrane represents the ultimate barrier to be overcome in nondividing cells, whereas pDNA primarily enters the nucleus in conjunction with the concomitant breakdown of the nuclear membrane structure during the M phase in mitotic cells.[14,15] The mutual transport of small molecules and nuclear localization signal (NLS)-possessing proteins between the cytosol and nucleus occurs via the nuclear pore complex (NPC).[16] One of the most well-defined NLS signals is the peptide (PKKKRKV) derived from the SV40 T-antigen (NLS_{SV40}).[17] In light of these findings, numerous attempts have been made to improve nuclear delivery processes by conjugation to pDNA itself[18−20] or pDNA-condensing polymers.[21,22] However, it is likely that electrostatic interactions between cationic NLS peptides and pDNA prevent the topology and the surface density of the NLS from being controlled. To overcome this issue, we previously modified the lipid envelope of a MEND with

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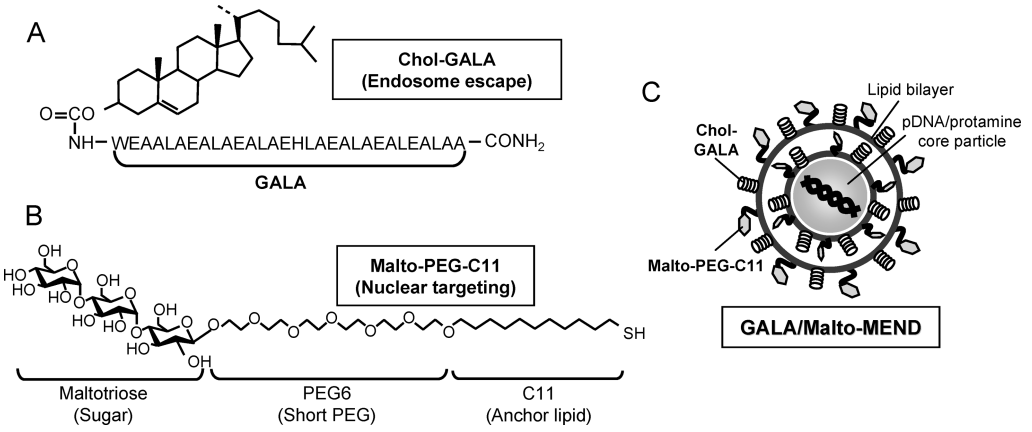

Figure 1. Structures of Chol-GALA (A), Malto-PEG6-C11 (B) and Malto/GALA-MEND (C). The GALA peptide and maltotriose were conjugated with hydrophobic groups to permit incorporation into the lipid envelope of a MEND.

a NLS by inserting a lipid derivative of a NLS. This design permits the spontaneous display of the NLS oriented outward from the particle surface, and results in the transfection of nondividing cells (i.e., primary dendritic cell cultures).[23]

Meanwhile, the function of noncationic, water-soluble sugar molecules as a nuclear transport signal has been clarified in recent years.[24−26] Glycosylated polymers, such as lactosylated polylysine and lactosylated polyethyleneimine, have recently been reported to function as efficient gene carriers.[27,28] Very recently, we found that the surface modification of fluorescent nanoparticles (quantum dots) or albumin with a trisaccharide (maltotriose) enhanced their nuclear accumulation, while monosaccharides (α-glucose) or disaccharides (maltose) did not function as nuclear transporters.[29] Thus, maltotriose has a unique function as a nuclear localization signal for nanoparticles, as well as proteins.

We wish to report herein on the development of an efficient gene carrier for the liver via iv administration from the point of view of improving intracellular trafficking by modifying the devices in a manner that permits them to overcome the endosomal and nuclear membranes via the combined use of GALA and maltotriose.

■ MATERIALS AND METHODS

General. Protamine sulfate salmon milt was purchased from Calbiochem (Darmstadt, Germany). Before use, the protamine solution was filtered through a cellulose acetate filter (DISMIC-13 cP: 0.2 μm pore size) obtained from ADVANTECH (Chiba, Japan). 1,2-Dioleyl-3-trimethylammonium propane (DOTAP), *N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium (DOTMA) and cholesterol (Chol) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). [*cholesteryl*-1,2-^{3}H(N)]-Cholesteryl hexadecyl ether ([^{3}H]CHE) was purchased from PerkinElmer Life Science Japan (Tokyo, Japan). The reporter plasmid pcDNA3.1(+)-luc (7,037bp) encoding the firefly luciferase gene was purified with a Qiagen Endofree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). Cholesteryl-GALA (Figure 1A)[12] was purchased from Kurabo Industries, Ltd. (Osaka, Japan). Maltotriose-lipid(C11) conjugate (Malto-PEG6-C11)[29] (Figure 1B) was conjugated as described previously.[30] Tetra-(ethylene)glycol (TEG)-conjugated cholesterol was prepared as reported previously.[31]

Male, five-week-old ICR mice (26−28 g) were purchased from Japan SLC (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals". In all experiments, the animals were used without fasting.

All other organic reagents and solvents were commercially available reagents of extrapure grade and were used without further purification.

Preparation of MEND. The MENDs were prepared by the lipid hydration method, as reported previously[32,33] with minor modifications, to prepare a highly concentrated MEND solution for *in vivo* use. In a typical run, 125 μL of pDNA (0.3 mg/mL) was condensed with 125 μL of protamine (0.20 mg/mL) in 10 mM HEPES (pH 7.4), at a nitrogen/phosphate (N/P) ratio of 1.0. A lipid film was prepared in a glass test tube by evaporating a chloroform solution of lipids, containing a cationic lipid (DOTAP or DOPE), Chol and TEG-Chol (cationic lipid/cholesterol/TEG-Chol = 30:40:30, total lipid amount: 412.5 nmol). To prepare the GALA-MENDs, 2 mol % Chol-GALA (Figure 1A) was added to the lipid film composition. For the preparation of the GALA/Malto-MEND (Figure 1C), a part of TEG-Chol (sixth part of total TEG-Chol, corresponding to 5% of the total lipid content) was replaced with Malto-PEG6-C11 (Figure 1B). The prepared lipid film was then hydrated with the condensed DNA solution for 10 min at room temperature. After hydration, the tube was sonicated for 1 min in a bath-type sonicator (AU-25C; Aiwa Co., Tokyo, Japan) to complete the lipid coating of the condensed DNA. The diameter and ζ-potential of the MENDs were determined using an electrophoretic light-scattering spectrophotometer (Zetasizer; Malvern Instruments Ltd., Malvern, WR, U.K.).

***In Vivo* Pharmacokinetics Study of MENDs.** The lipid bilayers of MENDs were labeled with a tracer amount of [^{3}H]CHE (0.5 μCi per 412.5 nmol of total lipid) by incorporating [^{3}H]CHE as a lipid film component. The [^{3}H]MENDs were administered to male ICR mice (5−6 weeks old, Japan SLC) via the tail vein, at a dose of 40 μg of DNA (0.44 μmol of lipids) in 350 μL of a 5% glucose solution. At 6 h postinjection, the radioactivity in the liver was measured as described previously.[34] Briefly, at the indicated times, the mice were sacrificed, and 0.2−0.3 g of liver tissue was solubilized in 1 mL of Soluene-350 (Perkin-Elmer Life Sciences) overnight at 50 °C. The radioactivity was determined by liquid scintillation counting, after adding 10 mL of scintillation fluid (Hionic fluor), followed by incuba-

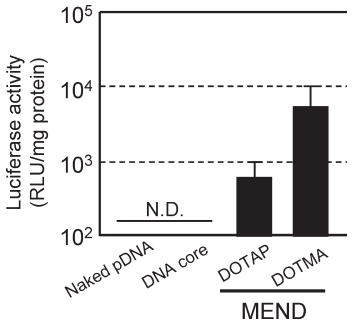

Figure 2. *In vivo* transfection activity of naked DNA and MENDs unmodified with a functional device. Naked pDNA, pDNA/protamine core and MENDs were intravenously administered at a dose of 40 μg/mouse with 350 μL of a 5% glucose solution. *In vivo* hepatic gene expression was evaluated at 6 h postadministration. Each point and vertical bar represents the mean ± SD of quadruplicate experiments.

tion overnight at 4 °C. Hepatic accumulation of the MENDs was represented as the % of injected dose (%ID) per 1 g of tissue (%ID/g tissue).

2.4. *In Vivo* Luciferase Reporter Assay. Hepatic gene expression was evaluated as described previously.[13] The MENDs were injected into male, five-week-old ICR mice via the tail vein, at a dose of 40 μg of DNA in 350 μL of a 5% glucose solution. At 6 h postinjection, the mice were sacrificed and the liver specimens were collected. The collected organs were then washed with saline, weighed, and minced with scissors. Approximately 0.2 g samples of liver tissue were completely homogenized using a POLYTRON homogenizer (KINEMATICA, Littan, Switzerland) in 1 mL of lysis buffer (100 mM Tris−HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.8). After centrifugation at 13000 rpm for 10 min at 4 °C, a 20 μL aliquot of the supernatant was used for a luciferase assay using the Luciferase Assay System (Promega, Madison, WI). Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). Luciferase activities are expressed as relative light units (RLU) per mg of protein.

As a reference, the transgene expression of a conventional lipoplex composed of DOTAP/DOPE (50:50) was prepared as reported previously.[35] pDNA was also condensed with *in vivo* jetPEI-Gal (Polyplus-transfection Inc. NY, USA) following the manufacturer's protocol at an N/P ratio of 10. pDNA, condensed with the lipoplex and *in vivo* jetPEI-Gal, was injected via the tail vein at a dose of 50 μg of pDNA/mouse and 40 μg of pDNA/mouse, respectively.

RESULTS AND DISCUSSION

***In Vivo* Transfection Activity of Prototype MENDs.** *In vivo* transfection activity of naked pDNA, pDNA/protamine core particles and MENDs were first evaluated (Figure 2). It was previously demonstrated that liver undergoes significant transgene expression (>10^8 RLU/mg protein) when pDNA is rapidly administered with a large volume of saline.[36,37] Thus, we minimized the injection volume to 350 μL by preparing a MEND solution with a maximum concentration, and then slowly injected the solution (350 μL/>5 s). As shown in Figure 2, the transfection activity of naked pDNA was negligible, most probably due to enzymatic degradation[38] and/or clearance by macrophages via scavenger receptors.[39] Furthermore, no transgene expression was found for the pDNA/protamine core particles. Thus, the hepatic gene expression observed in subsequent experiments can be attributed to the function of the MENDs, and not to nonspecific activity as the result of hydrodynamic conditions.

Table 1. Physicochemical Characteristics of the MENDs Used in This Study

		size (nm)	zeta-potential (mV)
DOTAP	none	201	52.5
	GALA	275	44.3
	GALA/maltotriose	222	35.3
DOTMA	none	217	52.8
	GALA	282	42.6
	GALA/maltotriose	246	37.5
	GALA/C6	249	44.8

Cationic lipids such as DOTAP and DOTMA are conventionally used in gene delivery, because of their serum resistance.[40,41] Thus, we used these lipids as key components of the lipid envelope. However, preliminary experiments showed that MENDs prepared with DOTAP/1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE)/Chol (3:4:3) showed only negligible amounts of hepatic gene expression (data not shown). In the absence of PEG modification, MENDs that contain fusogenic DOPE might be inactivated by irreversible binding to erythrocytes[42,43] or serum proteins.[44−46] Thus, DOPE can be excluded as a component of the lipid envelope. Furthermore, we previously demonstrated that a MEND prepared using TEG-Chol, as the lipid component, is advantageous for the formation of smaller-sized, homogeneous particles.[31] Thus, we added 30% TEG-Chol as a lipid component of the MEND.

After the administration of MENDs prepared with cationic lipids (DOTAP or DOTMA)/Chol/TEG-Chol = 3:4:3, the luciferase activity was only slightly above background (<10^4 RLU/mg protein) (Figure 2). On the other hand, the iv administration of radiolabeled [^{3}H]MENDs showed that more than 70% of the injected dose accumulated in the liver (see Figure 4, below). Thus, the poor *in vivo* transfection activity cannot be explained from a pharmacokinetic point of view. We then focused on improving intracellular trafficking.

Improving *in Vivo* Transfection Activity by Modification with GALA and Maltotriose. We first modified GALA by incorporating Chol-GALA into the lipid envelope. Modification by GALA resulted in an increase in the average sizes of the MENDs from approximately 200−210 nm to 275−285 nm (Table 1), presumably because of reversible particle-to-particle binding as the result of electrostatic interactions between the negatively charged GALA and the positively charged lipid envelope. As shown in Figure 3, modification with GALA enhanced the transfection activity by 1 and 2 orders of magnitude in MENDs prepared with DOTAP and DOTMA, respectively.

Then, nuclear transfer of pDNA was challenged. When Malto-PEG6-C11 was used to modify the MENDs, the average particle size was reduced (Table 1), probably because the thick hydrophilic layer, which is composed of a sugar and PEG6, prevented interparticle electrostatic interactions between GALA and the cationic lipid envelope. As shown in Figure 3, modification with Malto-PEG6-C11 enhanced the transfection activity by approximately 10-fold in both MENDs. It is generally considered that electrostatic interactions between particles and negatively charged heparin sulfate proteoglycans (HSPGs) are a crucial driving force in cellular uptake.[47,48] However, the ζ-potentials

were decreased as the result of modification with GALA and the sugar. This suggests that the cellular uptake process cannot explain the stimulating transfection activity in the case of the GALA-MEND and the GALA/Malto-MEND. In the structure of Malto-PEG6-C11, maltotriose was conjugated with the hydrophobic chain (C11) by means of a hexa(ethylene)glycol (PEG6) spacer. Thus, we evaluated the transfection activity of the DOTMA-based MEND modified with GALA and sugar-unmodified PEG6-C11 (GALA/PEG6-MEND), as a more strict control, in order to evaluate the effect of maltotriose. The transfection activity of the GALA/PEG6-MEND was comparable to the GALA-MEND, strongly indicating that maltotriose plays a key role in improving transfection activity in GALA/Malto-MEND.

Overall, transfection activities of DOTMA-based MENDs exceed those prepared with DOTAP-based ones (Figures 2 and 3). These results are consistent with a previous report that lipoplexes prepared with DOTMA and DOTAP exhibit a 10-fold difference in the level of transgene expression in the lung after intravenous administration.[35,49] Similar phenomena were also observed in MEND.[50] As proposed previously, difference of linkage bonds between cationic headgroup and two hydrocarbon chains (two ether bonds in DOTMA and two ester bonds in DOTAP) might affect the stability or biodegradability of liposome structure in blood circulation and/or in the cells.[35,51] As to the tissue specificity, hepatic gene expression of DOTMA-based GALA/Malto-MEND was comparable to those observed in lung ($2-4 \times 10^5$ RLU/mg protein), and moreover, it exceeds those in spleen (2×10^4 RLU/mg protein) (data not shown). Thus, the GALA/Malto-MEND is a more liver-oriented gene carrier than conventional DOTMA-based lipoplex, whose transfection activity was dominantly observed in lung (2 orders of magnitude more than liver),[35,51]

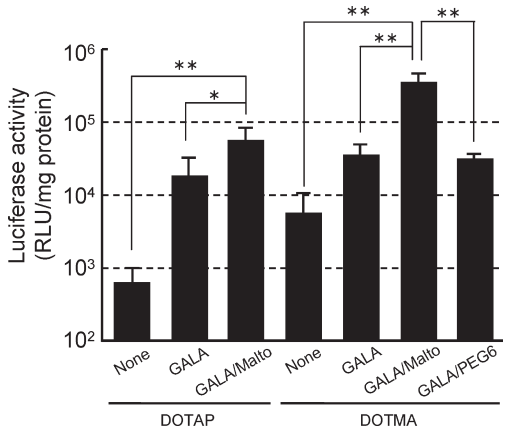

Figure 3. *In vivo* transfection activity of GALA-MEND and GALA/Malto-MEND. MENDs whose lipid envelope was composed of cationic lipids (DOTAP or DOTMA)/cholesterol/TEG-Chol = 30:40:30 were modified with Chol-GALA (GALA-MEND) and with Malto-PEG6-C11 (GALA/Malto-MEND). *In vivo* hepatic gene expression was evaluated at 6 h postadministration. Each point and vertical bar represents the mean ± SD. Statistical differences among unmodified MEND, GALA-MEND and GALA/Malto-MEND were evaluated by one-way ANOVA, followed by the Student−Newman−Keuls test (*$p < 0.05$, **$p < 0.01$).

Sugars are generally used as a tool in gene delivery to enhance the efficiency of cellular uptake and/or hepatic clearance. It was previously demonstrated that the hepatic uptake of nanoparticles was enhanced by modification with certain types of sugars (i.e., galactose and mannose).[52] Thus, the accumulations of the MEND, GALA-MEND and GALA/Malto-MEND in the liver were quantified to distinguish the contribution of pharmacokinetics (PK) and intracellular pharmacokinetics (iPK) to the overall improvement in *in vivo* transfection activity. The focus of our studies was on DOTMA-based MENDs since they showed a higher gene expression than those prepared with DOTAP as described above. As shown in Figure 4A, the extent of liver accumulation was comparable, or slightly decreased as the result of modification with GALA and maltotriose. Thus, the drastic enhancement in transfection activity can be largely attributed to an improved iPK, and not to PK. Figure 4B shows the transfection activities normalized by the amount of hepatic accumulation, which reflect the contribution of GALA and maltotriose to the improvement in the iPK processes. The values increased synergistically by approximately 1 order of magnitude in a stepwise manner. This provides support for our hypothesis that the use of GALA and maltotriose results in an improved intracellular trafficking because of their effect on different processes (endosomal escape and nuclear delivery, respectively).

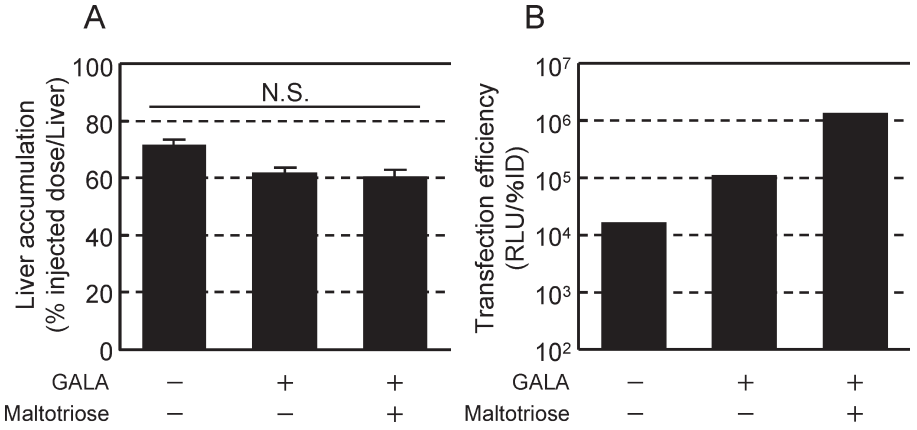

Figure 4. Liver accumulation of [^{3}H]MENDs. [^{3}H]MENDs whose lipid envelope, composed of DOTMA/cholesterol/TEG-Chol = 30:40:30, was modified with Chol-GALA and Malto-PEG6-C11. The radioactivity in the liver was measured at 6 h postinjection (A). Hepatic accumulation of the MENDs was represented as the % of injected dose (% ID) per 1 g of tissue (% ID/g tissue). Liver accumulation was comparable (not significant) regardless of modification with Chol-GALA and Malto-PEG6-C11. (B) Transfection activities were normalized by the amount of hepatic accumulation to quantify the contribution of intracellular pharmacokinetics (iPK) on the transfection activity of MENDs.

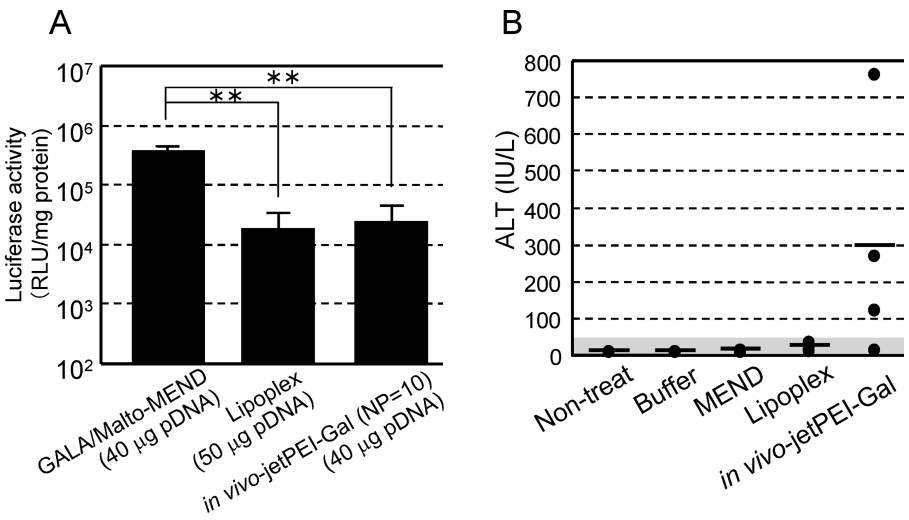

Figure 5. Comparison of transfection activity and hepatic toxicity among GALA/Malto-MEND, lipoplex and *in vivo* jetPEI-Gal. pDNA encapsulated in the GALA/Malto-MEND (40 μg) or condensed by lipoplex (50 μg) and *in vivo* jetPEI-Gal (40 μg) were injected with 350 μL of a 5% glucose solution. *In vivo* hepatic gene expression (A) and serum ALT levels (B) were evaluated at 6 h postadministration. Each point and vertical bar represents the mean ± SD. Statistical differences among GALA/Malto-MEND, lipoplex and *in vivo* jetPEI-Gal were evaluated by one-way ANOVA, followed by the Student−Newman−Keuls test ($^*p < 0.05$, $^{**}p < 0.01$).

Comparative Studies with Other Types of Vectors. Finally, the transfection activity of the GALA/Malto-MEND and its toxic effect against the liver were compared with a conventional lipoplex and a commercially available polyplex (*in vivo* jetPEI-Gal). As shown in Figure 5A, the GALA/Malto-MEND (40 μg of pDNA/mouse) exhibited a 20.8- and 15.8-fold higher efficiency than the lipoplex (50 μg of pDNA/mouse) and the *in vivo* jetPEI-Gal (40 μg pDNA/mouse). Furthermore, high levels of alanine aminotransferase (ALT), a marker for hepatic injury, were observed in plasma in 3 mice out of 4 after an iv injection of *in vivo* jetPEI-Gal, while the levels of ALT remained within normal levels (below 37 IU/L) even after an injection of the GALA/Malto-MEND (Figure 5B). Thus, the GALA/Malto-MEND confers efficient hepatic gene expression without detectable liver toxicity.

Collectively, the GALA/Malto-MEND satisfies the requirements for both high efficiency and safety as an *in vivo* hepatic gene carrier. Moreover, the control of intracellular trafficking (i.e., endosomal escape and nuclear delivery) is an important aspect in improving transfection activity for the *in vivo* hepatic delivery of pDNA.

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Author Contributions

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