Targeted Delivery of siRNA to Hepatocytes and Hepatic Stellate Cells by Bioconjugation

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Previously, we successfully conjugated galactosylated poly(ethylene glycol) (Gal-PEG) to oligonucleotides (ODNs) via an acid labile ester linker (Zhu et al., *Bioconjugate Chem.* **2008***, 19,* 290−8). In this study, sense strands of siRNA were conjugated to Gal-PEG and mannose 6-phosphate poly(ethylene glycol) (M6P-PEG) for targeted delivery of siRNAs to hepatocytes and hepatic stellate cells (HSCs), respectively. These siRNA conjugates were purified by ion exchange chromatography and verified by gel retardation assay. To evaluate their RNAi functions, the validated siRNA duplexes targeting firefly luciferase and transforming growth factor beta 1 (TGF-β1) mRNA were conjugated to Gal-PEG and M6P-PEG, and their gene silencing efficiencies were determined after transfection into HepG2 and HSC-T6 cells. The disulfide bond between PEG and siRNA was cleaved by dithiothreitol, leading to the release of intact siRNA. Both Gal-PEG-siRNA and M6P-PEG-siRNA conjugates could silence luciferase gene expression by about 40% without any transfection reagents, while the gene silencing effects reached more than 98% with the help of cationic liposomes at the same dose. Conjugation of TGF-β1 siRNA with Gal-PEG and M6P-PEG could silence endogenous TGF-β1 gene expression as well. In conclusion, these siRNA conjugates have the potential for targeted delivery of siRNAs to hepatocytes and hepatic stellate cells for efficient gene silencing in vivo.

INTRODUCTION

Significant progress has been made in enhancing the potency and specificity of RNA interference (RNAi) molecules, which include double-stranded RNA (dsRNA), small interfering RNA (siRNA) (19−23 base pairs), short hairpin RNA (shRNA), and micro RNA (miRNA). Although they are structurally different, all of these molecules induce sequence-specific gene silencing by either degrading or inhibiting the target mRNA via perfect or mismatched binding in the presence of RNA-induced silencing complex (RISC) (*1, 2*). The simplest and most effective strategy of gene silencing is to synthesize siRNAs based on the target mRNA sequence. siRNA has been shown to have the potential to treat numerous diseases, including cancer, fibrosis, genetic disorders, and viral infection (*3*−*5*). However, siRNAs have not been successfully used as clinical therapeutics due to several obstacles, including the poor enzymatic stability, short circulation time in the bloodstream, nonspecific tissue or cell targeting, and insufficient intracellular transport (*6*).

Various cationic lipids and polymers are being used as transfection reagents for siRNA delivery. siRNAs could be condensed by these carriers via electrostatic interaction into nanosized particles which are easy for cells to take up via endocytosis, membrane fusion, or both. After complex formation, siRNAs are protected from enzymatic digestion (*7*). However, cationic carriers are not suitable for in vivo delivery of siRNAs since they may bind to negatively charged serum proteins and undergo aggregation resulting in undesired distribution, and induction of inflammatory cytokines release and activation of the complement system (*8, 9*).

PEGylation is known to increase the in vitro and in vivo stability of drug molecules, as well as improve their pharmacokinetic profiles. We previously reported that the conjugation of galactose (*10*)−poly(ethylene glycol) (PEG) to oligonucleotide (ODN) using an acid-labile ester linker successfully stabilized ODN, prolonged its circulation time, and specifically delivered ODN to hepatocytes after systemic administration (*11*). The linkage between ODN and PEG could be cleaved at pH 5.5 (endosomal pH), and free ODN could be released. In a separate study, we have also demonstrated that mannose-6-phosphate (M6P) as a ligand could significantly increase the accumulation of ODN in hepatic stellate cells (HSCs) in rats.

Here, we used a similar strategy to Gal-PEG-ODN conjugate to design two siRNA conjugates, Gal-PEG-siRNA and M6P-PEG-siRNA, for targeted delivery of siRNAs to hepatocytes via asialoglycoprotein receptor-mediated endocytosis and HSCs via M6P/insulin-like growth factor-II receptor (M6P/IGF2R) mediated endocytosis, respectively. In this study, the heterobifunctional PEG, (*ortho*-pyridyl) disulfide−poly(ethylene glycol)−*N*-hydroxysuccinimidyl ester (OPSS-PEG-NHS), was used as a backbone followed by modification with either Gal or M6P at its NHS end and with the 3′-sense strand of siRNA at its OPSS end via disulfide bond. To make sure there is no loss in gene silencing due to bioconjugation, Luciferase siRNA and TGF-β1 siRNA were used as model siRNA sequences.

MATERIALS AND METHODS

Materials. OPSS-PEG-NHS (MW: 3400 Da) was purchased from Creative PEGWorks (Winston Salem, NC). Palladium (10 wt % on activated carbon), thiophosgene, dithiothreitol (DTT), L-glutathione (GSH) reduced, *p*-aminophenyl β-D-galactopyranoside, ethidium bromide (EtBr), *p*-nitrophenyl-α-D-mannopyranoside, dimethylformamide (DMF), and deuterium oxide (D_2O) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Sodium chloride, acetic acid, and HPLC-grade water were purchased from Fisher Scientific (Fair Lawn, NJ). Luciferase assay kit, TE buffer, 6× gel loading buffer, and Tris-hydrochloride were purchased from Promega (Madison, WI).

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Human/mouse TGF-β1 ELISA kit was purchased from eBioscience (San Diego, CA). Dialysis tubing (MWCO 1000 Da) was purchased from Spectrum Laboratories, Inc. (Houston, TX). Zeba Spin Desalting Columns (MWCO: 7000 Da) was purchased from Thermo Scientific (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM), penicillin streptomycin solution (100×), phosphate buffered saline (PBS), trypsin-EDTA, agarose, and Lipofectamine 2000 were purchased from Invitrogen Corporation (Carlsbad, CA). Bovine serum albumin (BSA) (fraction V, purity >98%) was purchased from Atlanta Biologicals (Lawrenceville, GA). Pyridinium cationic liposomes containing pyridinium lipid (C16:1, amide linker, trans)/DOPE (1:1 mol/mol) cationic liposomes were prepared by Zhu et al. (*12*).

Small Interfering RNAs (siRNAs). Luciferase siRNA (sense strand: CUUACGCUGAGUACUUCGA[dT][dT], antisense strand: UCGAAGUACUCAGCGUAAG[dT][dT]) was purchased from Invitrogen Corporation. TGF-β1 siRNA (*13*) (sense strand: GCUGUACAUUGACUUUAGG[dT][dT], antisense strand: CCUAAAGUCAAUGUACAGC[dT][dT]) (Accession no.: NM_021578) and the scramble siRNA were purchased from Invitrogen Corporation (Carlsbad, CA). For conjugation purposes, the sense strand of the siRNA was modified with a thiol group (-$(CH_2)_3$-S-S-$(CH_2)_3$-OH-3′) at its 3′ end by Invitrogen Corporation.

Methods. *Synthesis of p-Aminophenyl β-D-Galactopyranoside and p-Aminophenyl-6-phospho-α-D-mannopyranoside. p*-Aminophenyl β-D-galactopyranoside was synthesized according to our previous study (*11*). Briefly, p-nitrophenyl β-D-galactopyranoside was reduced with 10% palladium on activated carbon under hydrogen (1 atm) in a 1:1 (v/v) ethanol−water mixture for 2 h. The product, *p*-aminophenyl β-D-galactopyranoside, was concentrated by solvent evaporation and characterized by ^{1}H NMR and mass spectrometry after dissolving in D_2O.

p-Aminophenyl-6-phospho-α-D-mannopyranoside was synthesized according to our previous study (*14*) by dissolving *p*-nitrophenyl-α-D-mannopyranoside (pnpM) (0.3 g, 1 mmol) in the mixture of acetonitrile, pyridine, and water (1/0.4/0.04, v/v/v). Phosphorus oxide chloride (0.4 mL, 4.4 mmol) was added and stirred for 1 h on an ice−water bath. The reaction mixture was poured onto 20 g of ice. After the ice melted, pH was adjusted to 7.0 by slowly adding 2.5 M NaOH, and the solution was evaporated to dryness. The solid material was dissolved in water and crystallized at 4 °C overnight. The crystals were filtered and washed with 5 mL of absolute ethanol. The compound was recrystallized in a mixture of 1 mL of water and 10 mL of ethanol, redissolved in water, and lyophilized to give *p*-nitrophenyl-6-phospho-α-D-mannopyranoside (pnpM6P). PnpM6P was reduced with 100 mg of 10% palladium on activated carbon under H_2 (1 atm) in a 4:1 (v/v) methanol−water mixture for 2 h. After filtration, the residue was redissolved in water and lyophilized to give *p*-aminophenyl-6-phospho-α-D-mannopyranoside (papM6P). The product was characterized using ^{1}H NMR and mass spectrometry.

Synthesis of Gal-PEG-OPSS and M6P-PEG-OPSS. OPSS-PEG-NHS (50 mg) and *p*-aminophenyl β-D-galactopyranoside (50 mg) were dissolved separately in 0.4 mL of dimethylformamide (DMF), mixed together, and stirred for 6 h at room temperature under N_2 protection in the dark. The reaction mixture was dialyzed against distilled water for 48 h with MWCO 1000 to remove the unreacted *p*-aminophenyl β-D-galactopyranoside. The product Gal-PEG-OPSS was freeze−dried and characterized by ^{1}H NMR after dissolving it in D_2O.

OPSS-PEG-NHS (50 mg) were dissolved in 0.4 mL of DMF, and papM6P (50 mg) was dissolved in 0.4 mL of distilled water. Then, they were mixed and stirred for 6 h at room temperature under N_2 protection in the dark. The reaction mixture was dialyzed against distilled water for 48 h with MWCO 1000 to remove the unreacted papM6P. The product M6P-PEG-OPSS was freeze−dried and characterized by ^{1}H NMR after dissolving it in D_2O.

Synthesis of Gal-PEG-siRNA and M6P-PEG-siRNA. The lyophilized 3′ thiol-siRNA was reconstituted in TE buffer of pH 8.3−8.5 at a concentration of approximately 100 A260 units/mL. siRNA solution was then treated with 0.1 M dithiothreitol (DTT) aqueous solution at room temperature for 30 min. The excess DTT was removed by gel filtration and 3′-sulfhydryl siRNA was stored under N_2 to avoid oxidative dimerization to the disulfide before conjugation. 3′-Sulfhydryl siRNA and Gal-PEG-OPSS (or M6P-PEG-OPSS) were dissolved in 0.5 mL PBS (pH7.5) at a molar ratio of 1:100, and stirred under nitrogen at room temperature for 6 h. The reaction mixture was analyzed and purified by ion exchange high-performance liquid chromatography, which was carried on a Resource Q ion exchange column (GE Healthcare Life Sciences, Piscataway, NJ) by an HPLC system (Waters, Milford, MA) with detection at 260 nm using a gradient starting from 0% B to 80% B (A, 20 mM Tris-HCl buffer; B, 20 mM Tris-HCl buffer containing 0.1 M sodium chloride) at a flow rate of 1 mL/min at 25 °C. The purified Gal-PEG-siRNA and M6P-PEG-siRNA were desalted using Zeba Spin Desalting Column by centrifugation at the speed of 2000 *g* and freeze−dried.

Characterization of Gal-PEG-siRNA and M6P-PEG-siRNA. The concentrations of siRNA and siRNA conjugate solutions were quantitated by a UV spectrophotometer. To determine the cleavage of the disulfide bond and siRNA dissociation from Gal-PEG-siRNA and M6P-PEG-siRNA, 2 μg siRNA and the conjugates containing 2 μg siRNA were incubated in 50 mM DTT aqueous buffer for 40 min at room temperature. The DTT-treated samples and controls (nontreated siRNA and siRNA conjugate) were mixed with 6× gel loading buffer and applied on the 1% agarose gel and run at 10 V/cm for 60 min at room temperature followed by ethidium bromide (EtBr) staining and visualization under UV light.

Cell Cultures and Transfections. HepG2 cells were maintained in a complete growth medium containing DMEM medium supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. HSC-T6 cells, kindly provided by Dr. Scott Friedman (Mount Sinai School of Medicine, New York), were maintained in the same condition as HepG2 cells. Before transfection, the cells were seeded in 24-well plates and maintained in 0.5 mL of complete growth medium at 37 °C for 24 h. The cell number was about 4×10^4 cells per well.

Luciferase expression plasmid (pDNA3-Luc) was first transfected with cells by pyridinium cationic liposomes (*12*). Briefly, 0.2 μg/well luciferase plasmid and pyridinium cationic liposomes were diluted to 25 μL by Opti-MEM I medium, respectively, and incubated for 5 min at room temperature. Lipoplexes were prepared by mixing plasmid and liposomes in equal volume and incubated for 20 min at room temperature to allow complex formation. Immediately before transfection, the medium was removed, and cells were washed gently with sterile phosphate buffered saline. Lipoplexes were added to each well and diluted to 0.5 mL by Opti-MEM I medium, and the plate was incubated under 5% CO_2 at 37 °C for 6 h. Following transfection, the culture medium was replaced with complete growth medium and maintained overnight before further treatment.

To transfect luciferase siRNA and its conjugate, two methods were used. In the first method, siRNA and its conjugates were added directly into cell cultures without the assistance of cationic liposomes. In the second method, siRNA and its conjugates were mixed with cationic liposomes to form the lipoplexes before

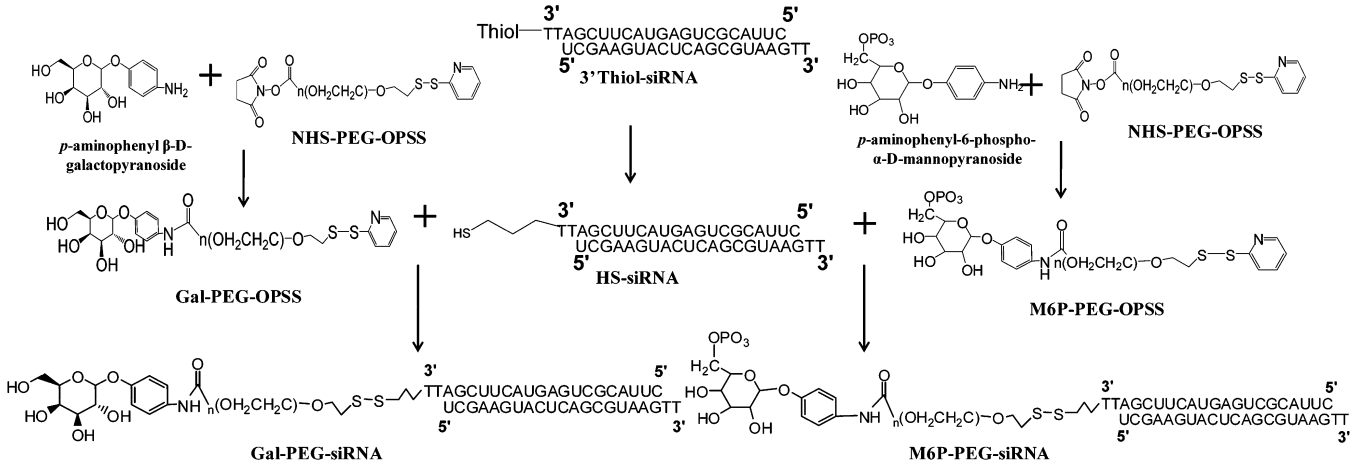

Figure 1. Synthesis scheme of Gal-PEG-siRNA and M6P-PEG-siRNA.

adding into cell cultures. After 6 h of transfection, the medium was replaced with the complete growth medium and incubated for an additional 42 h.

To determine luciferase gene expression, the cells were collected and lysed. The cell lysate was measured for luciferase gene expression by Luciferase Assay Kit (Promega, Madison, WI). The total protein concentration was measured by bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Luciferase activity was normalized by the total protein content and expressed as relative light unit (RLU) per microgram of protein (RLU/μg protein).

To determine their gene silencing effects, TGF-β1 siRNA and its conjugates were mixed with the media and added into HepG2 cells and HSC cells. After 6 h of transfection, the medium was replaced with the complete growth medium and incubated for additional 42 h. To determine TGF-β1 gene expresstion, the supernatant was collected and measured by Human/Mouse TGF-β1 ELISA kit.

RESULTS

Synthesis of Gal-PEG-siRNA and M6P-PEG-siRNA. The synthesis scheme of Gal-PEG-siRNA and M6P-PEG-siRNA was shown in Figure 1. Before conjugation, *p*-nitrophenyl β-D-galactopyranoside was reduced to *p*-aminophenyl β-D-galactopyranoside. ESI-MS and ^{1}H NMR spectra of this intermediate product were not shown but could be found in our previous paper (*11*). *p*-Nitrophenyl-α-D-mannopyranoside (pnpM) was phosphorylated to *p*-nitrophenyl-6-phospho-α-D-mannopyranoside (pnpM6P) and pnpM6P was reduced to *p*-aminophenyl-6-phospho-α-D-mannopyranoside (papM6P). ESI-MS and ^{1}H NMR results of this intermediate product are shown in Figure 2.

Gal-PEG-OPSS and M6P-PEG-OPSS were synthesized from heterofunctional PEG derivative, NHS-PEG-OPSS and identified by ^{1}H NMR. After reaction with Gal or M6P, the two peaks (a and b positions) of the benzene ring were found in the NMR spectra. The data indicated that Gal and M6P reacted with NHS group and linked with PEG (Figure 3). Gal-PEG-OPSS and M6P-PEG-OPSS were then conjugated to 3′-sulfhydryl siRNA by disulfide exchange reaction between sulfhydryl and OPSS groups. Ion exchange HPLC was used to monitor the conjugation reaction and purify the final products. The ion exchange HPLC chromatograms of siRNA, M6P-PEG-OPSS, and M6P-PEG-siRNA were shown in Figure 4. M6P-PEG-OPSS eluted first, since they have no charge and cannot bind to anion exchange resin; free siRNA is a negatively charged molecule and bound to anion exchange resin and eluted last. After conjugation, the charge of siRNA was partially blocked by the flexible long chain of PEG and eluted between PEG and siRNA. The chromatograms of Gal-PEG-OPSS and Gal-PEG-siRNA were not shown, since they are similar to those of M6P-PEG-OPSS and M6P-PEG-siRNA. Gal-PEG-siRNA and M6P-PEG-siRNA conjugates were also identified by 1% agarose gel electrophoresis and visualized under UV light (Figure 5).

Dissociation of siRNAs from Gal-PEG-siRNA and M6P-PEG-siRNA Conjugates. To determine whether the disulfide bond between Gal-PEG or M6P-PEG and siRNA is cleavable and free siRNA can be released, siRNA and its conjugates were treated with DTT aqueous buffer for 40 min and analyzed by gel retardation assay. Gal-PEG-siRNA and M6P-PEG-siRNA conjugates migrated more slowly than free siRNA. However, after treatment with DTT for 40 min, the disulfide bond was completely cleaved and free siRNA was released and migrated at the same speed as the siRNA control (Figure 5).

Gene Silencing Study. Firefly luciferase expression plasmid was transfected into both HepG2 and HSC-T6 cells using previously optimized pyridinium cationic liposomes at the charge ratio of 3:1 (N/P) (*12*). Gene silencing efficiencies of siRNA conjugates were determined after transfection of luciferase expression plasmid. In the absence of cationic liposomes, Gal-PEG-siRNA conjugate showed luciferase gene silencing up to about 45% at the dose of 400 nM compared to the naked siRNA group and the control group (plasmid group) without siRNA treatment (Figure 6A). Addition of an excessive amount of Gal-PEG-OPSS into HepG2 cells before transfection of siRNA conjugate abolished the gene silencing ability of Gal-PEG-siRNA conjugate. Similarly, pretreatment of HSC-T6 cells with an excess amount of M6P-PEG-OPSS before the transfection of M6P-PEG-siRNA conjugate abolished the gene silencing ability of this conjugate. Target cells were pretreated with an excess amount of Gal-PEG-OPSS or M6P-PEG-OPSS to saturate the surface receptors and inhibit the receptor-mediated cellular uptake of siRNA conjugates. Since the molecular weights of galactose or mannose 6-phosphate are too small and may not be effective in saturating the target receptors, the conjugates containing the ligands are commonly used (*11*). To keep data comparable, we used 400 nM siRNA conjugates in Gal-PEG+ conjugate and M6P-PEG+conjugate groups.

Figure 6B showed the luciferase gene silencing efficiency of siRNA and Gal-PEG-siRNA. After complex formation with pyridinium cationic liposomes, the gene silencing of Gal-PEG-siRNA significantly increased and reached up to 90% even at the dose of 100 nM, while the gene silencing effect of luciferase siRNA was just about 60% at the same dose. Figure 6C showed the gene silencing ability of M6P-PEG-siRNA in HSC-T6 cells without pyridinium cationic liposomes. M6P-PEG-siRNA could

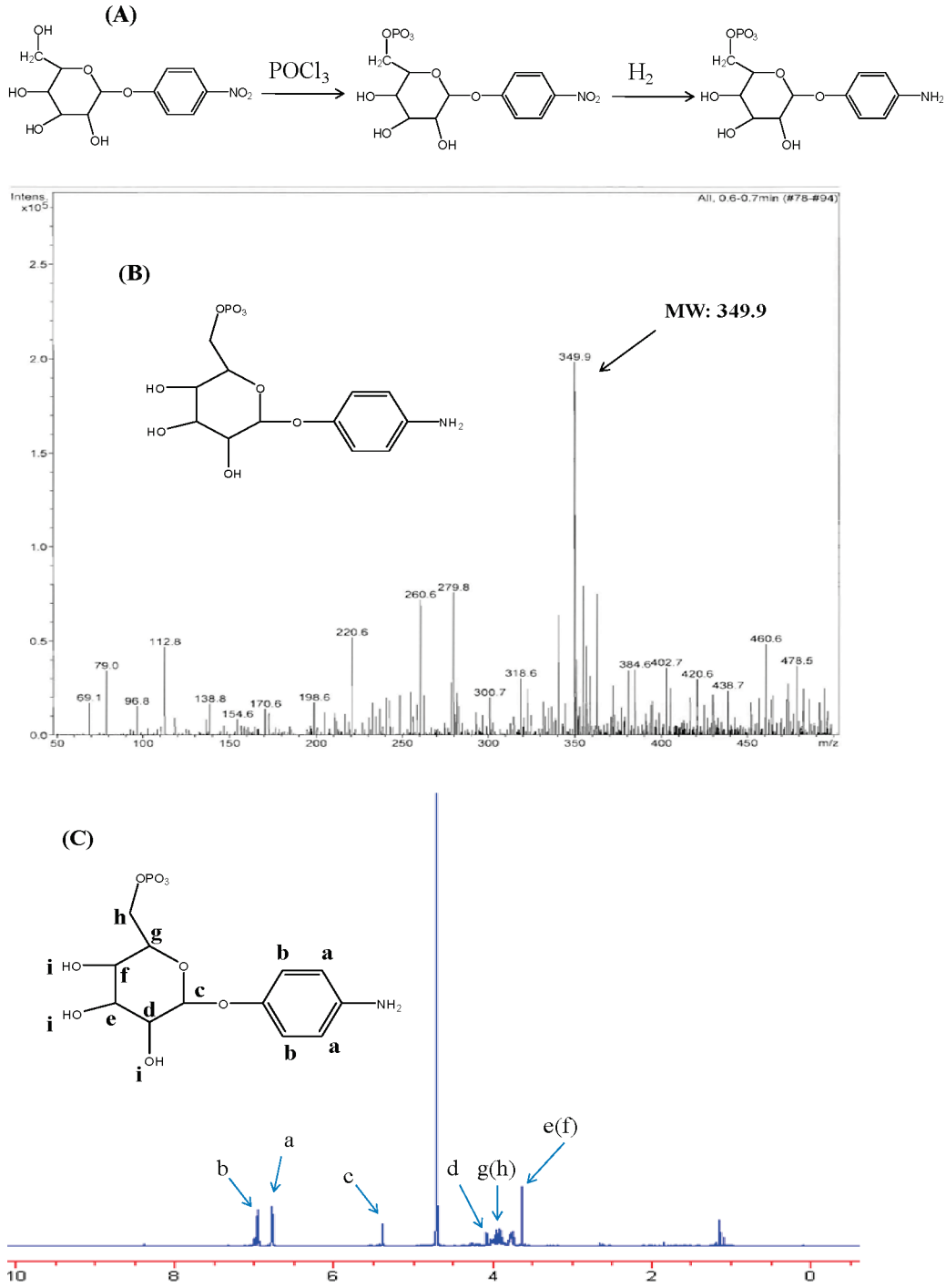

Figure 2. Synthesis and characterization of *p*-aminophenyl-6-phospho-α-D-mannopyranoside with ESI-MS and ^{1}H NMR. (A) Synthesis scheme of *p*-aminophenyl-6-phospho-α-D-mannopyranoside. (B) Electron spray ionization mass spectra were obtained after dissolving the product in a 4:1 (v/v) methanol−water mixture on an ESQUIRE-LC ion trap LC/MS sy stem in negative mode. (C) For ^{1}H NMR, samples were dissolved in D_2O, and the spectra were recorded on a Bruker ARX-500 MHz NMR spectrometer at 25 °C.

silence luciferase gene expression at a similar level to Gal-PEG-siRNA. Luciferase gene silencing was dependent on Gal-PEG-siRNA and M6P-PEG-siRNA dose in both the absence and presence of pyridinium cationic liposomes.

To determine the effect of bioconjugation on TGF-β1 gene silencing in HepG2 and HSC-T6 cells, the sense strand of a potent TGF-β1 siRNA duplex was conjugated with either Gal-PEG-OPSS or M6P-PEG-OPSS to form TGF-β1 siRNA conjugates. After transfection, the level of TGF-β1 protein in the cell medium was measured by ELISA kit. The gene silencing effects of siRNA conjugates were shown in Figure 7. Without transfection reagents, conjugation of TGF-β1 siRNA with Gal-PEG-OPSS or M6P-PEG-OPSS significantly enhanced TGF-β1 gene silencing effects up to 40% of the nontreatment group compared to TGF-β1 siRNA.

DISCUSSION

Effective delivery of siRNA to specific cell types in vivo is critical for the successful therapeutic use of siRNA. The current studies of siRNA delivery systems are focused on the electrostatic interaction with cationic liposomes and polymers, since negatively charged siRNAs are poorly taken up by the cells. Although the use of these cationic carriers is effective in vitro, an ideal siRNA carrier should not bear any positive charge, since

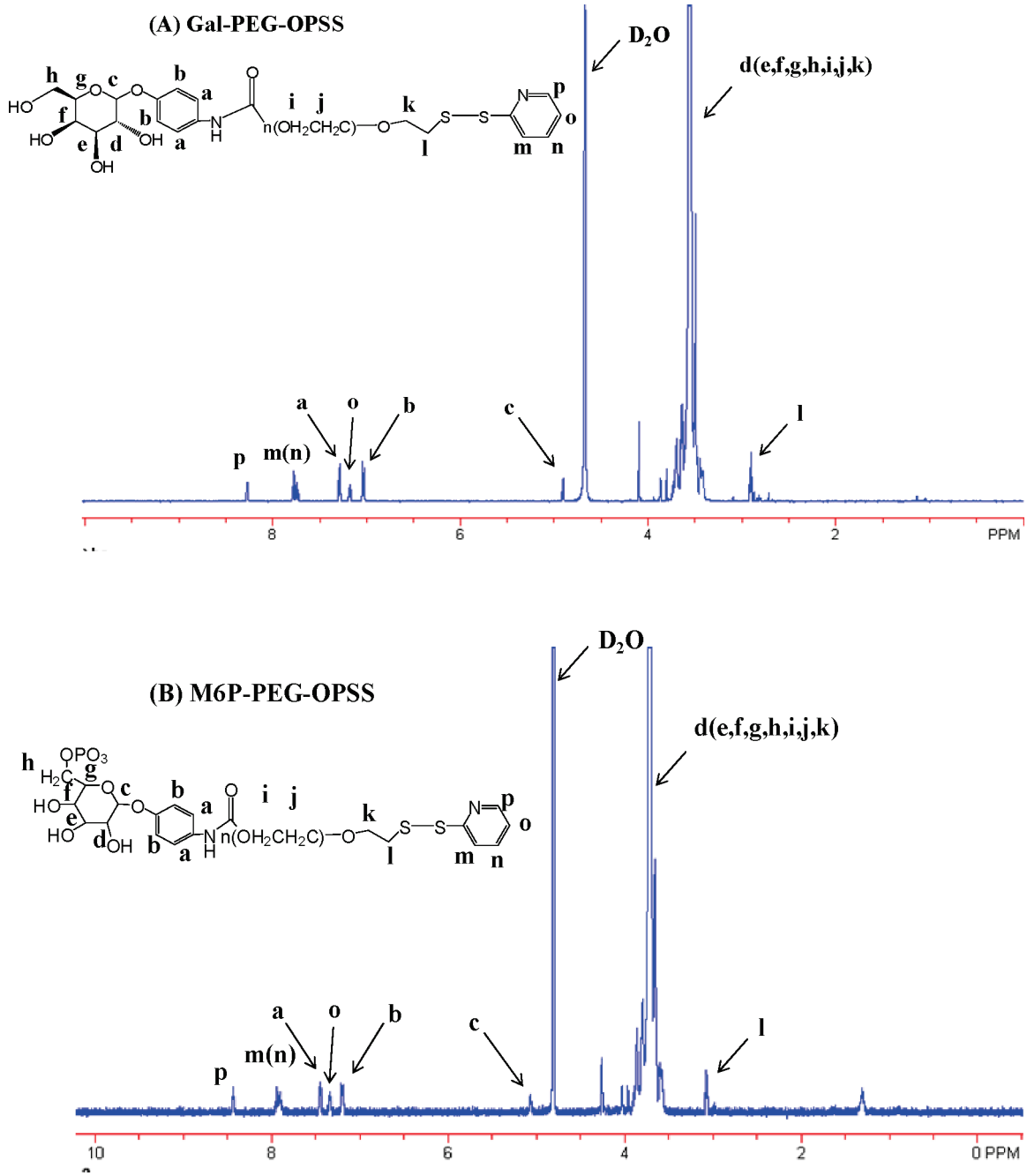

Figure 3. Characterization of Gal-PEG-OPSS (A) and M6P-PEG-OPSS (B) with ^{1}H NMR. The samples were dissolved in D_2O, and the spectra were recorded on a Bruker ARX-500 MHz NMR spectrometer at 25 °C.

it may cause an immune response and unexpected distribution due to nonspecific ionic interaction with plasma proteins. To avoid the use of polycations, Rajue et al. directly conjugated asialoglycoprotein to ODNs via disulfide bonds to target hepatocytes (*15*). Nakagawa et al. directly conjugated the small-molecule ligand, anisamide, to ODNs, to target the σ receptor on the surface of human lung cancer cells (*16*). The lipophilic molecule, α-tocopherol, was also covalently conjugated to the antisense strand of siRNA to target the liver (*17*). Although direct conjugation of ligands to ODN or siRNA increased their targeting efficiencies, it might not significantly improve the stability or dissociation of loaded ODN and siRNA. PEGylation is known to significantly enhance the stability of ODNs and siRNAagainst exonucleases and reduce renal clearance compared to unmodified ones (*18*−*20*), which improves their bioactivity by increasing plasma residence time and decreasing nuclease degradation. The flexible PEG chains also shield the inherent negative charge of these ODNs and siRNA, thereby possibly facilitating cellular uptake of the conjugated ODNs and siRNA (*21*).

Oligonucleotides and siRNA are cleared rapidly from the blood circulation and accumulated in the peripheral tissues, especially in the liver and kidney (*22*−*24*). Their in vivo activity depends on their pharmacokinetic profiles, especially the rate of excretion and degradation. In a previous study, we successfully conjugated ODN to Gal-PEG via an acid-labile linkage and demonstrated that systemic administration of Gal-PEG-ODN significantly increased accumulation in hepatocytes via galactose/asialoglycoprotein receptor-mediated endocytosis (*11*). In a separate study, we synthesized M6P-BSA and conjugated this to ODN. Systemic administration of M6P-BSA-ODN conjugate increased the accumulation of ODNs in HSCs via M6P/IGF2R mediated endocytosis compared to other types of liver cells in rats (*14*).

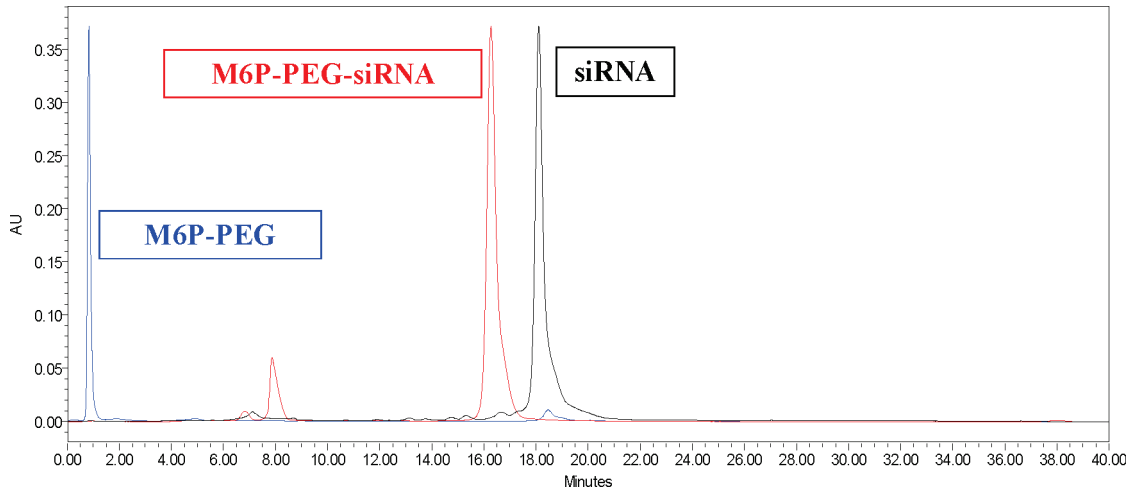

Figure 4. Purification of M6P-PEG-siRNA conjugate by ion-exchange chromatography. The reaction mixture was carried on a Resource Q ion exchange column (GE Healthcare Life Sciences, Piscataway, NJ) by an HPLC system (Waters, Milford, MA) with detection at 260 nm using a gradient starting from 0% B to 80% B (A, 20 mM Tris-HCl buffer; B, 20 mM Tris-HCl buffer containing 0.1 M sodium chloride) at a flow rate of 1 mL/min at 25 °C.

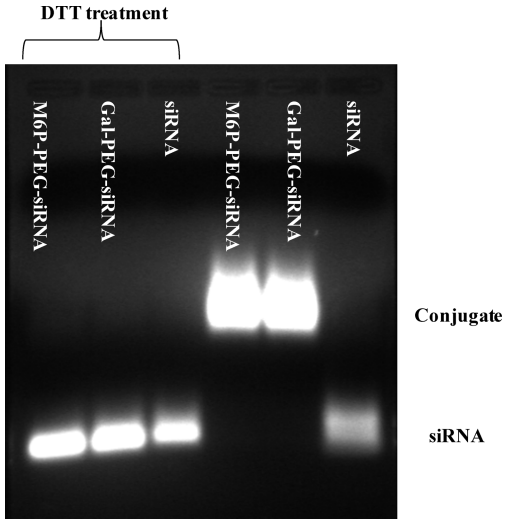

Figure 5. Identification of siRNA conjugates by gel retardation assay. Two microgram aliquots of siRNAs, Gal-PEG-siRNA, and M6P-PEG-siRNA were incubated with 50 mM DTT at room temperature for 40 min. The treated samples and nontreated controls were applied on 1% agarose gel and run at 10 V/cm for 60 min followed by EtBr staining, then visualized under UV light.

siRNAs can be covalently linked with functional molecules via either noncleavable or cleavable linkages, such as acid-labile ester (*11, 25*) and reducible disulfide bond (*19, 26*). For single-stranded ODNs, both 3′- and 5′-termini of these molecules are reported to be used as functional sites for the conjugation reaction (*11, 27*). For the siRNA duplex, however, the antisense strand of siRNA plays a key role and works as the template in RISC to perform RNAi. Improper modification of antisense strands may cause the loss of RNAi function (*28*). It was further shown that the 5′-terminus of the antisense strand was more important than the 3′-terminus and determined RNAi activity (*29*−*31*). In comparison to the antisense strand, manipulation of the sense strand of siRNA is relatively safe and effective. Both 3′- and 5′-ends of the sense strand can be used for conjugation (*32*).

In the present study, we investigated the application of a PEG-based macromolecular delivery system using two different ligands for siRNA delivery. Since the disulfide bond has been proven to be cleaved by reducing agents in the cytoplasm and was extensively used in drug delivery systems (*19, 33*−*35*), we used cleavable disulfide bonds as the linkage between siRNA and PEG to ensure the release of intact siRNA molecules from the conjugates in the reducing environment in the cytoplasm where RNAi takes action after endocytosis. This is expected to be better than the use of PEGylated cationic liposomes and PEI, where the PEG chain may influence the endosomal escape, as the PEG chain often cannot be released from the carriers.

Chronic liver injury and inflammation of hepatocytes or other hepatic cells cause liver fibrosis, resulting in excessive accumulation of extracellular matrix (ECM) proteins and activation and transformation of HSCs to proliferative myofibroblast-like cells. Many inflammatory cytokines were up-regulated during liver fibrosis. Among them, TGF-β1, which is mainly expressed by HSCs, plays a key role in initiation and maintenance of liver fibrosis (*36*). TGF-β1 gene silencing has been shown to inhibit the ECM production as well as accelerate its degradation resulting in resolution of fibrogenesis (*37*). In our previous study, we demonstrated siRNA sequence and dose-dependent TGF-β1 gene silencing and consequent inhibition of collagen synthesis (*13*). Therefore, hepatocytes and HSCs are often chosen as targets to deliver the therapeutics to treat liver fibrosis. Excessive ECM causes the loss of sinusoidal fenestrae (*38*), suggesting that liposomal and other particulate delivery systems may not be suitable for siRNA delivery to hepatic cells. In comparison to particulate delivery systems, the water-soluble delivery systems, Gal-PEG-siRNA and M6P-PEG-siRNA, are much more attractive for siRNA delivery to hepatocytes and HSCs in fibrotic liver. In this study, the 3′-thiol modifier was first reduced to a 3′-sulfhydryl group, which was active and could form a disulfide bond with an (*ortho*-pyridyl)-disulfide (OPSS) group by disulfide exchange reaction in PBS. The disulfide bond was cleaved, and free siRNAs were released after treatment with a reducing agent DTT. The gel retardation assay confirmed that, after conjugation with PEG, the migration rate of siRNA conjugates was significantly slower than that of free siRNA, but after DTT treatment, the linker between siRNA and PEG was cleaved, and the released siRNA showed a similar migration rate to unconjugated siRNA (Figure 5). We did not use mass spectrophotometry to identify the siRNA conjugates, because siRNA may get degraded during the MALDI-TOF MS test, and it requires a large amount of siRNA to get the signal (*39, 40*).

Luciferase gene silencing of siRNA conjugates indicated that siRNAs did not lose their RNAi function during conjugation and after dissociation from siRNA conjugates (Figure 6). siRNA

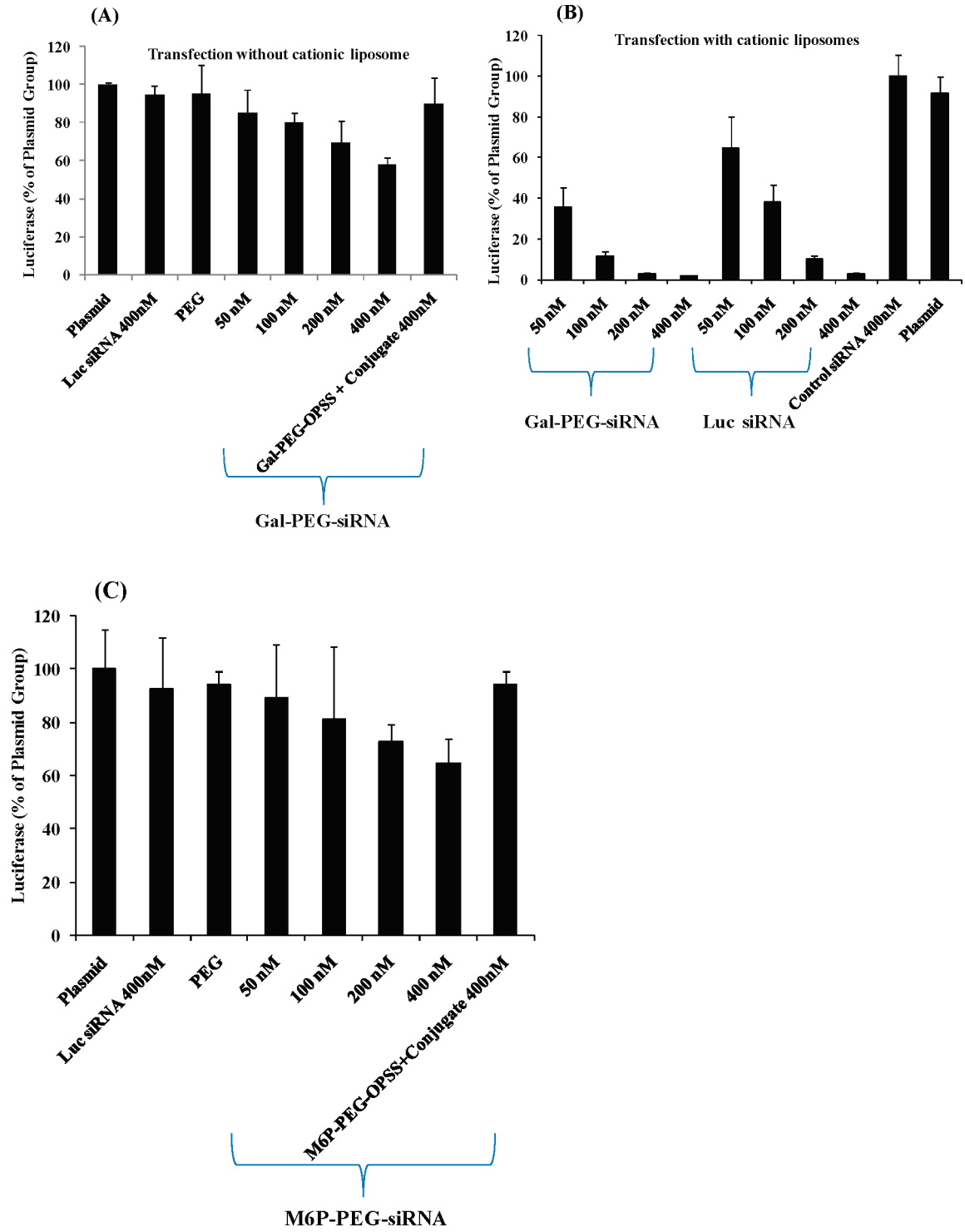

Figure 6. Luciferase gene silencing effects of Gal-PEG-siRNA and M6P-PEG-siRNA. Before the gene silencing study, luciferase plasmid was transfected into both HepG2 and HSC-T6 cells using lipoplexes formed between luciferase plasmid and pyridinium cationic liposomes at the charge ratio of 3/1 (±). The dose of plasmid was 0.2 μg per well in a 24-well plate. To study the gene silencing ability of Gal-PEG-siRNA, luciferase siRNA and its Gal-PEG-siRNA conjugate were added into HepG2 cells without (A) and with (B) the formation of siRNA/liposome complexes using pyridinium cationic liposomes. For M6P-PEG-siRNA, luciferas siRNA and its M6P-PEG-siRNA conjugate were added directly into HSC-T6 cells without cationic liposomes (C). After 6 h of transfection, the medium was replaced with the complete growth medium and incubated for additional 42 h. The cell number was about 4×10^4 cells per well in a 24-well plate.

conjugates had about 40% down-regulation of luciferase gene at the dose of 400 nM in both HepG2 and HSC-T6 cells in the absence of cationic liposomes (Figure 6A,C), while the luciferase gene was almost completely silenced in the presence of cationic liposomes at the same condition (Figure 6B). Without condensation with cationic liposomes, the flexible hydrophilic PEG chain surrounds and shields siRNA or the ligand on it from accessing the cell membrane, thereby decreasing both specific and nonspecific endocytosis. After complex formation with cationic liposomes, the lipoplexes were slightly positively charged and easy for cells to take up (nonspecific) and had high gene silencing effects. Furthermore, the combination of ligand−receptor-mediated specific endocytosis, siRNA conjugates showed higher gene silencing effect compared to naked

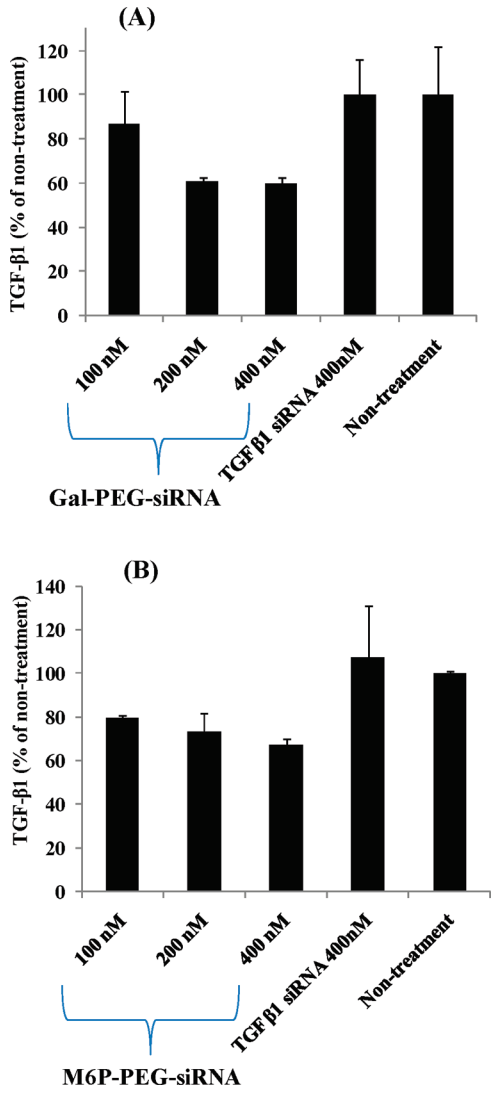

Figure 7. TGF-β1 gene silencing effects of Gal-PEG-siRNA and M6P-PEG-siRNA. (A) TGF-β1 siRNA and Gal-PEG-siRNA were added directly into HepG2 cells without cationic liposomes. (B) TGF-β1 siRNA and M6P-PEG-siRNA were added directly into HSC-T6 cells without cationic liposomes. After 6 h of transfection, the medium was replaced with the complete growth medium and incubated for an additional 42 h. There were about 4×10^4 cells per well in a 24-well plate.

siRNAs (Figure 6B). However, in the in vivo situation, PEGylated siRNAs will be retained in blood circulation and cleared very slowly compared to naked siRNAs. Our previous studies showed that pegylated ODN could significantly prolong the elimination half-life ($t_{1/2\beta}$) of ODN up to 118.61 $\pm$ 22.06 min (*11*) compared to only 34.60 $\pm$ 4.32 min of naked ODN after i.v. injection in rats (*14*). The longer residence will provide sufficient time for siRNA conjugates to access their target cells and initiate ligand−receptor-mediated endocytosis. In Figure 6A,C, preaddition of an excessive amount of Gal-PEG or M6P-PEG significantly inhibited the gene silencing effects of corresponding siRNA conjugates, which indicated that the cellular uptake of siRNA conjugates was ligand−receptor-mediated endocytosis, not nonspecific endocytosis.

To verify the function of PEG-based siRNA conjugate, we used TGF-β1 siRNA as the model sequence. The similar gene silencing effects were observed when TGF-β1 siRNA was conjugated to Gal-PEG-OPSS and M6P-PEG-OPSS (Figure 7A,B). On the basis of these results, we can predict that cation-free Gal-PEG-siRNA and M6P-PEG-siRNA conjugates will be specifically accumulated in hepatocytes or HSCs and minimize the nonspecific distribution and immunostimulatory effect in vivo.

In conclusion, we successfully conjugated siRNA to Gal-PEG-OPSS and M6P-PEG-OPSS via a disulfide bond. The gene silencing effects of these siRNA conjugates were observed in HepG2 and HSC-T6 cells. These siRNA conjugates have the potential to be used for targeted delivery of siRNAs to hepatocytes and hepatic stellate cells in vivo.

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