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Reducible Hyaluronic Acid–siRNA Conjugate for Target Specific Gene Silencing

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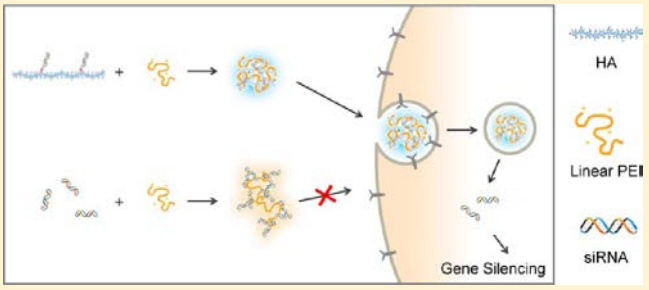
ABSTRACT: Despite wide applications of polymer–drug conjugates, there are only a few polymer–siRNA conjugates like poly(ethylene glycol) conjugated siRNA. In this work, reducible hyaluronic acid (HA)–siRNA conjugate was successfully developed for target specific systemic delivery of siRNA to the liver. The conjugation of siRNA to HA made it possible to form a compact nanocomplex of siRNA with relatively nontoxic linear polyethyleneimine (LPEI). After characterization of HA–siRNA conjugate by size exclusion chromatography (SEC) and gel electrophoresis, its complex formation with LPEI was investigated with a particle analyzer. The HA–siRNA/LPEI complex had a mean particle size of *ca.* 250 nm and a negative or neutral surface charge at physiological condition. The reducible HA–siRNA/LPEI complex showed a higher *in vitro* gene silencing efficiency than noncleavable HA–siRNA/LPEI complex. Furthermore, after systemic delivery, apolipoprotein B (ApoB) specific HA–siApoB/LPEI complex was target specifically delivered to the liver, which resulted in statistically significant reduction of ApoB mRNA expression in a dose dependent manner. The HA–siRNA conjugate can be effectively applied as a model system to the treatment of liver diseases using various siRNAs and relatively nontoxic polycations.



INTRODUCTION

Since the RNA induced interfering phenomenon was reported in 1998, small interfering RNA (siRNA) has been spotlighted as a new paradigm therapeutic.[1] Despite wide investigation for further development, however, there is no clinically available product, most likely due to difficulties in the delivery of siRNA to target cells.[2−4] To date, various nonviral vectors, such as peptides,[5] proteins,[6] natural or synthetic polymers,[7−9] and lipid-like carriers,[10,11] have been investigated for siRNA delivery. In addition, it has been reported that direct conjugation of peptides,[12,13] proteins,[14] lipids,[15] or polymers[16,17] to siRNA resulted in significant improvement of *in vivo* siRNA delivery and gene silencing efficiency. Furthermore, siRNA–poly(ethylene glycol) (PEG) conjugate system has been developed just like PEGylated protein drugs with a market of tens of billion dollars to enhance serum stability and minimize nonspecific interactions with blood components.[18,19]

Most conventional siRNA delivery carriers have been originally applied to plasmid DNA delivery because they have similar delivery barriers.[4] However, siRNA has a lower density of negative charge and a higher stiffness than plasmid DNA, resulting in inefficient siRNA delivery.[20] To circumvent these problems, long double-stranded RNAs with more than 21 base pairs were prepared and exhibited an enhanced gene-silencing efficiency with prolonged RNA interference effects.[21] Unexpectedly, long RNA molecules caused undesirable immune responses due to the innate immunity by toll-like receptor (TLR) binding. Bolcato-Bellemin et al. reported gene-like-siRNA using short complementary overhangs to increase the complex stability.[22] Recently, the self-cross-linked and multimerized siRNA with cleavable disulfide bonds was reported to increase charge density and flexibility.[23] By interleaving the disulfide bonds, lengthened siRNA splits back into single molecules in the cytosol with a high concentration of glutathione, which makes it easy for siRNA to interact with RISC proteins.

We previously reported reducible polyethyleneimine (rPEI)–hyaluronic acid (HA) conjugate to make a complex with siRNA for the treatment of tumor[24] and liver cirrhosis.[25] The polyanionic HA prevented the nonspecific interaction between serum protein and polycationic rPEI. Furthermore, HA receptors at target tissues, especially abundant in the liver, facilitated the target specific delivery of siRNA/rPEI-HA complex by HA receptor mediated endocytosis.[26] In this work, reducible HA–siRNA conjugate was developed for target specific systemic delivery of hepatic siRNA. Since HA is a natural polyanion in the body, the conjugation of siRNA to HA can make it possible to form a compact nanocomplex of siRNA with relatively nontoxic weak polycations like linear polyethyleneimine (LPEI). HA–siRNA conjugate was characterized by size exclusion chromatography (SEC) and gel electrophoresis, and its complex formation with LPEI was characterized with a particle analyzer. After *in vitro* gene silencing tests, systemic delivery of apolipoprotein B (ApoB) specific HA-siApoB/LPEI complex was carried out for target specific gene silencing in the liver.

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Finally, the HA−siRNA conjugate was discussed as a model system for applications in the treatment of liver diseases.

MATERIALS AND METHODS

Materials. Hyaluronic acid (HA) with a molecular weight (MW) of 100 kDa was purchased from Shiseido (Tokyo, Japan). Linear polyethyleneimine (LPEI) with a MW of 25,000 was obtained from Polyscience (Warrington, PA). Tris(2-carboxyethyl)phosphine (TCEP), dimethyl sulfoxide (DMSO), and 1,4-diaminobutane (DAB) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Hydroxybenzotriazole monohydrate (HOBt) was obtained from Daejung Chemicals & Metals (Shiheung, Korea). Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and N-ε-malemidocaproyl-oxysuccinimide ester (EMCS) were purchased from Thermo Scientific (Rockford, IL), and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) hydrochloride was purchased from Tokyo Chemical Industry (Tokyo, Japan). The modified siRNA was purchased from Bioneer (Daejeon, Korea). The sequences of siLuc were 5′-CCA CAC UAU UUA GCU UCU UdTdT-3′ (sense) and 5′-AGA AGC UAA AUA GUG UGG dTdT-3′ (antisense). The sequences of siPRK2 were 5′-UCA AAG AAG GAG CUG AAA AUU dTdT-3′ (sense) and 5′-AAU UUU CAG CUC CUU CUU UGA dTdT-3′ (antisense). The sequences of siApoB were 5′-GUC AUC ACA CUG AAU ACC AAU dTdT-3′ (sense) and 5′-AUU GGU AUU CAG UGU GAU GAC-3′ (antisense). The 3′- and 5′-ends of sense strand were modified to thiol group and Cy3, respectively. All reagents were used without further purification.

Synthesis and Characterization of HA−siRNA Conjugate. For the synthesis of HA−siRNA conjugate, HA was chemically modified with DAB as reported elsewhere.[27] Briefly, 1 g of HA with a MW of 100 kDa was dissolved in 200 mL of distilled water. DAB at 10 molar ratio of HA repeating unit was added to the HA solution. Then, EDC and HOBt at 4 molar ratio of HA were added to activate the carboxyl groups of HA. The pH of the mixed solution was adjusted to 4.8 with 1 N HCl. After reaction at room temperature for 20 min, the resulting HA-DAB conjugate was dialyzed against distilled water for 5 days. The purified conjugate solution was lyophilized for 3 days. The degree of HA modification was analyzed by ^{1}H NMR (DRX-500, Bruker, Germany). Then, the synthesized HA-DAB was dissolved in water. SPDP or EMCS at 2 molar ratio of amine groups of HA-DAB was dissolved in DMSO and added to the HA-DAB solution. After reaction at room temperature for 2 h, the resulting solution was dialyzed against distilled water for 12 h and lyophilized for 3 days. The degree of modification was analyzed by measuring UV absorbance and ^{1}H NMR. After that, siRNA with thiol group was added to HA-DAB-SPDP or HA-DAB-EMCS solution at various molar ratios of pyridyl or maleimide group and reacted for 6 h. The final crude products were purified by the fractionation using SEC with the following system: Waters 717 plus autosampler, Waters 1525 binary HPLC pump, Waters 2487 dual λ absorbance detector, and Superdex200 10/300 GL (GE Healthcare) column. The detection wavelengths were 210 nm for HA and 260 nm for siRNA. The conjugation was also analyzed by gel electrophoresis. HA−siRNA conjugates were loaded in the well of 1.0 wt % agarose gel containing ethidium bromide at 0.1 μg/mL of siRNA concentration, which was applied to 150 V electrodes in 50 mM borate buffer (pH 8.98) for 20 min. To check the release of siRNA from the conjugate in response to a reducing agent, 1 μg/mL of TCEP solution was mixed with the conjugate solution before loading. The siRNA was visualized by bromide staining and the gel image was taken under UV. For the serum stability test, HA−siRNA conjugates at a concentration of 0.1 μg/mL of siRNA were incubated with 95% fetal bovine serum (FBS) at 37 °C for 12, 24, and 36 h.

Preparation and Characterization of HA−siRNA/LPEI Complex. HA−siRNA/LPEI complex was prepared by mixing HA−siRNA conjugate with the specified amount of LPEI solution (10 mg/mL) followed by incubation in 150 mM of NaCl solution for 15 min. The N/P ratio of LPEI to siRNA varied from 5 to 20. The hydrodynamic volume and surface charge of HA−siRNA/LPEI complex were measured at 25 °C with a particle analyzer (Zetasizer Nano, Malvern Instrument Co., UK) after dilution of the complex solution with 1 mL of PBS. Each formulation was made in triplicate and analyzed independently. Then, HA−siRNA/LPEI complex at an N/P ratio of 10 was used for the following experiments.

Cell Culture and Cytotoxicity Test. MDA-MB-231, MCF7, and HSC-T6 cells were cultured in 5% CO_2 incubator at 37 °C. DMEM was supplemented with 10 vol % FBS and 10 IU/mL of antibiotics (penicillin). The cytotoxicity of HA−siRNA/LPEI complex was evaluated by MTT assay. The different cells at a population of 5×10^3 were dispensed in each well of 96 well-plate. After incubation for a day, fresh medium containing various concentrations of HA−siRNA/LPEI, siRNA/LPEI, or siRNA/bPEI complexes was added and incubated for 24 h. Then, 20 μL of 2 mg/mL MTT solution in DMEM was added to each well and incubated at 37 °C for 2 h. After removal of the medium containing MTT, 100 μL of DMSO was added to dissolve the formazan crystal formed by live cells. The optical density was measured at 540 nm with an absorbance microplate reader (EMax microplate reader, Bucher Biotec AG, Basel, Switzerland). Cell viability (%) was calculated by the following equation: Cell viability (%) = $[OD_{540(sample)}/OD_{540(control)}] \times 100$, where $OD_{540(sample)}$ represents the optical density from the wells treated with samples and $OD_{540(control)}$ represents that from the wells treated with PBS.

Confocal Microscopy for the Cellular Uptake. The intracellular delivery of HA−siRNA/LPEI complex was assessed by con-focal microscopy. For the analysis, MDA-MB-231 cells were placed on culture slides (Bedford, MA) at a density of 1.0×10^4 cells/well and incubated for 24 h. Then, the culture medium was replaced with fresh nonserum medium containing 10 μg/mL of chloropromazine, 50 μg/mL of nastatin, or 50 μg/mL of wortmannin. After incubation for 3 h, Cy3 labeled HA−siRNA/LPEI complex was added and further incubated for longer than 3 h. Then, the cells were washed with cold PBS and fixed with 4 wt % paraformaldehyde. The cells were observed with a confocal laser scanning microscope (LSM 510, Carl-Zeiss Inc., Thornwood, NY).

***In Vitro* Gene Silencing of HA−siRNA/LPEI Complex.** *In vitro* gene silencing efficiency of HA−siRNA/LPEI complex was evaluated in MDA-MB-231, MCF7 cells, which stably express the luciferase gene, and HSC-T6 cells. The cells were dispensed on 96-well plate at a density of 5×10^3 cells/well and incubated for 24 h. Then, the culture medium was replaced with 100 μL of DMEM containing HA−siRNA/LPEI complex at various N/P ratios. After 24 h, transfected MDA-MB-231 and MCF7 cells were lysed with a lysis buffer (1% Triton X-100) and 10 μL of the lysed solution was mixed with 25 μL of luciferin solution. The gene silencing efficiency of PRK2 siRNA at HSC-T6 cell was assessed by RT-PCR. HSC-T6 cells were dispensed on 6-well plate at a density of 2×10^5 and incubated for 24 h. Then, various concentrations of HA−

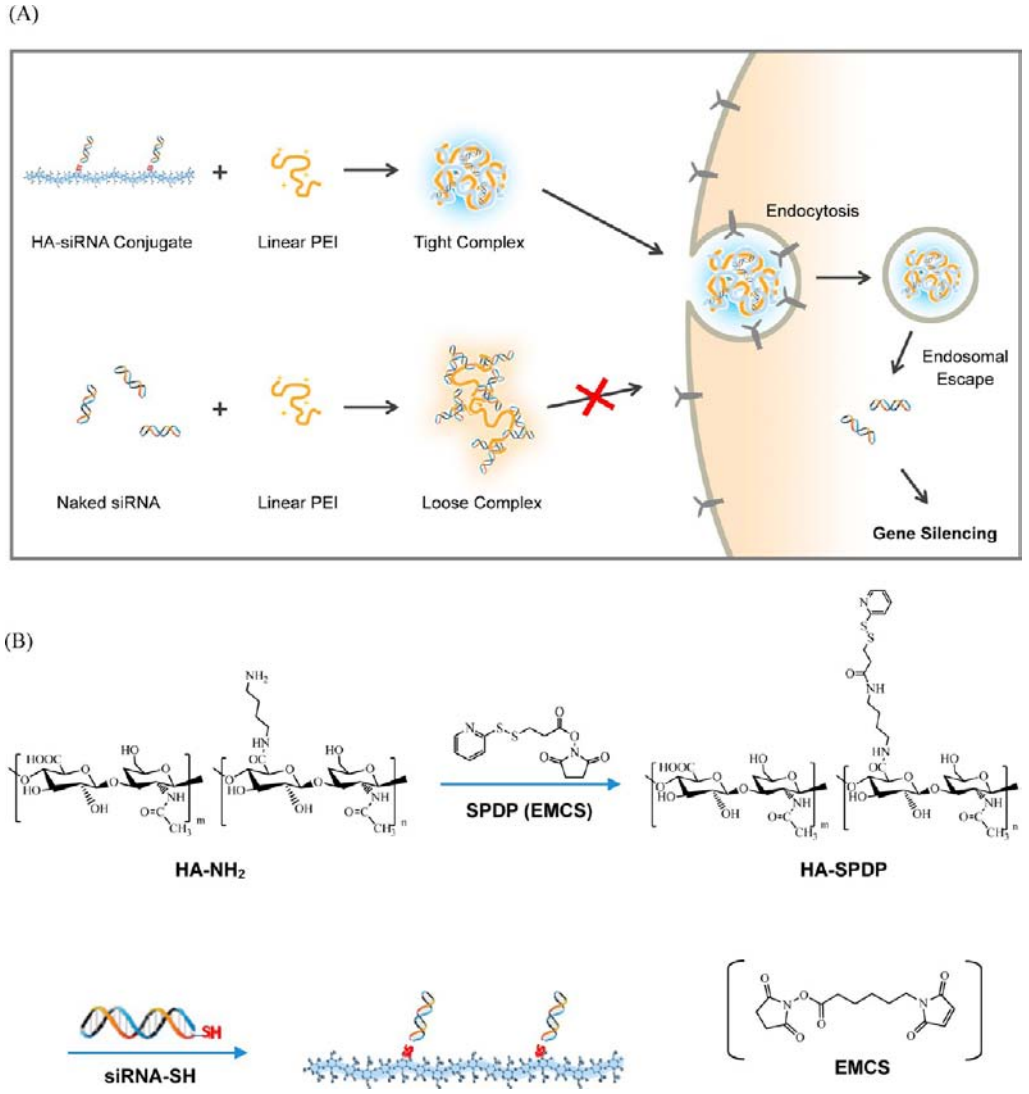

Figure 1. (A) Schematic illustration of the complex formation of HA−siRNA conjugate and naked siRNA with linear polyethyleneimine (LPEI). (B) Schematic representation of the synthesis of reducible and noncleavable HA−siRNA conjugate using SPDP and EMCS.

siRNA/LPEI or siRNA/LPEI complexes were added to cells. To confirm the HA receptor mediated endocytosis, HA−siRNA/LPEI complex was incubated in the DMEM with and without 20 μg/mL of free HA. After 24 h incubation, the total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The total RNA (1 μg) was reverse-transcribed to single strand cDNA using random primers and the Exscript RT reagent kit (TaKaRa, Shiga, Japan). Quantitative RT-PCR was performed using the icycler system (Bio-Rad laboratories). The amount of amplified *PRK2* gene was normalized with that of amplified *GAPDH* gene. In order to investigate the mechanism of cellular uptake of HA−siRNA/LPEI complex, MDA-MB-231 cells were cultured for 24 h and the medium was replaced with DMEM containing 10 μg/mL of chloropromazine, 50 μg/mL of nastatin, or 50 μg/mL of wortmannin. After incubation for 3 h, HA−siRNA/LPEI complexes were treated for gene silencing at the specified concentrations.

***In Vivo* Gene Silencing of HA−siRNA/LPEI Complex.** ApoB specific siRNA was conjugated to HA and complexed with LPEI as described above. HA−siApoB/LPEI complexes at various doses of 10, 20, and 30 μg/head in 200 μL of PBS were systemically delivered to Balb/c mice by tail vein injection. The 30 μg/head dose of naked siApoB/LPEI complex was also administrated as a control group. After 24 h, the mice were sacrificed and the dissected liver was homogenized with a RNA extraction kit according to the manufacturer's protocol. The extracted RNA was reverse-transcribed into cDNA by using Superscript III First-Strand Synthesis System for RT-PCR. The synthesized cDNA was analyzed by RT-PCR (CFX96 Real-Time PCR Detection System, Bio-Rad, CA) with SYBR premix. The sequences of primers were as follows: 5′-TTT TCC TCC CAG ATT TCA AGG-3′ (sense) and 5′-TCC AGC ATT GGT ATT CAG TGT G-3′ (antisense) for *ApoB*, and 5′-CCT TCA TTG ACC TCA ACT AC-3′ (sense) and 5′-GGA AGG CCA TGC CAG TGA GC-3′ (antisense) for *GAPDH*. PCR parameters were as follows: initial denaturation for 5 min at 95 °C followed by 40 cycles of 30 s at 95 °C and 30 s at 53 °C. The amount of amplified *ApoB* gene was normalized with that of amplified *GAPDH* gene.

Statistical Analysis. The data are expressed as means ± SD from several separate experiments. Statistical analysis was carried out via the two-way analysis of variance (ANOVA) tests using the software of SigmaPlot 10.0. * $P < 0.05$ and ** $P < 0.01$ were considered statistically significant.

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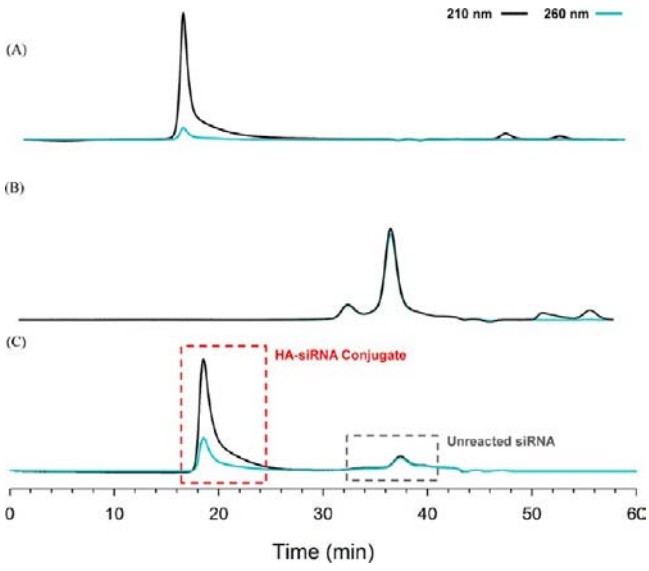

Figure 2. Size exclusion chromatograms of (A) HA-SPDP, (B) siRNA, and (C) HA–siRNA conjugate.

Table 1. Characteristics of HA–siRNA Conjugates

	siRNA per HA chain	substitution ratio (%)	conjugation efficiency (%)
Cleavable HA–siRNA	6.9	2.76	65.1
Noncleavable HA–siRNA	7.2	2.89	70.2

RESULTS AND DISCUSSION

Synthesis and Characterization of HA–siRNA Conjugate. Figure 1 shows a schematic illustration for the preparation of HA–siRNA/LPEI complex for gene silencing applications. Due to the low charge density and high stiffness,[23] siRNA does not make a tight complex with weak polycations like LPEI (Figure 1A). Accordingly, siRNA was complexed with strong cationic polymers, which limited *in vivo* applications of siRNA therapeutics causing a severe cytotoxicity. To alleviate this problem, we conjugated siRNA to flexible and polyanionic HA. Figure 1B shows a schematic representation for the synthesis of HA–siRNA conjugate. Because of the electrostatic repulsion, the conjugation of both negatively charged siRNA and HA was not efficient. Thus, HA was chemically modified with positively charged DAB. The binding affinity of HA to the receptors can be controlled by changing the chemical modification degree of HA with DAB.[26] For target specific systemic delivery, slightly modified HA–DAB conjugates with a modification degree of 19 mol % was used to synthesize HA–siRNA conjugates in the following experiments. Then, HA–DAB was modified with SPDP or EMCS, which was conjugated with thiolated siRNA to prepare cleavable (reducible) or noncleavable HA–siRNA conjugate (Figure 1B). As is well-known, the disulfide bond can be reduced by glutathione in the cytosol.

The successful synthesis of HA–siRNA conjugate was confirmed by SEC and agarose gel electrophoresis. As shown in Figure 2A, the peak of HA–DAB appeared at a retention time of 19 min. The absorbance intensity of HA–DAB was not significant at 260 nm. The peak of siRNA appeared at the retention time of *ca.* 37 min (Figure 2B). After conjugation, however, the absorbance peak of siRNA at 260 nm appeared at the same retention time of HA–DAB (Figure 2C). The unreacted siRNA was removed by fractionation. The siRNA contents in HA–siRNA conjugates were determined by measuring the peak area of naked siRNA detected at 260 nm, which revealed the bioconjugation efficiency up to *ca.* 75%. The characteristics of

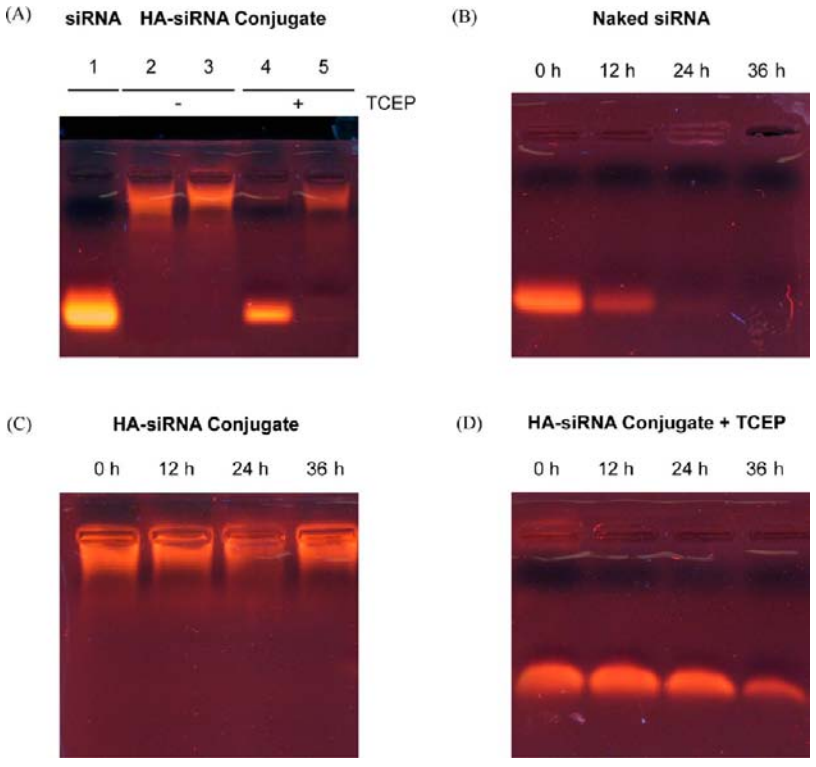

Figure 3. (A) Comparative gel electrophoresis (GE) of HA–siRNA conjugates with cleavable (2 and 4 lanes) and noncleavable linkages (3 and 5 lanes). The GE of (B) naked siRNA, (C) HA–siRNA conjugate, and (D) HA–siRNA conjugate with a reducing agent of TCEP in the serum, respectively.

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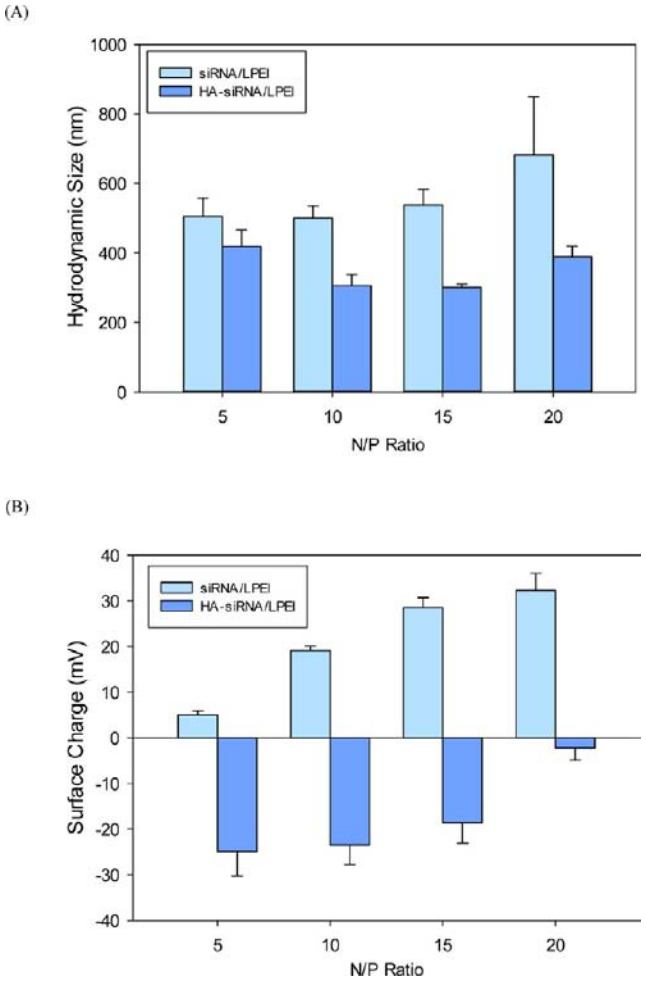

Figure 4. (A) Hydrodynamic particle sizes and (B) surface charges of siRNA/LPEI and HA−siRNA/LPEI complexes with increasing N/P ratios.

HA−siRNA conjugates, used for the following experiments, were summarized in Table 1.

The HA−siRNA conjugate was also characterized by agarose gel electrophoresis (Figure 3). A reducing agent of TCEP was added to confirm the cleavable linkage between HA and siRNA in the conjugate (Figure 3A). While noncleavable HA−siRNA conjugate remained stable, reducible HA−siRNA conjugate was effectively cleaved in the presence of the reducing reagent. Figure 3B,C shows the enhanced stability of HA−siRNA conjugate in the serum. The naked siRNA degraded to *ca.* 3% in 36 h, but *ca.* 80% of siRNA conjugated to HA remained stable even after incubation with FBS for 36 h. After treatment with TCEP, the released siRNA from HA−siRNA conjugate degraded like the naked siRNA (Figure 3B,D). The stability against enzymatic degradation by the conjugation of siRNA to HA might be advantageous for *in vivo* systemic delivery.

Preparation and Characterization of HA−siRNA/LPEI Complex. LPEI has a lower positive charge and a lower cytotoxicity than branched PEI.[28,29] Although LPEI has been widely used for plasmid DNA delivery,[30,31] it does not form a compact complex with short and rigid siRNA. As schematically shown in Figure 1A, the loose complex cannot pass the cell membrane effectively. Accordingly, we conjugated siRNA to flexible and polyanionic HA to make a tight complex with LPEI, which was thought to facilitate the cellular uptake.

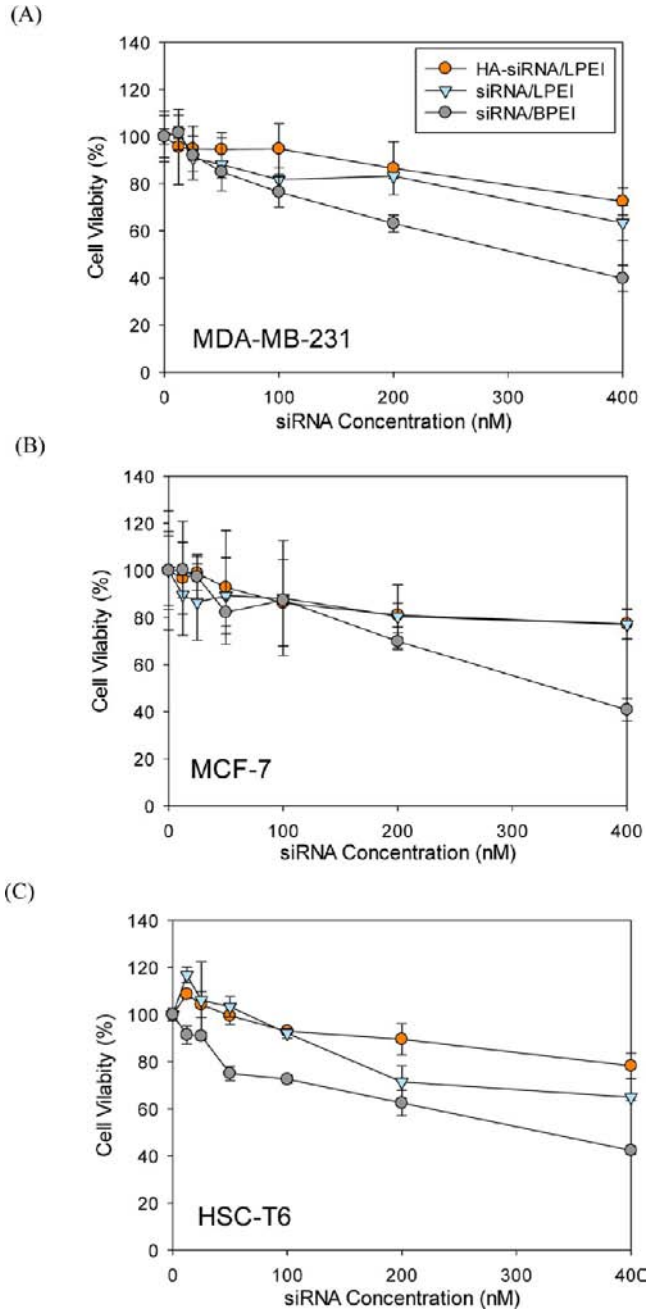

Figure 5. Cell viability (%) with increasing concentration of siRNA after treatment with HA−siRNA/LPEI, siRNA/LPEI, and siRNA/BPEI complexes in (A) MDA-MB-231, (B) MCF-7 cells, and (C) HSCs-T6 (n = 3).

The structure of HA−siRNA/LPEI complex was analyzed by dynamic light scattering (Figure 4A). The hydrodynamic volume size of siRNA/LPEI complex was relatively large over 400 nm at every N/P ratio. On the other hand, the particle sizes of HA−siRNA/LPEI complexes at the N/P ratios of 10 and 15 were in the range of 250−300 nm, reflecting a tight complex formation. The surface charge of the complex was also analyzed by measuring the zeta-potential value (Figure 4B). While all siRNA/LPEI complexes had a high positive charge, HA−siRNA/LPEI complexes had a negative or neutral surface charge. This negative or neutral surface charge was expected to reduce nonspecific interaction with blood serum components and the following falloff in delivery efficiency.[32,33] The HA−siRNA/LPEI complex

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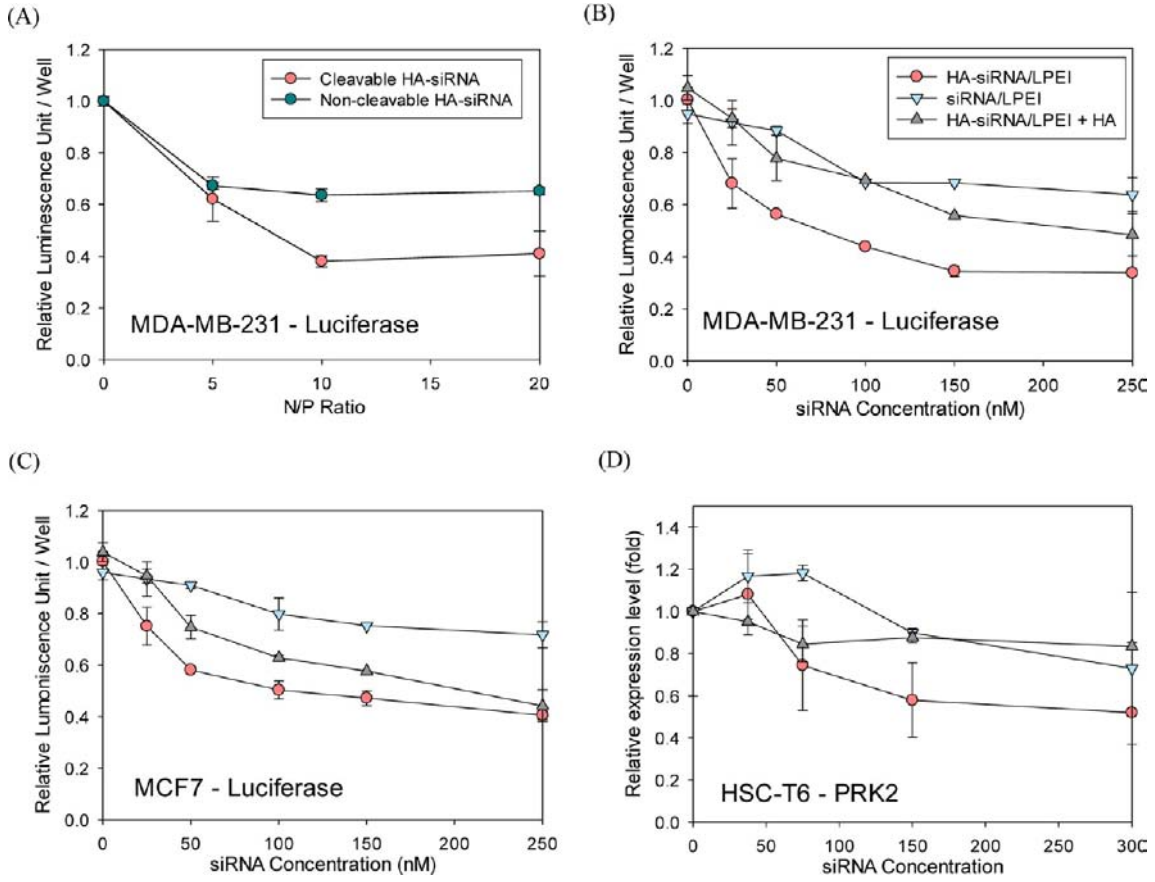

Figure 6. (A) Luciferase gene silencing in MDA-MB-231 cells by cleavable (reducible) and noncleavable HA−siRNA conjugates complexed with LPEI. Data are presented as means ± SD. * $P < 0.05$ and ** $P < 0.01$ versus the control group ($n = 4$). The gene silencing of luciferase in (B) MDA-MB-231 and (C) MCF7 cells, and (D) PRK2 in HSCs-T6.

at the N/P ratio of 10 was used for the following *in vitro* and *in vivo* gene silencing applications.

To compare the cytotoxicity of HA−siRNA conjugate after making a complex with conventional LPEI or BPEI, MTT assay was performed using various cell lines of MDA-MB-231 breast cancer cells with a high content of HA receptors, MCF7 breast cancer cells with a low content of HA receptors, and HSCs-T6 in the liver tissue (Figure 5). According to the analysis, HA−siRNA/LPEI complex showed the lowest toxicity in three cell lines, followed by siRNA/LPEI complex and siRNA/BPEI complex. The relatively lower cytotoxicity of HA−siRNA/LPEI complex than siRNA/LPEI complex might be ascribed to the shielding effect of positive charge of LPEI by the surrounding HA. The conjugation of siRNA to HA was thought to alleviate the cytotoxicity of siRNA carrier and prevent the nonspecific interaction with serum components.

***In Vitro* Gene Silencing of HA−siRNA/LPEI Complex.** The effect of cleavable and noncleavable linkages was comparatively analyzed on gene silencing efficiency in MDA-MB-231 cells which stably express luciferase gene. The MDA-MB-231 breast cancer cell line has a representative HA receptor of CD44. As shown in Figure 6A, cleavable HA−siRNA/LPEI complex reduced the luciferase gene expression down to 60%, whereas noncleavable HA−siRNA/LPEI complex showed a gene silencing efficiency in the range of 30−40%. The disulfide bond between siRNA and HA chain might be cleaved in the cytosol after endosomal escape, which certainly enhanced the gene silencing efficiency of the HA−siRNA/LPEI complex.

The gene silencing efficiency of HA−siRNA/LPEI complex was compared in MDA-MB-231, MCF7, and HSC-T6 cells at various siRNA concentrations. While the gene silencing efficiency of siRNA/LPEI complex was very low in three cell lines, the HA−siRNA/LPEI complex showed a relatively high gene silencing efficiency. The result could be ascribed to the tight complex formation by conjugating siRNA to the HA, facilitating the efficient intracellular delivery. The gene silencing efficiency of HA−siRNA/LPEI complex was higher in MDA-MB-231 cells than MCF7 cells (Figure 6B,C). In addition, when free HA molecules were added for competitive binding to HA receptors, the gene silencing efficiency decreased more severely in MDA-MB-231 cells than MCF7 cells. The result might be explained by the different expression levels of CD44 in MDA-MB-231 and MCF7 cells. Generally, MDA-MB-231 cells are known to have a higher expression level of CD44 than MCF7 cells. Furthermore, HA−siRNA/LPEI complex efficiently reduced the expression of PRK2 gene, which plays a critical role in replication of hepatitis virus in HSC-T6 cells (Figure 6D). The inhibition effect of free HA was observed by competitive binding in every cell line, which reflected the HA receptor mediated endocytosis of HA−siRNA/LPEI complex.

Intracellular Uptake of HA−siRNA/LPEI Complex. In order to clarify the intracellular uptake mechanism of HA−siRNA/LPEI complex, the MDA-MB-231 cells were treated with three different inhibitor drugs (Figure 7). Chloropromazine, nystatin, and wortmannin are the inhibitors for clathrin, caveolae, and macropinocytosis mediated endocytosis, respectively.[34]

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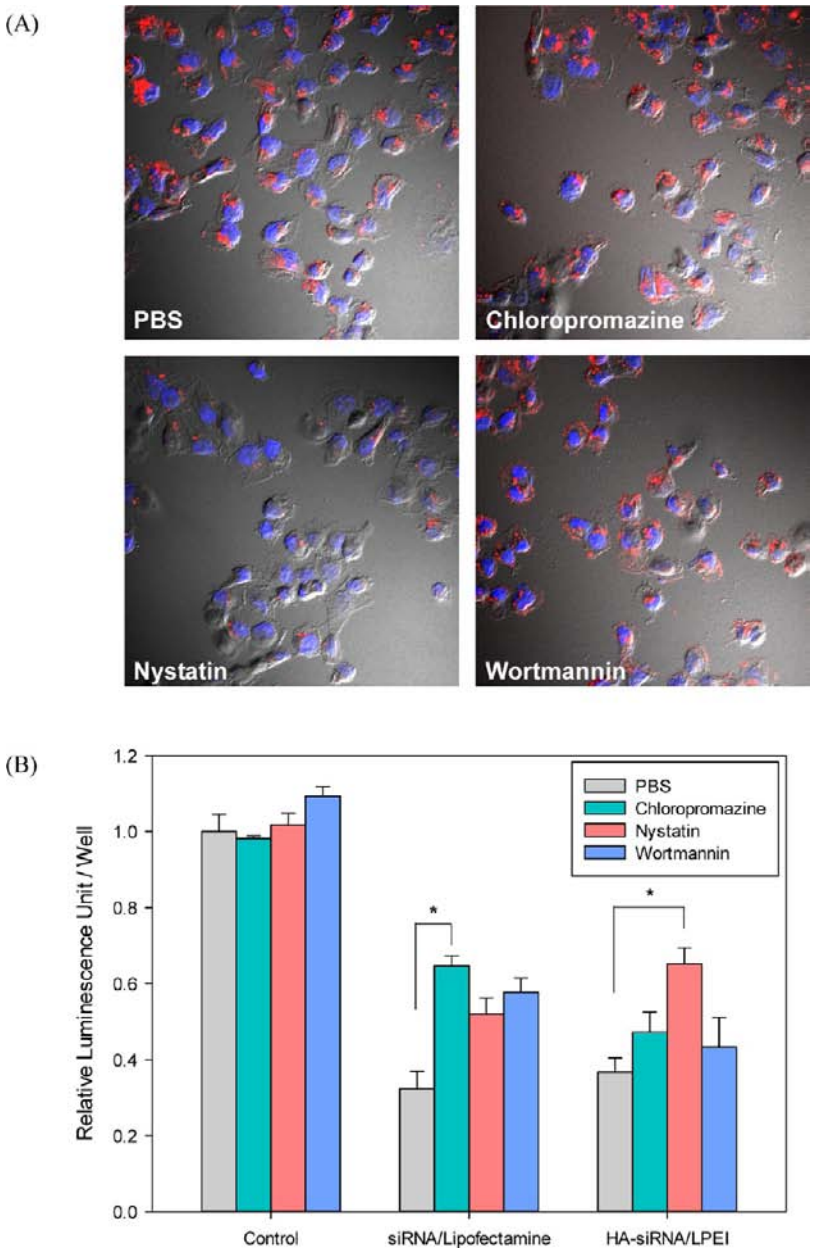

Figure 7. (A) Confocal microscopic images of MDA-MB-231 cells after treatment with Cy3 labeled HA−siRNA/LPEI complex in the presence of various endocytosis blocking agents: PBS as a control, chloropromazine, nystatin, and wortmannin. The red fluorescence represents Cy3-siRNA and the blue fluorescence, DAPI. (B) Effect of various endocytosis blocking agents on the gene silencing efficiency. Data are presented as means ± SD. * $P < 0.05$ versus the control group ($n = 4$).

According to confocal microscopic analysis, the internalization of cy3 labeled HA−siRNA/LPEI complex was more significantly inhibited by nystatin than chloropromazine and wortmannin (Figure 7A). The result matched well with previous reports on the internalization mechanism of HA,[35] confirming the HA receptor mediated endocytosis of HA−siRNA/LPEI complex. The results were further corroborated by gene silencing tests (Figure 7B). While chloropromazine reduced the gene silencing efficiency of siRNA/lipofectamine complex, nystatin affected the gene silencing efficiency of HA−siRNA/LPEI complex the most significantly. The competitive binding tests in the presence of free HA also confirmed the HA-receptor mediated endocytosis of HA based siRNA delivery systems.[26] Taken together, we could confirm the target specific intracellular delivery of HA−siRNA/LPEI complex by HA receptor mediated endocytosis.

Target Specific Systemic Gene Silencing of HA−siRNA/LPEI Complex. Encouraged by the *in vitro* results, we decided to carry out the target specific systemic delivery of HA−siRNA/LPEI complex using ApoB specific siRNA as a model siRNA. The target specific systemic delivery of HA derivatives was previously confirmed by the bioimaging studies[32,36] and the therapeutic efficacy assessment of HA-based biopharmaceutical delivery systems.[26,37,38] In accordance, after tail vein injection of HA−siApoB/LPEI complex, the ApoB mRNA expression level was significantly reduced with a gene silencing efficiency of *ca.* 60% in a dose-dependent manner (Figure 8). However, none of knockdown efficiency was discovered in the case of naked siApoB/LPEI complex. Taken together, the HA−siRNA/LPEI complex was thought to be successfully applied to the target specific systemic delivery of therapeutic siRNA for the

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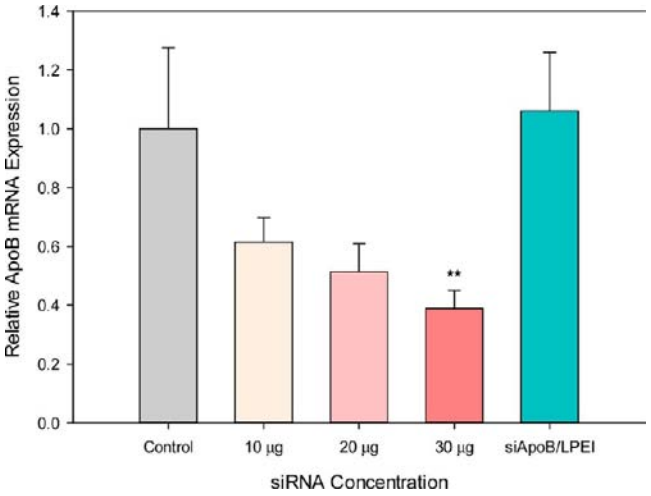

Figure 8. Apolipoprotein B (ApoB) mRNA expression levels in the liver after tail-vein injection of HA−siApoB/LPEI complex with increasing doses of siRNA up to 30 μg/head. The gene silencing efficiency was compared with the control of PBS and naked siRNA/LPEI complex. ** $P < 0.01$ versus the control group ($n = 5$).

treatment of liver diseases with a minimal cytotoxicity of the carrier system.

CONCLUSIONS

A target specific systemic siRNA delivery system was successfully developed using reducible HA−siRNA conjugate complexed with relatively noncytotoxic weak polycations. SEC and electrophoresis confirmed the synthesis of HA−siRNA conjugate by disulfide exchange reaction between HA-SPDP and siRNA-SH. The highly negative and flexible HA−siRNA conjugate could make a tight complex with a weak polycation of LPEI. The conjugation of siRNA to HA enhanced the resistance to RNase and facilitated the receptor mediated endocytosis. *In vitro* gene silencing efficiency of HA−siRNA/LPEI complex was in the range of 60−70%. Moreover, the caveloae mediated endocytosis of HA−siRNA/LPEI complexes was corroborated by the endocytosis inhibition assay. Finally, the target specific systemic delivery of HA−siApoB/LPEI complex resulted in efficient down-regulation of ApoB mRNA levels in the liver. The target specific HA−siRNA conjugate system can be effectively applied to the treatment of liver diseases.

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Notes

The authors declare no competing financial interest.

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