Hepatocyte-Targeted Delivery of siRNA Polyplex with PEG-Modified Lactosylated Dendrimer/Cyclodextrin Conjugates for Transthyretin-Related Amyloidosis Therapy

Yuya Hayashi, Taishi Higashi, Keiichi Motoyama, Hirofumi Jono, Yukio Ando, Risako Onodera, and Hidetoshi Arima

INTRODUCTION

RNA interference (RNAi) induced by small interfering RNA (siRNA) is a fundamental pathway in mammalian cells and specifically suppressed the target gene expression. Therefore, RNAi-mediated gene silencing is expected as an effective therapy for various intractable diseases such as cancer, viral infection, and hereditary disorder. However, siRNA molecules have several physicochemical and biological drawbacks properties for efficient in vivo application such as a relatively large size, hydrophilicity, negative charge, enzymatic degradation by ribonuclease (RNase), immune response activation and rapidly clearance from the blood, resulting in poor transfection efficiency and biological stability. Thus, development of the efficient siRNA delivery system to achieve the RNAi therapy is required.

Heredity transthyretin (TTR)-related amyloidosis is a fetal, autosomal dominant, systemic amyloidosis caused by amyloidogenic variant TTR. The most common treatment of TTR amyloidosis is liver transplantation, because TTR is mainly expressed in hepatocytes. Liver transplantation rapidly decreases the amyloidogenic variant TTR concentration in blood and improves the clinical symptoms and survivals. Recently, RNAi therapy for TTR amyloidosis have been developed and undergoing clinical trials. Patisiran, TTR targeted siRNA (siTTR)-encapsulated lipid nanoparticles, was approved by U.S. Food and Drug Administration (FDA) in 2018 and showed great efficacy and safety for TTR-related amyloidosis patients. In order to develop a novel siRNA delivery system using polymer-based nanoparticles, we have developed hepatocytes-specific starburst polyamidoamine (PAMAM) dendrimer (dendrimer)-based siRNA carriers.

Lately, we developed the lactose-appended dendrimer (generation 3; G3)/α-cyclodextrin (α-CyD) conjugate (Lac-α-CDE (G3)) as a hepatocyte-targeted siRNA delivery system. Lac-α-CDE (G3) efficiently uptake to the hepatocytes via asialoglycoprotein receptor (ASGPR)-mediated endocytosis because ASGPR is highly expressed on hepatocytes cell surface. Lac-α-CDE (G3)/siTTR polyplex induce the in vivo TTR silencing effect without cytotoxicity. Most recently, we have reported that the novel ternary polyplex system consisting of an amphotolytic polysaccharide sacran, Lac-α-CDE (G3) and siRNA as a hepatocyte-targeted siRNA delivery system.
the high level of siRNA accumulation and the gene silencing effect in liver rather than that of the binary polyplex. However, the in vivo gene silencing effect is not enough for the therapeutic use of TTR amyloidosis.

Polyethylene glycol (PEG) modification is a broadly used for siRNA delivery system in order to reduce the non-specific interaction of serum component and prolong the blood circulation of polyplex, resulting in in vivo siRNA transfer activity is improved. However, excess modification of PEG significantly decreases the cellular uptake and endosomal escaping ability, which is known as PEG dilemma.\(^{18,19}\) Previously, we newly prepared PEG-modified Lac-\(\alpha\)-CDE (PEG-L\(\alpha\)Cs, G3) (Fig. 1A) as a hepatocyte-targeted pDNA delivery system.\(^{20}\) As a result of optimization of a degree of substitution of PEG (DSP), PEG-L\(\alpha\)C (G3, DSP2)/pDNA polyplex showed higher gene expression in hepatic parenchymal cells than Lac-\(\alpha\)-CDE (G3, DSL1)/pDNA polyplex, while maintaining the hepatocyte-targeting ability. However, the utility of PEG-L\(\alpha\)C (G3) as a hepatocyte-targeted siRNA delivery system was not revealed yet. In this study, we examined the TTR silencing effect, physicochemical properties, serum stability, cellular uptake, pharmacokinetics and a safety profile of PEG-L\(\alpha\)Cs (G3)/siTTR polyplex both in vitro and in vivo.

**MATERIALS AND METHODS**

**Materials** PAMAM dendrimer (ethylenediamine core, generation 3) and asialofetuin (AF) from fetal calf serum (type I) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). \(\alpha\)-CyD was provided by Nihon Shokuhin Kako (Tokyo, Japan). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Lipofectamine\(^\text{\textregistered}2000\), fetal bovine serum (FBS) and TRIzol reagent were obtained from Thermo Fisher Scientific (Tokyo, Japan). \(\alpha\)-Amino-\(\omega\)-carboxyl PEG (SUNBRIGHT\(^\text{\textregistered}\) PA-020HC, MW = 2170) was purchased from NOF (Tokyo, Japan). JetPEI\(^\text{\textregistered}\)-Hepatocyte was obtained from Polyplus-Transfection (Bioparc, France). Scramble siRNA (siCont) and siTTR were supplied by Alnylam Pharmaceuticals (Cambridge, MA, U.S.A.). Other solvents and chemicals were of analytical re-
agent grade.

**Animals** Healthy male BALB/c mice (4 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and all animal experiment followed the guidelines of the Ethics Committee for Animal Care and Use of Kumamoto University (Approval ID: A27-138).

**Preparation of PEG-LaCs** PEG-LaCs (G3), having various DSP, were prepared as previously reported.\(^{20}\) \(\alpha\)-Amino-\(\omega\)-carboxyl PEG (MW = 2170) (DSP2, 9.5 mg; DSP4, 14.8 mg; DSP6, 46.6 mg) was reacted with \(N\)-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 0.2 M boric acid solution, and mixed for 2 h. Then, \(\alpha\)-CDE (G3, DSL1) (20 mg) was added to the solution, and mixed for 6 h. In the case of PEG-LaC (G3, DSP6), the solution was incubated for 48 h. All reaction performed at room temperature. The resulting conjugates were purified by a dialysis (MWCO = 3500). The DSP value of the PEG-LaCs were obtained from \(^{1}H\)-NMR calculation.

**Cell Culture** HepG2 cells, a human hepatocellular carcinoma cell line, were purchased from Riken Bioreresource Center (Tsukuba, Japan). HepG2 cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (0.1 mg/mL) in a 37°C humidified incubator with 5% CO₂.

**Gene Silencing Effect in Vitro** Lac-\(\alpha\)-CDE (G3, DSL1)/siRNA and PEG-LaCs (G3)/siRNA polyplexes were prepared at various charge ratios (carriers/siRNA) by mixing both components for 15 min at room temperature. The dose of siRNA was 100 nM. HepG2 cells were seeded in 24-well plates (100,000 cells per well) and incubated overnight in 500 μL of DMEM containing 10% FBS at 37°C. After washing the cells with serum-free medium, the cells were incubated with DMEM containing the siRNA polypeptide (50 μL) and DMEM (250 μL) with or without AF for 24 h at 37°C. The total RNA was isolated with TRIzol Reagent. The complementary DNA (cDNA) was synthesized using a total of 0.5 μg RNA using PrimeScript™ RT reagent Kit (TaKaRa Bio, Shiga, Japan). The RNA isolation and RT reaction were followed the manufacture's instruction.

**Quantification of TTR mRNA Expression by Real-Time PCR** The TTR mRNA expression was quantified by real-time PCR using Light Cycler 480™ (Roche, Penhburg, Germany) with TB Green™ Premix DimerEraser™ (TaKaRa Bio). The sequence of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), human TTR, murine β-actin and murine TTR specific primers were follows: human GAPDH forward, 5’-GCA CCG TCA AGG CTG AGA AC-3’; human GAPDH reverse, 5’-ATG GTG TGT AGG AAG CAC GTA-3’; human TTR forward, 5’-CAT TCT TGG CGA GAT GGC TCT-3’; human TTR reverse, 5’-CTC CGA GGT GTC ATC ACG AG-3’; human β-actin forward, 5’-TTG GCA TAG AGG TCT TTA CGA A-3’; murine β-actin reverse, 5’-GCA CCA CCC TCT CTA CAA TG-3’; murine TTR forward, 5’-CAT GAA TTC GCC GAT GTG T-3’; murine TTR reverse, 5’-GAT GGA TGT AGT GGC CAT G-3’.

The PCR parameters were initial denaturation for 15 min. Liver was collected 3 h after the administration of the samples and homogenized with lysis buffers (pH 7.5) using a ULTRA-TURRAX T-25 apparatus including Tris–HCl buffer. After the treatment of PEG-LaC (G3, DSP2)/siRNA polyplex for 24 h, the supernatant samples were mixed with equivalent volume of Laemmli sample buffer (Bio-Rad Laboratories, Tokyo, Japan). The samples was loaded on 10% gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer to nitrocellulose membranes (Bio-Rad Laboratories) was applied at 25 V for 1 h, and the blots were blocked overnight with 2.5% skim milk in phosphate buffered saline (PBS)–Tween at 4°C. The membranes incubated with primary antibody (1: 1000 dilution), a rabbit polyclonal anti-human prealbumin (Dako, Glostrup, Denmark), for 1 h at room temperature. And then, the membranes incubated with secondary antibody (1: 1000 dilution), polyclonal goat anti-rabbit immunoglobulin horseradish peroxidase (Dako), for 1 h at room temperature. ECL Plus Western blotting Detection Reagents (Amersham plc, Amersham, U.K.) were used to visualize the Western blotting.

**Cellular Association** Lac-\(\alpha\)-CDE (G3, DSL1), PEG-LaC (G3, DSP2) and Lipofectamine® 2000 mixed with BLOCK-it™ Alexa Fluor™ Red Fluorescent Control (Alexa Oligo, 0.4 μg) for 15 min at room temperature. Then, HepG2 cells were transfected with various Alexa Oligo polyplexes for 24 h. After washing the cells with PBS (pH 7.4) (Gibico, Tokyo, Japan) twice, the cells were immediately scraped with 1 mL PBS. Samples were filtered with nylon mesh and analyzed with Guava® easyCyte Flow Cytometers (EMD Millipore, MA, U.S.A.).

**Cellular Localization** To investigate the cellular localization of Alexa Oligo polyplex with PEG-LaC (G3, DSP2), HepG2 cells (100,000 cells per well) were incubated with PEG-LaC (G3, DSP2)/Alexa Oligo polyplex for 24 h. Then, the nuclei were stained with Hoechst33342 (DOJINDO, Kumamoto, Japan) for 10 min at 37°C. After a rinse with PBS twice, the cells were observed with fluorescence microscope (KEYENCE Biozero BZ-8000, Tokyo, Japan).

**Quartz Crystal Microbalance (QCM) Analysis** The interaction of peanut lectin (PNA) with PEG-LaC (G3, DSP2) and PEG-LaC (G3, DSP2)/siRNA polypeptide was examined using AffiNex QNü (Initium, Tokyo, Japan). The QCM cell was suffered 0.5 mL (for Affinix QNü) of running buffer (PBS, pH 7.4). The immobilization of PNA-Biotin (J-OIL MILLS, Tokyo, Japan) on the sensor was carried out using NeutrAvidin-Biotin interaction by the manufacturer's protocol. After replacement of running buffer, the solution containing PEG-LaC (G3, DSP2) alone or PEG-LaC (G3, DSP2)/siRNA polypeptide was injected into cuvette. A charge ratio and siRNA concentration were 5 and 400 nM, respectively. The interaction was measured until the frequency change reached equilibrium. The results were analyzed by Scatchard plot, and the kinetic parameters were calculated using an AQUA 2.0 software.

**Pharmacokinetic Properties of Polyplex** PEG-LaC/siRNA and Lac-\(\alpha\)-CDE/siRNA polyplexes at a dose of 10 μg of siRNA in 500 μL of 5% mannitol solution were injected into healthy male BALB/c mice (4 weeks old, approx. 20 g) via tail vein in 30 s under anesthesia. The charge ratios of PEG-LaC/siRNA and Lac-\(\alpha\)-CDE/siRNA polyplexes were 5 and 2, respectively. Blood samples were collected via orbital vein. Then, plasma was recovered after centrifugation at 3000 rpm for 15 min. Liver was collected 3 h after the administration of the samples and homogenized with lysis buffers (pH 7.5) using a ULTRA-TURRAX T-25 apparatus including Tris–HCl buffer.
creatinine (CRE), blood urea nitrogen (BUN), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) were performed by Scheffe’s test. In all statistical analysis, the mean ± standard error of the mean (S.E.M.) was calculated. Statistical analyses were performed by Scheffe’s test. In all statistical analysis, p-value <0.05 was considered significant.

RESULTS

TTR Silencing Effect of PEG-LaC (G3) at siRNA Polyplexes in Vitro To investigate whether the DSP value of PEG-LaC (G3) could influence the gene silencing effect, we examined the gene silencing efficiency of the PEG-LaC (G3)/siRNA polyplex in HepG2 cells (Fig. 1B). Here, we employed siCont, scrambled sequence siRNA, and siTTR, TTR mRNA targeted siRNA. As previously reported,16) Lac-α-CDE (G3, DSL1)/siTTR polyplex elicited TTR mRNA down-regulation. Importantly, PEG-LaC (G3, DSP2) also possessed siRNA transfer activity in vitro, although it has PEG chain in the molecule. In addition, the highest gene silencing efficiency was achieved by PEG-LaC (G3, DSP2)/siTTR polyplex, which was higher than that of the other PEG-LaCs (G3, DSP4 and 6)/siTTR polyplex, indicating that PEG-LaC (G3, DSP2)/siTTR polyplex has the greatest gene silencing effect among various PEG-LaCs (G3) used in this study. PEG-LaC (G3, DSP2)/siTTR polyplex also inhibited the TTR mRNA expression in a siTTR dose-dependent fashion, and roughly 40% of TTR mRNA silencing effect was shown at 400 nM siTTR (Fig. 1C). Among some charge ratios, PEG-LaC (G3, DSP2)/siTTR polyplex at the charge ratios of 20 and 50 (PEG-LaC (G3, DSP2)/siTTR) provided higher gene silencing effect than PEG-LaC (G3, DSP2)/siTTR polyplex at a charge ratio of 5 (Fig. 1D). Likewise, PEG-LaC (G3, DSP2)/siTTR polyplex down-regulated approx. 50% of the TTR protein concentration in the culture supernatant, compared with the siCont polyplex (Fig. 1E), suggesting that siTTR polyplex with PEG-LaC (G3, DSP2) has the gene silencing effect against TTR expression in vitro. Thus, PEG-LaC (G3, DSP2)/siTTR polyplex has the TTR silencing effect in vitro.

Next, to reveal the role of α-CyD and lactose in the PEG-LaC (G3, DSP2) molecule, we prepared PEG-Lac-dendrimer (G3, DSP2), which lacks an α-CyD molecule, and PEG-α-CDE (G3, DSP3), which lacks a lactose moiety, and then evaluated their gene silencing effects (Fig. 1F). PEG-LaC (G3, DSP2)/siTTR polyplex showed higher gene silencing effects compared to PEG-Lac-dendrimer (G3, DSP2)/siTTR polyplex and PEG-α-CDE (G3, DSP3)/siTTR polyplex. In our previous work, we reported that high gene or siRNA transfer activity of α-CDE (G3) is attributed to high endosomal escaping activity, resulting from the cooperative effect of the interaction between an α-CyD moiety and phospholipids in endosome membrane and the proton sponge effect of dendrimer.21–23) Thus, both α-CyD and lactose moieties of PEG-LaC (G3, DSP2) play important roles for the gene silencing effect.

Cellular Uptake and Cellular Localization of PEG-LaC (G3, DSP2)/siRNA Polyplex To clarify the mechanism for the ASGPR-mediated gene silencing effects of the siRNA polyplex with PEG-LaC (G3, DSP2), we investigated cellular uptake of PEG-LaC (G3, DSP2)/Alexa Oligo polyplex in HepG2 cells after 24 h of transfection using a flow cytometric analysis (Fig. 2). After treatment of HepG2 cells with PEG-LaC (G3, DSP2)/Alexa Oligo polyplex, uptake of the Alexa Oligo by HepG2 cells significantly reduced in the presence of AF, but not bovine serum albumin (BSA) (Figs. 2A, B).

Interaction between serum components and a carrier/siRNA polyplex causes low cellular uptake, dissociation and enzymatic degradation of siRNA in the polyplex, resulting in decrease in the siRNA transfer activity.24,25) Therefore, we next investigated the cellular uptake of various carriers/Alexa Oligo complexes in the presence and absence of FBS. Cellular uptake of PEG-LaC (G3, DSP2)/Alexa Oligo polyplex was maintained even in the presence of FBS condition (Fig. 2C). In contrast, cellular uptake of Lipofectamine® 2000/ Alexa Oligo lipoplex and Lac-α-CDE (G3, DSL1)/Alexa Oligo polyplex were significantly suppressed in the presence of FBS, suggesting that PEG-LaC (G3, DSP2)/siRNA polyplex has the high serum resistance.

To clarify the endocytosis mechanism of PEG-LaC (G3, DSP2)/siRNA polyplex, we investigated the effects of endocytosis inhibitors such as clathrin-dependent endocytosis inhibitor (chlorpromazine, sucrose), caveolae-dependent endocytosis inhibitor (nystatin) and micropinocytosis inhibitor (amiloride),26) on the cellular uptake of PEG-LaC (G3, DSP2)/Alexa Oligo polyplex in HepG2 cells. Cellular uptake of PEG-LaC (G3, DSP2)/Alexa Oligo polyplex was decreased by

(20 mM), Triton X-100 (0.05%) and ethylenediaminetetraacetic acid (EDTA) (2 mM). Lysates were centrifuged at 5000 rpm for 10 min, and 100 µL supernatants were collected. Fluorescent intensity was determined using fluorescent microplate reader Infinite® M1000 PRO (Tecan Group Ltd., Männedorf, Switzerland).

Gene Silencing Effects of Polyplex in Vivo PEG-LaC/siRNA polyplexes at a dose of 20 µg (1 mg/kg) or 100 µg (5 mg/kg) of siRNA in 500 µL of 5% mannitol solution were injected into healthy male BALB/c mice (4 weeks old, approx. 20 g) via tail vein in 30 s under anesthesia. A charge ratio of the polyplex was 5 or 20. The mice were sacrificed 72 h after polyplex administration, and then liver was collected and snap frozen in liquid nitrogen. In the case of repeated administration, the polyplexes were intravenously administrated at day 0 and day 3, and the mice were sacrificed 1 week after the first administration. The frozen liver and 1 mL TRIzol Reagent was added to the Lysing Matrix D (MP Biomedicals Japan, Tokyo, Japan), and the sample was homogenized using a FastPrep instrument for 20 s at a speed setting of 6.0. Homogenized sample was centrifuged at 12000 rpm for 5 min at 4°C, and then the supernatant was transferred to 1.5 mL tube. Total RNA extraction from liver samples using TRIzol Reagent, and the RNA was subsequently reverse-transcribed to cDNA using PrimeScript™ RT reagent Kit. Each procedure was followed the manufacture’s instruction.

Blood Chemistry Parameter For the measurement of blood chemistry parameters, blood samples were collected from the vital artery 72 h after the administration of PEG-LaC (G3, DSP2)/siRNA polyplex, at a dose of siRNA was 20 µg. A charge ratio of PEG-LaC (G3, DSP2)/siRNA was 5. A charge ratio of the polyplex was 5. The blood was centrifuged and serum sample was stored at −80°C until measurement. Serum creatinine (CRE), blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) values were determined using the JEOL BioMajesty™ JCA-BM2250 (Tokyo, Japan).

Statistical Analysis All results are presented as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed by Scheffe’s test. In all statistical analysis, p-value <0.05 was considered significant.
the addition of sucrose and chlorpromazine (Fig. 2D). However, cellular uptake of PEG-LaC (G3, DSP2)/siRNA polyplex was also decreased by amiloride treatment. Most recently, we found that cellular uptake of α-CDE (G3, DS2) may be associated with caveolea-dependent endocytosis and macropinocytosis in human carcinoma of the nasopharynx KB cells (unpublished data). Therefore, endocytosis properties of α-CDE (G3, DS2) may be maintained in the PEG-LaC (G3, DSP2) system. These results indicate that PEG-LaC (G3, DSP2)/siRNA polyplex is mainly internalized via clathrin-mediated endocytosis.

Next, we observed the cellular localization of PEG-LaC (G3, DSP2)/Alexa Oligo polyplex using a fluorescence microscope in HepG2 cells, because the RNA-induced silencing complex (RISC) is known to be in cytoplasm.27) (Fig. 3). The siRNA polyplex with PEG-LaC (G3, DSP2) was uniformly found in cytoplasm after transfection. In our previous work, α-CDE (G3)/siRNA polyplex and Lac-α-CDE (G3, DSL1)/siRNA polyplex also localized in cytoplasm.16,28) Therefore, PEG-LaC (G3, DSP2) is likely to maintain endosomal escaping ability of α-CDE (G3) and Lac-α-CDE (G3, DSL1).

Cell Viability of PEG-LaC (G3, DSP2)/siRNA Polyplex

Cytotoxicity of cationic carriers/siRNA polyplexes are seri-
ous drawbacks for siRNA delivery.\cite{9,29} Thus, we investigated the cell viability of the various carrier/siRNA polyplexes by WST-1 method. PEG-LαC (G3, DSP2)/siRNA polyplex did not induce severe cytotoxicity in HepG2 cells even in any charge ratio range in transfection condition (Fig. 4A). In contrast, jetPEI\textsuperscript{TM}-Hepatocyte/siRNA polyplex and Lipofectamine\textsuperscript{®} 2000/siRNA lipoplex significantly decreased cell viability in a carrier dose-dependent fashion (Figs. 4B, C). These results indicate that PEG-LαC (G3, DSP2) is an in vitro safer carrier than the commercial available transfection reagents used in this experimental condition.

**Physicochemical Characterization of PEG-LαC (G3, DSP2)/siRNA Polyplex** To determine physicochemical properties of PEG-LαC (G3, DSP2)/siRNA polyplex, we firstly performed a agarose gel retardation assay (Fig. S1). The bands of siRNA were observed at lower charge ratios because of the negative net charge of the polyplexes. Complexation of siRNA with PEG-LαC (G3, DSP2) prevented siRNA migration at a charge ratio of 5, indicating PEG-LαC (G3, DSP2) completely formed polyplexes with siRNA at a charge ratio of 5.

We next measured the particle sizes, polydispersity index and ζ-potential values of PEG-LαC (G3, DSP2) and PEG-LαC (G3, DSP2)/siRNA polyplex (Table S1). The particle size of PEG-LαC (G3, DSP2)/siRNA polyplex at a charge ratio of 20 (72.2 nm) was smaller than that at a charge ratio of 5 (142.0 nm) with somewhat high polydispersity index values. The ζ-potential value of PEG-LαC (G3, DSP2)/siRNA polyplex showed 4.08 mV at a charge ratio of 20.

To make sure whether PEG-LαC (G3, DSP2) alone and PEG-LαC (G3, DSP2)/siRNA polyplex bind to ASGPR, a dissociation constant of PEG-LαC (G3, DSP2) with PNA, a galactose binding lectin, was determined by QCM method (Table 1). The dissociation constant ($K_d$) of PEG-LαC (G3, DSP2)/siRNA polyplex showed significantly smaller than that of PEG-LαC (G3, DSP2) alone, suggesting that PEG-LαC (G3, DSP2)/siRNA polyplex is strongly recognized by ASGPR, compared to PEG-LαC (G3, DSP2) alone.

**Pharmacokinetic Properties of PEG-LαC (G3, DSP2)/siRNA Polyplex** To reveal the pharmacokinetics properties of PEG-LαC (G3, DSP2)/siRNA polyplex, we measured fluorescein isothiocyanate (FITC)-siRNA and TRITC-PEG-LαC (G3, DSP2) levels in the plasma (Figs. 5A, B). After the intravenous administration to mice, the plasma FITC-siRNA levels decreased to below 10% of the total concentration within 10 min. Meanwhile, elimination from blood circulation of TRITC-PEG-LαC (G3, DSP2) was slower than that of TRITC-Lac-α-CDE (G3, DSL1).

Next, we evaluated the liver accumulation of TRITC-PEG-LαC (G3, DSP2)/FITC-siRNA polyplex (Figs. 5C, D). When mice were intravenously administered with polyplex, the liver accumulation levels of FITC-siRNA and TRITC-PEG-LαC (G3, DSP2) in the PEG-LαC (G3, DSP2) system were higher than those in the Lac-α-CDE (G3, DSL1) system, respectively. Three h after the administration of TRITC-PEG-LαC (G3, DSP2)/FITC-siRNA polyplex, approximately 15% and 8% of TRITC-PEG-LαC (G3, DSP2) and FITC-siRNA to the dose were found in liver, respectively. These results indicate that both TRITC-PEG-LαC (G3, DSP2) and FITC-siRNA accumulate in liver of mice after intravenous administration.

**Gene Silencing Effects of PEG-LαC (G3, DSP2)/siRNA Polyplex in Vivo** To investigate the in vivo gene silencing effect of PEG-LαC (G3, DSP2)/siTTR polyplex, we examine the TTR silencing effect of the carriers/siTTR polyplexes in BALB/c mice after intravenous administration (Fig. 6A). Here, we employed the relatively low charge ratio of 5 as a safer system for in vivo experiments. After 72 h administration, PEG-LαC (G3, DSP2)/siTTR polyplex (1 mg/kg siRNA) did not show TTR mRNA suppression. Also, jetPEI\textsuperscript{TM}-Hepatocyte/siTTR polyplex (1 mg/kg siRNA) did not show the RNAi effect in liver (data not shown). At a dose of 5 mg/kg of siRNA, PEG-LαC (G3, DSP2)/siTTR polyplex, not Lac-α-CDE (G3, DSL1)/siTTR polyplex, elicited TTR silencing effect in the mice liver compared to siCont group. Then, we examined the effects of repeated intravenous administra-

![Fig. 4. Cell Viability of the siRNA Polyplexes with (A) PEG-LαC (G3, DSP2), (B) jetPEI\textsuperscript{TM}-Hepatocyte and (C) Lipofectamine\textsuperscript{®} 2000 in HepG2 Cells](image-url)
tion on TTR silencing effect of PEG-LaC (G3, DSP2)/siRNA polyplex in mice (Fig. 6B). Importantly, two-times intravenous administration of PEG-LaC (G3, DSP2)/siRNA polyplex induced roughly 50% TTR silencing effect in mice liver. These results indicate the potential of PEG-LaC (G3, DSP2)/siRNA polyplex as in vivo systemic RNAi effect agent for TTR-related amyloidosis.

To reveal the safety profiles in vivo, we determined the blood chemistry parameters, such as CRE, BUN, AST, ALT and LDH of PEG-LaC (G3, DSP2)/siRNA polyplex (1 mg/kg siRNA) after intravenous administration (Table 2). These blood chemistry values were almost the same as those of the control, suggesting that PEG-LaC (G3, DSP2)/siRNA polyplex shows in vivo safety profiles.

**DISCUSSION**

Recently, we have demonstrated that the utility of Lac-α-CDE (G3, DSL1)/siRNA/polysaccharide sacran ternary polyplex as a hepatocyte-targeted siRNA delivery system. However, the serum stability and in vivo gene silencing effects were not enough. Also, we previously reported the utility of PEG-LaC (G3)/pDNA polyplex as a hepatocyte-specific pDNA delivery system. It is well understood that physicochemical properties, efficacy, intracellular trafficking and cytotoxicity is extremely different between siRNA polyplex and pDNA.

**Fig. 5.** Pharmacokinetic Properties of PEG-LaC (G3, DSP2)/siRNA Polyplex in BALB/c Mice

(A, B) Blood level profile of Lac-α-CDE (G3, DSL1)/siRNA and PEG-LaC (G3, DSP2)/siRNA Polyplex. Mice were intravenously administrated with TRITC-carrier/FITC-siRNA polyplex. Blood was collected via retro-orbital vein 5, 10, 30, 60 and 180 min after the administration of polyplex. The dose of siRNA was 10 μg. Data are presented as mean ± S.E.M. (n = 3). *p < 0.05, compared with Lac-α-CDE (G3, DSL1)/siRNA polyplex. (C, D) Liver accumulation of Lac-α-CDE (G3, DSL1)/siRNA and PEG-LaC (G3, DSP2)/siRNA Polyplex. Liver was collected 3 h after the administration of polyplex. The dose of siRNA was 10 μg. Data are presented as mean ± S.E.M. (n = 3). *p < 0.05, compared with Lac-α-CDE (G3, DSL1)/siRNA polyplex.

**Fig. 6.** Gene Silencing Effects of PEG-LaC (G3, DSP2)/siRNA Polyplex in Vivo

Expression of murine TTR mRNA in BALB/c mice following (A) single or (B) two-times intravenous administration of PEG-LaC (G3, DSP2)/siRNA polyplexes at charge ratio of 5. Data are presented as mean ± S.E.M. (n = 3–4). *p < 0.05, compared with siCont.
polyplex, and optimization of polyplex formulation procedure is required for effective siRNA or pDNA delivery.\cite{26,27} Therefore, in this study, we evaluated the utility of PEG-LaCs (G3)/siRNA polyplex as novel hepatocyte-specific siRNA delivery system to improve stability, half-life in blood circulation and the in vivo gene silencing effect of the polyplex.

PEG-LaC (G3, DSP2)/siTTR polyplex showed much higher gene silencing effect than those of the other PEG-LaCs (G3, DSP4 and 6) polyplexes in HepG2 cells. This could be due to a weak interaction of PEG-LaCs (G3, DSP4 and 6) with siRNA, resulting from reduce the number of a positive charge of amino group of dendrimer. In addition, PEG modification is acknowledged to reduce in vitro cellular uptake of the polyplex.\cite{28} In fact, cellular uptake of PEG-LaC (G3, DSP6) was markedly decreased compared to that of PEG-LaC (G3, DSP2 and 4) (data not shown). Of various charge ratios, PEG-LaC (G3, DSP2)/siTTR polyplex induced greatest gene silencing effect at a charge ratio of 20 (Fig. 1D). This is probably due to a formation of a stable polyplex,\cite{29,30} high cellular uptake, efficient endosomal escape and negligible cytotoxicity. Moreover, PEG-LaC (G3, DSP2)/siTTR polyplex also suppressed TTR expression in protein level (Fig. 1E). Generally, in the treatment of amyloidosis such as AA amyloidosis and AL amyloidosis, more than 50% of reduction of the amyloidogenic protein is associated with substantial survival benefit.\cite{31,32} Thereby, PEG-LaC (G3, DSP2)/siTTR polyplex could be expected to be the potent new approach for siRNA-based TTR related amyloidosis therapy under in vitro experimental conditions.

The remarkable gene silencing effects of PEG-LaC (G3, DSP2)/siTTR polyplex are attributed to the high cellular uptake activity via ASGPR (Figs. 2A, B), localization of siRNA in cytoplasm (Fig. 3) and negligible cytotoxicity (Fig. 4). Notably, the endosomal escaping ability of PEG-LaC (G3, DSP2) can be attributed to the cooperative effect of the proton sponge effect of dendrimer and interaction between phospholipids of endosomal membranes and α-CyD as reported previously.\cite{33} In fact, PEG-LaC (G3, DSP2)/siTTR polyplex elicited higher RNAi effect than that with PEG-Lac-dendrimer (G3, DSP2), which lacks an α-CyD molecule (Fig. 1F). Additionally, after treatment with PEG-LaC (G3, DSP2)/siRNA polyplex to HepG2 cells, siRNA was located in cytoplasm (Fig. 3). Intracellular trafficking analysis previously demonstrated that RISC loading efficacy is extremely low, only less than 1% of siRNA can load to the RISC after cationic lipid/siRNA lipoplex transfection.\cite{34,35} Therefore, a detailed study of PEG-LaC (G3, DSP2)/siRNA polyplex trafficking should be performed. High serum resistance of PEG-LaC (G3, DSP2)/siRNA polyplex could be also important for its remarkable RNAi effects (Fig. 2C). In general, PEG modification of carriers increases the stability of the polyplexes through the reduced interaction with blood components.\cite{36} For these reasons, PEG-LaC (G3, DSP2)/siRNA polyplex may show serum resistance.

The mean diameter and ζ-potential values of PEG-LaC (G3, DSP2)/siRNA polyplexes were about 70 nm and 4 mV, respectively (Table S1). Herein, Wisse et al. reported that liver sinusoidal endothelial cells has approx. 100 nm fenestrae.\cite{37} In addition, Mahato et al. demonstrated that excess cationic charged polyplexes interact with blood protein, erythrocytes and vessel endothelia.\cite{38} Thereafter, these results indicate that PEG-LaC (G3, DSP2)/siRNA polyplexes has the desirable physicochemical properties for systemic administration in vivo.

PEG-LaC (G3, DSP2)/siRNA and PEG-LaC (G3, DSP2)/siRNA polyplexes were recognized by ASGPR (Table 1). In addition, ASGPR recognition of PEG-LaC (G3, DSP2)/siRNA polyplex was markedly stronger than that of PEG-LaC (G3, DSP2) alone. It is well known that polypeptide subunit of the ASGPR can associate with at least a single terminal galactose N-acetylgalactosamine residue,\cite{39} and ASGPR strongly recognize the multivalent galactose residues compared to single galactose residue.\cite{40} Moreover, the studies using asialoglycopeptides from naturally occurring glycopeptides,\cite{41,42} as well as synthetic cluster glycosides\cite{43,44} have revealed that clustering galactose residues significantly enhances the association with ASGPR. In PEG-LaC (G3, DSP2)/siRNA polyplex, the lactose density of particle surface should be higher than that of PEG-LaC (G3, DSP2) alone, suggesting that PEG-LaC (G3, DSP2)/siRNA polyplex is strongly recognized by ASGPR than PEG-LaC (G3, DSP2) alone.

In vivo study demonstrated that PEG-LaC (G3, DSP2)/siTTR polyplex (5 mg/kg siRNA) elicited higher in vivo gene silencing effect than Lac-α-CDE (G3, DSL1)/siTTR polyplex after intravenous administration (Fig. 6). This could be related to the high serum stability (Fig. 2C) and liver accumulation (Fig. 5) of PEG-LaC (G3, DSP2)/siTTR polyplex, compared to that of Lac-α-CDE (G3, DSL1)/siTTR polyplex. By suppressing the production of TTR from the liver, a reduction of amyloid deposition and delay of progression are expected. Importantly, the blood chemistry parameters of PEG-LaC (G3, DSP2)/siTTR polyplex treatment were almost the same compared to control (Table 2). This in vivo safety profile of PEG-LaC (G3, DSP2)/siTTR polyplex corresponds with the negligible cytotoxicity in vitro (Fig. 4). Roberts et al. demonstrated that higher generation dendrimers cause severe cytotoxicity, compared to lower generation dendrimers to Chinese hamster lung fibroblasts. In this study, we employed relatively low generation (G3) dendrimer, that is PEG-LaC (G3, DSP2), which PEG, α-CyD and lactose moieties were introduced to the amino groups molecule of the dendrimer. For these reasons, PEG-LaC (G3, DSP2)/siTTR polyplex was exhibited a high in vitro and in vivo safety profile.

Table 2. Blood Chemistry Parameters in BALB/c Mice after the Treatment of PEG-LaC (G3, DSP2)/siRNA Polyplex

| Carrier          | CRE (mg/dL) | BUN (mg/dL) | AST (U/L) | ALT (U/L) | LDH (U/L) |
|------------------|-------------|-------------|-----------|-----------|-----------|
| Control          | 0.11 ± 0.01 | 22.8 ± 2.08 | 65.8 ± 13.2 | 34.0 ± 8.61 | 388 ± 79.7 |
| jetPEI\textsuperscript{30}-Hepatocyte | 0.15 ± 0.01 | 23.2 ± 2.47 | 50.5 ± 2.08 | 34.7 ± 2.28 | 238 ± 11.1 |
| PEG-LaC (G3, DSP2) | 0.12 ± 0.01 | 16.5 ± 0.65 | 47.8 ± 2.39 | 23.5 ± 0.01 | 298 ± 35.3 |

Mice were intravenously administrated with PEG-LaC (G3, DSP2)/siRNA or jetPEI\textsuperscript{30}-Hepatocyte/siRNA polyplex. Blood was collected via vital artery 72 h after the administration of polyplex. The charge ratios of PEG-LaC (G3, DSP2)/siRNA and jetPEI\textsuperscript{30}-Hepatocyte/siRNA were 5 and 1, respectively. Data are presented as mean ± S.E.M. (n = 3–8).
CONCLUSION

We demonstrated that PEG-LαC (G3, DSP2)/siRNA polyplex induces the potent gene silencing effects in vitro due to high cellular uptake ability via ASGPR, serum stability, efficient endosomal escape and cytoplasm localization without cytotoxicity. Furthermore, PEG-LαC (G3, DSP2)/siRNA polyplex elicited significant in vivo gene silencing effects without side effects after intravenous administration, resulting from increase of the polyplex serum stability and liver accumulation. Therefore, these findings indicate that PEG-LαC (G3, DSP2) has utility as a hepatocyte-specific siRNA delivery system in vitro and in vivo. Moreover, PEG-LαC (G3, DSP2)/siTTR polyplex has possibility as a novel polymer-based approach for TTR-related amyloidosis therapy.

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Conflict of Interest The authors declare no conflict of interest.

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