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Cell Type-Specific Delivery of RNAi by Ligand-Functionalized Curdlan Nanoparticles: Balancing the Receptor Mediation and the Charge Motivation

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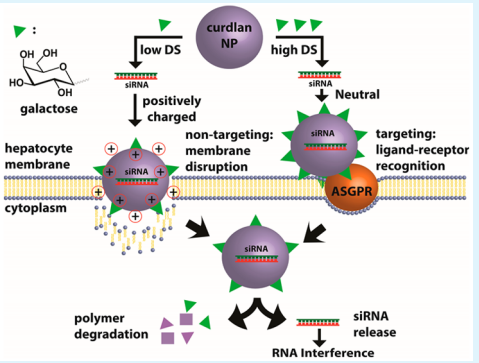
ABSTRACT: Tissue-specific delivery of therapeutic RNAi has great potential for clinical applications. Receptor-mediated endocytosis plays a crucial role in targeted delivery of biotherapeutics including short interfering RNA (siRNA). Previously we reported a novel Curdlan-based nanoparticle for intracellular delivery of siRNA. Here we designed a nanoparticle based on ligand-functionalized Curdlan. Disaccharides were site-specifically conjugated to 6-deoxy-6-amino Curdlan, and the cell line specificity, cellular uptake, cytotoxicity, and siRNA delivery efficiency of the corresponding disaccharide-modified 6-deoxy-6-amino-Curdlan were investigated. Observation by fluorescence microscopy as well as flow cytometry showed that galactose-containing Curdlan derivatives delivered fluorescently labeled short nucleic acid to HepG2 cells expressing ASGPR receptor but not in other cells lacking surface ASGPR protein. Moreover, highly galactose-substituted Curdlan derivatives delivered siRNA specifically to ASGPR-expressing cells and induced RNAi activities, silencing endogenous GAPDH gene expression. Our data demonstrated that galactose-functionalized 6-deoxy-6-amino-Curdlan is a promising carrier for short therapeutic nucleic acids for clinical applications.



KEYWORDS: *siRNA delivery, Curdlan, receptor-mediated delivery, ASGPR, galactose*

INTRODUCTION

RNA interference (RNAi) is an important cellular mechanism for regulation of gene expression in most eukaryotes. This post-transcriptional gene silencing process is highly efficient in cleavage of target mRNA, due to highly organized protein complex called RNA-induced silencing complex (RISC) containing Argonaute 2 (Ago2) endonuclease.[1]

The evolutionally reserved process of immunity to invasion of exogenous nucleic acids, RNAi has now been extensively investigated for therapeutical purpose by designing synthetic short interference RNAs (siRNAs) to target mRNAs of specific genes that play key role in pathogenic developments in the cells.[2,3]

RNAi has great potential for clinical applications.[4,5] A decade of efforts by scientists worldwide have achieved tremendous progress in research for breakthrough in application of RNAi therapeutics in life-threatening diseases such as cancer and virus infection.[6] One of the bottlenecks of application of RNAi therapeutic in clinic has been lack of efficient delivery system.[7] Designing and screening for nontoxic, cell-specific siRNA carrier has been the focus for development of RNAi therapeutic. Various inorganic and organic material-based nanoparticles that were investigated as nonviral siRNA carriers have shown their advantages over "naked" siRNA molecules in terms of siRNA stability in the body and efficiency of organ targeting and tissue penetration. While the synthetic lipids,[8−10] peptides,[11,12] and polymers[13−15] have been predominantly explored for potential carrier for siRNA, naturally occurring molecules such as chitosan[16] have also been investigated for their possible advantages for in vitro and in vivo siRNA delivery. Chemically modified Curdlan has been explored for a variety of biomedical applications including antiviral material,[17,18] immune modulation,[19] and anticoagluant.[20] Recently, we reported a potential siRNA carrier based on chemically modified Curdlan (6-deoxy-6-amino Curdlan, 6AC-100) nanoparticles.[21] 6AC-100 efficiently delivered siRNA to multiple cell lines and substantially knocked down endogenous gene expression. The cell penetration of 6AC-100 might be attributed to membrane disruption, like other cationic polymers do. Therefore, further chemical modification is necessary for cell-type specificity of 6AC-100.

Receptor-mediated siRNA delivery is an attractive approach for siRNA delivery to specific cell types. Small-molecule ligands,[22] aptamers,[23] peptides,[24] and antibodies[25] have been conjugated either directly to siRNA or to the carrier for tissue-specific delivery of siRNA. Asialoglycoprotein receptor (ASGPR) on hepatocytes has been proved to be efficient target for liver targeted siRNA delivery. Small molecular ligands such as galactose and *N*-acetylgalactosamine specifically bind to ASGPR, prompting the subsequent cellular uptake of siRNA/

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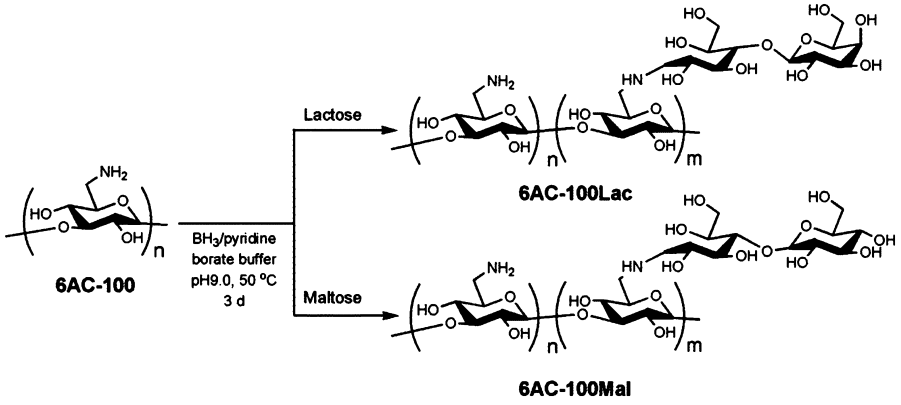
Scheme 1. Synthetic Scheme for Preparation of 6AC-100Lac and 6AC-100Mal



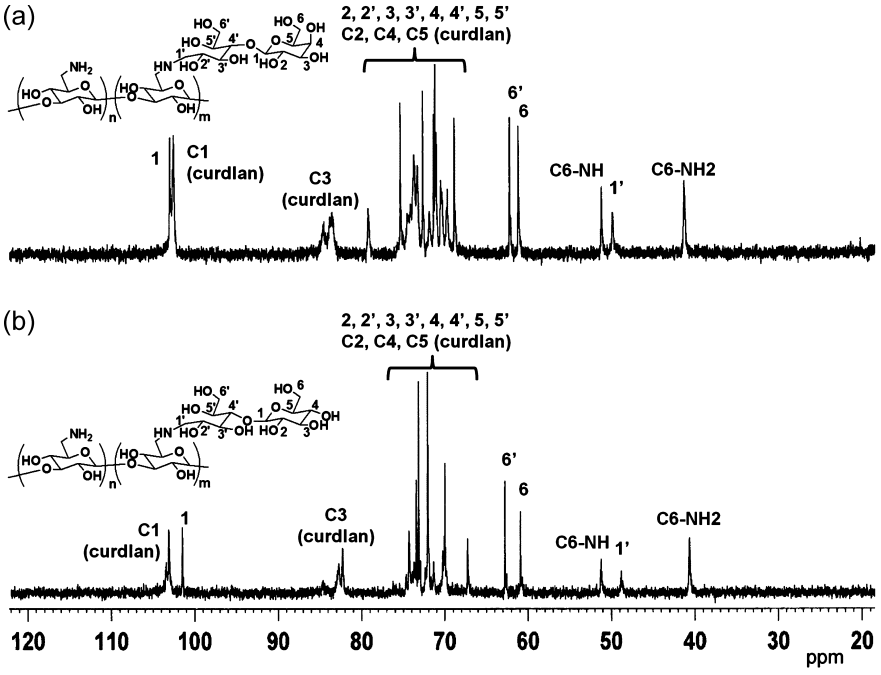


Figure 1. ^{13}C NMR spectra of (a) 6AC-100Lac and (b) 6AC-100Mal in D_2O.

carrier complex conjugated with such ligands, or chemically stabilized siRNA directly conjugated with multivalent ligands for ASGPR.[26−28] Here we report the design of nanoparticles based on disaccharides modified 6AC-100 as biocompatible carrier for cell surface targeted siRNA delivery.

RESULTS AND DISCUSSION

Synthesis and Characterization of 6AC-100Lac and 6AC-100Mal. Previously we reported the preparation of 6-deoxy-6-amino-Curdlan (6AC-100) as a potential carrier for siRNA delivery.[21] The goal of the present study is to further functionalize 6AC-100 for cell-type specific delivery of siRNA through receptor-mediated cellular internalization. To do this, we conjugated lactose to C6-amino groups of 6AC-100 through reductive amination to provide galactose units as targeting moiety for hepatocytes specific siRNA delivery (Scheme 1). Our strategy was to introduce galactose to 6AC-100 (designated as 6AC-100Lac) as multivalent ligands to recognize and bind to cell surface receptor ASGPR on hepatocytes and promote galactose-ASGPR mediated endocytosis. ASGPR, which specifically binds to its natural ligands *N*-acetyl-D-glucosamine and galactose, is abundantly present on the hepatocytes cell surface and has been extensively investigated for receptor-mediated delivery of biotherapeutics including siRNA. To investigate the influence of abundance of galactose on the particle size, surface charge, and cellular entrance of complex formed from the resulting polymer and siRNA, we adjusted the degree of substitution (DS) of galactose branches on the resulting Curdlan backbone so that ∼20% (DS: 0.2), 10% (DS: 0.1), and 6% (DS: 0.06) of C6-amino glucose units of 6AC-100Lac were covalently linked with galactose. The structures of the resulting polymers were confirmed by ^{13}C NMR spectrometry, which shows signals from both parental polymer 6AC-100 and galactose branches that were used to calculate approximate DS of 6AC-100Lac (Figure 1b ^{13}C NMR). The weight-average molecular weight (Mw) of 6AC-100Lac increased with the increasing DS (79 897, 81 805, and 100 052 for DS 0.06, 0.1, and 0.2, respectively), as revealed by GPC analysis. Meanwhile, we prepared maltose-conjugated 6AC-100 (designated as 6AC-100Mal) with different DS as controls for nontargeted siRNA delivery system. Since maltose conjugation produces glucose branches instead of galactose, the

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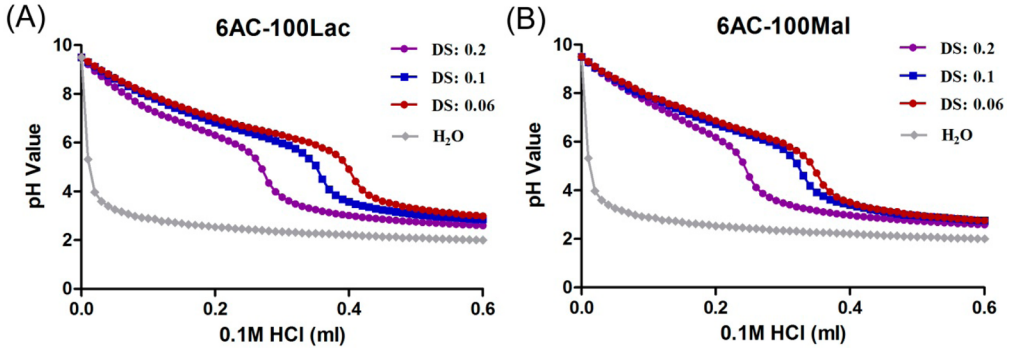

Figure 2. Buffering capacity titration. The concentration of (a) 6AC-100Lac and (b) 6AC-100Mal was 1.0 mg/mL and was titrated by using 0.1 M HCl in 10 μL increments at room temperature.

resulting 6AC-100Mal would lack receptor recognition, and hence would not show cell-type specificity when complexed to siRNA (Figure 1b).

Buffering Capacity. Efficient endosomal escape is one of the key processes for successful cytoplasmic delivery of siRNA by polymeric carriers. Cationic polymers with proton sponge function can promote osmotic swelling of endosome, effectively disrupt endosomal membrane, and facilitate cargo release in the cytoplasm. To investigate the effect of lactose or maltose conjugation on the proton absorption capacity of the resulting 6AC-100 derivatives, we measured buffering efficiency of the 6AC-100Lac and 6AC-100Mal with different DS. At DS 0.06, the buffering efficiency of 6AC-100Lac was as strong as parental polymer (6AC-100). An increased DS of 0.1 slightly reduced buffering effect of 6AC-100Lac. At DS 0.2, an apparent decrease in buffering effect was observed (Figure 2a). Although the content of amine in 6AC-100Lac was consistent with the parent molecule 6AC-100, the result of reductive amination produced secondary amine, which may have less proton absorption. Moreover, the bulky branches of lactose derivative may also contribute to the diminished effect of proton sponge of 6AC-100Lac. A similar buffering behavior was also observed for 6AC-100Mal with different DS (Figure 2b).

Electrophoretic Mobility Shift Assay. Since the electrostatic interaction is essential for cationic polymers to efficiently complex with nucleic acids, we next investigated siRNA binding ability of the 6AC-100 derivatives by gel shift retardation assay (Figure 3). Conjugation of lactose to 6AC-100 slightly

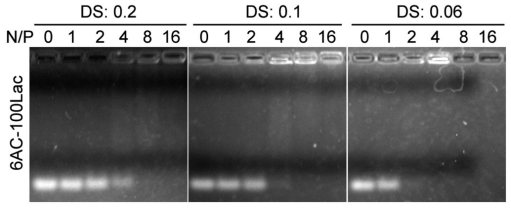

Figure 3. 6AC-100Lac readily binds to siRNA. Gel shift assay of 6AC-100Lac complexed with siRNA at amine-to-phosphate ratio (N/P) ranging from 1 to 16.

decreased the binding efficiency of the resulting 6AC-100Lac with siRNA. For 6AC-100Lac with a DS of 0.2, an amine-to-phosphate charge ratio (N/P ratio) of higher than 4 was needed to stably complex siRNA; at DS 0.1, N/P ratio of 4 showed efficient binding of siRNA; at DS 0.06, N/P ratio of 2 induced strong siRNA binding activity, indicating that DS plays important role for 6AC-100Lac to complex with nucleic acids. Highly substituted polymer has increased molecular mass-to-charge ratio as well as steric hindrance, leading to a decreased nucleic acid access and binding. Similar binding profile was observed for 6AC-100Mal (data not shown).

Cytotoxicity Assay. Conjugation of small molecular ligands may modulate the cytotoxicity of the resulting polymers. To test the cytotoxicity of 6AC-100 derivatives, we treated HepG2 cells with different concentrations of 6AC-100 derivatives (Figure 4). Conjugation of lactose dramatically decreased the cytotoxicity of the resulting polymers: at 60 μg/mL concentration of 6AC-100Lac with a DS of 0.2, ~85% of cells survived, compared to 37% cell survival of the unmodified 6AC-100 treated cells, indicating that conjugation of lactose significantly decrease cytotoxicity of 6AC-100. Similar cytotoxicity was also observed for 6AC-100Mal (Figure 4).

Physiochemical Characterization of siRNA/6AC-100Lac Complex. Ideal surface charge and particle size are crucial for nucleic acid/cationic polymer complex to efficiently enter the cells through receptor-mediated endocytosis.[29] Strongly positively charged complex may enter the cytoplasm by membrane disruption, while the neutral or moderately charged complex may bind to the cell surface through ligand–receptor recognition, which may eventually facilitate cellular uptake of the whole complex. To test the potential receptor-mediated cellular uptake of galactose-functionalized 6AC-100Lac, we formulated siRNA with 6AC-100Lac or 6AC-100Mal by carefully adjusting the amine-to-phosphate ratio so that the resulting complex varies on physicochemical properties such as particle size and zeta potential. Three distinct complexes were prepared to provide slightly negatively charged, neutral, or slightly positively charged nanoparticles for both 6AC-100Lac and 6AC-100Mal (Table 1).

Cell-Type Specific Uptake of siRNA/6AC-100Lac Complex. To evaluate the ability of 6AC-100Lac for the receptor-mediated delivery of nucleic acids, we complexed an FAM-labeled siRNA with 6AC-100Lac or 6AC-100Mal, respectively, and treated HepG2 cells, which express abundant cell surface ASGPR, and subsequently analyzed fluorescence signals using fluorescence microscopy and flow cytometry. Since highly positively charged nanoparticles may penetrate the cells by membrane disruption regardless of cell surface receptors, we first choose a slightly positively charged (zeta potential 5.93 ± 0.25 mV, Table 1) FAM-siRNA/6AC-100Lac complex so that the receptor binding and subsequent cellular internalization might predominate cellular uptake of the nanoparticle. After 4 h of treatment, cellular uptake of FAM-siRNA was observed under fluorescence microscopy. FAM-

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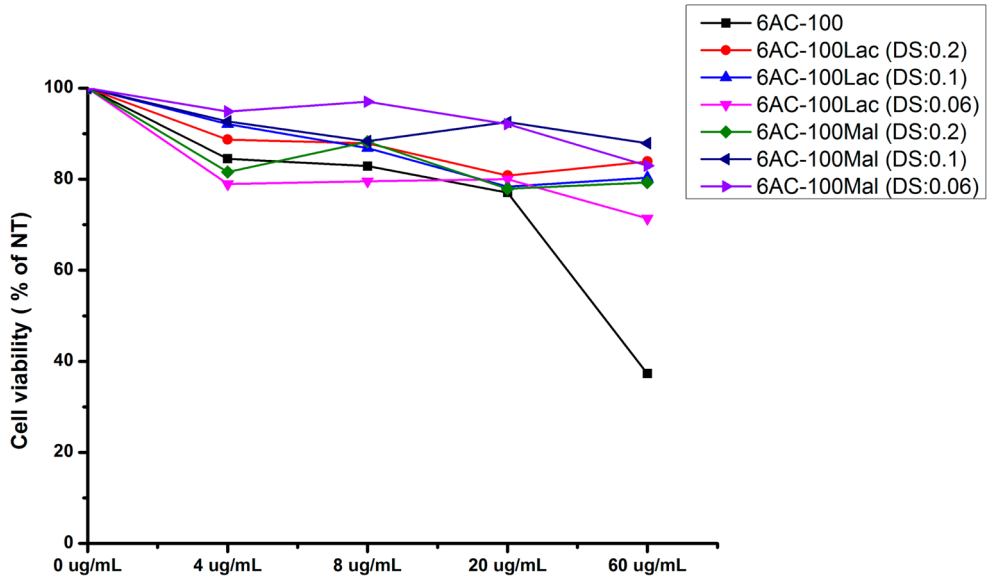

Figure 4. Measurement of cytotoxicity of 6AC-100 derivatives. HepG2 cells remain viable 24 h after treatment with 6AC-100Lac and 6AC-100Mal. Cells were treated with four different concentrations of 6AC-100 derivatives. Final concentrations of 6AC-100Lac or 6AC-100Mal were 0, 4, 8, 20, and 60 μg/mL. Cell toxicity levels are expressed as percent of control (no treatment).

Table 1. Particle Size and Zeta Potential of siRNA-Complexed 6AC-100Lac (DS: 0.2) and 6AC-100Mal (DS: 0.2)[a]

sample	weight ratio	charge ratio (∓)	particle size (nm)	polydispersity index ([(μ_2/Γ^2)])	zeta potential (mV)
siRNA/6AC-100Lac	1:4.7	1:2	288.9	0.124	−4.50 ± 0.7
	1:5.4	1:2.5	440.2	0.136	0.35 ± 0.15
	1:6.2	1:2.9	233.6	0.054	5.93 ± 0.25
siRNA/6AC-100Mal	1:5	1:2.4	386.6	0.296	−4.30 ± 0.325
	1:5.7	1:2.7	467.2	0.162	0.13 ± 0.105
	1:8.6	1:4	217.2	0.002	5.59 ± 0.625

[a]Complex of siRNA/6AC-100Lac and siRNA/6AC-100Mal was prepared in 1X PBS, pH 7.2, and incubated at room temperature for 20 min before performing the measurement.

siRNA/6AC-100Lac treated cells showed significant fluorescence signal (Figures 5a and 6a), while FAM-siRNA/6AC-100Mal treated cells showed apparently less cellular uptake, indicating that galactose-functionalized 6AC-100Lac may bind to the ASGPR on the surface of HepG2 cells and subsequently promotes cellular uptake of the complex, while a glucose-functionalized 6AC-100Mal (zeta potential 5.59 mV, Table 1) cannot. Moreover, pretreatment of HepG2 cells with *N*-acetyl-D-glucosamine substantially reduced cellular uptake of FAM-siRNA/6AC-100Lac complex (Figure 6b) but not FAM-siRNA/6AC-100Mal complex (Figure 6c), confirming that the cellular entrance of FAM-siRNA/6AC-100Lac was indeed mediated by the ASGPR receptor binding. Similar results were observed on another ASGPR expressing cell line Huh7 cells (data not shown). A non-ASGPR expressing cell line A549 did not show apparent fluorescence signal with both FAM-siRNA/6AC-100Lac and FAM-siRNA/6AC-100Mal treatment (Figure 5b), suggesting that the entrance of these nanoparticles is merely charge-driven, not mediated by cell surface receptors. Negatively charged (zeta potential −4.50 ± 0.7 mV, Table 1) or neutral (zeta potential 0.35 ± 0.15 mV, Table 1) FAM-siRNA/6AC-100Lac nanoparticles also showed receptor-mediated cellular entrance to HepG2 cells only, to a lesser extent (data not shown).

Hepatocytes Specific siRNA Delivery by 6AC-100Lac. Fluorescence signals could be generated from FAM-siRNA/6AC-100Lac complexes binding on the cell surface, trapped in endosomes, or released into cytoplasm. To test the receptor-mediated cellular internalization and cytosol siRNA delivery efficacy of 6AC-100Lac nanoparticles, we treated HepG2 cells and A549 cells with 6AC-100 derivatives complexed with siGAPDH. Twenty-four hours later, total RNA was extracted from the cells, and mRNA level of GAPDH was measured by RT-qPCR. A single transfection of HepG2 cells with siGAPDH/6AC-100Lac (DS: 0.2) induced 45% decrease of GAPDH mRNA level (Figure 7a), while siGAPDH/6AC-100Mal complex gave a lower knock down of 35% (Figure 7b), indicating that highly galactose-substituted 6AC-100Lac nanoparticles enter the cells through receptor-mediated cellular internalization, release siRNA in the cytoplasm, and induce RNAi activities. However, stronger knock down of endogenous GAPDH was achieved by 6AC-100Lac with lower DS (85% and 95% decrease of mRNA level for DS 0.1 and 0.06, respectively; Figure 7a). 6AC-100Mal with lower DS also gave relatively high siRNA delivery efficiency (70% and 90% decrease of mRNA level for DS 0.1 and 0.06, respectively; Figure 7b), indicating that 6AC-100 derivatives with low DS may enter the cells through membrane disruption by strong cationic backbone, regardless of cell surface receptors. Moreover, treatment of A549 cells with both 6AC-100 derivatives did not show any difference in terms of siRNA delivery efficiency (Figure 7c,d).

CONCLUSION

Galactose-functionalized 6-amino-6-deoxy-Curdlan (6AC-100) has been successfully synthesized by one-step reductive amination. Measurement of buffering capacity revealed that 6AC-100Lac with different DS showed excellent proton

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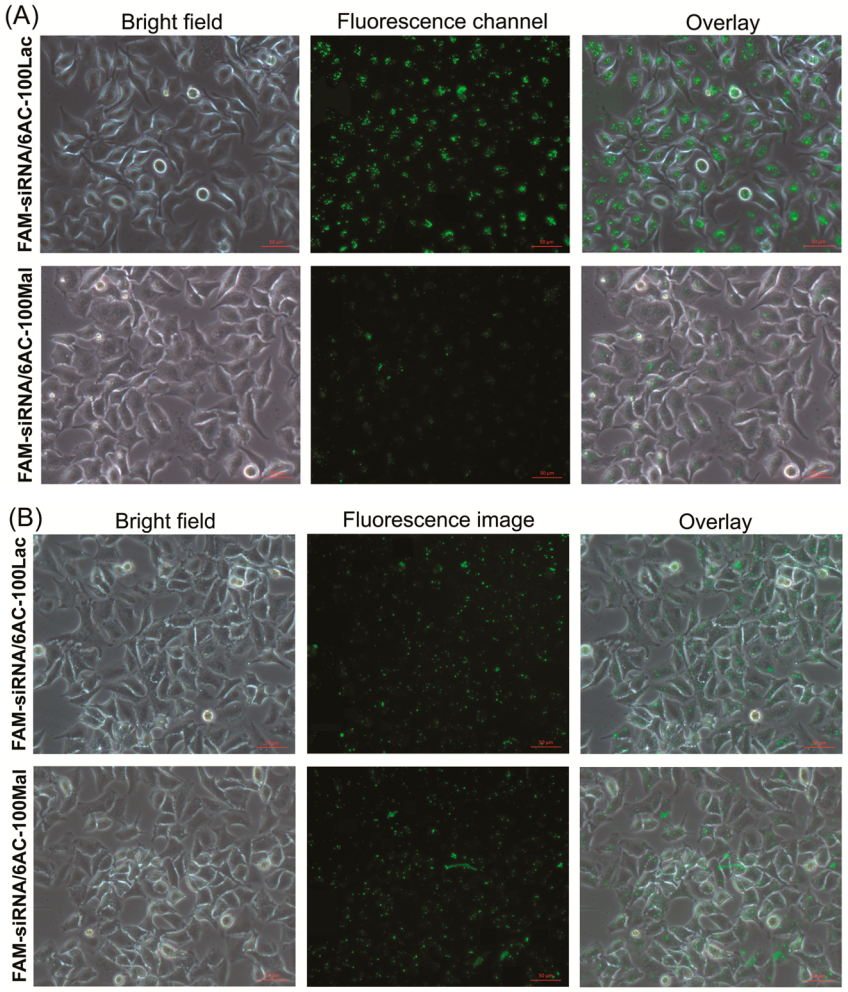

Figure 5. Fluorescence microscopy observation of FAM-siRNA/6AC-100Lac or FAM-siRNA/6AC-100Mal in (a) HepG2 cells and (b) ASGPR-negative A549 cells after 4 h of culture at 37 °C. FAM-siRNA fluorescence signal was shown as green color. Cells were observed using a Zeiss Axio Vert.A1 fluorescence microscope. Doses of FAM-siRNA were 100 nM.

absorption, which may contribute to endosomal escape after cell penetration. The electrophoresis mobility shift assay showed that 6AC-100Lac can strongly complex to nucleic acids even at very low amine-to-phosphate ratio. 6AC-100Lac was less toxic than the parent molecule 6AC-100, as demonstrated by MTT assay. Highly substituted 6AC-100Lac showed efficient nucleic acid delivery to cells expressing ASGPR receptor (HepG2 cells) only, as confirmed by both fluorescence microcopy and flow cytometry analysis. Pretreating HepG2 cells with GalNAc partially blocked cellular uptake of siRNA/6AC-100Lac complex. The 6AC-100Lac-based nanoparticle delivered siRNA preferably to HepG2 cells and substantially knocked down endogenous gene. Our results collectively demonstrated that 6AC-100Lac nanoparticle enters cells through receptor-mediated internalization. Therefore, 6AC-100Lac is a promising delivery agent for targeted siRNA.

EXPERIMENTAL SECTION

Chemicals and General Methods. Curdlan was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D-Lactose monohydrate, D-maltose monohydrate, and borane pyridine complex were purchase from Aladdin (Shanghai, China). Dialysis tube was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Chemical shifts in ^{13}C NMR (Bruker 500 NMR spectrometer) were referred to tetramethylsilane with the residual proton of the deuterated solvent.

Synthesis of 6AC-100Lac. 6-Amino-6-dexoy-Curdlan (6AC-100, 80 mg) was dissolved in 3 mL of borate buffer (0.1 M, pH = 9.0), and borane−pyridine complex (15 μL) was added, followed by stirring at 50 °C for 20 min. Then lactose (35.8 mg) was added, and the reaction mixture was refluxed at 50 °C for 3 d. The product was then dialyzed using a dialysis tube (MWCO 3500) and was obtained by freeze-drying from water with a yield of 65 mg. 6AC-100Lac with low DS was synthesized in the same way by reducing the amount of lactose.

Synthesis of 6AC-100Mal. 6AC-100 (80 mg) was dissolved in 3 mL of borate buffer (0.1 M, pH = 9.0), and borane−pyridine complex (15 μL) was added, followed by stirring at 50 °C for 20 min. Then maltose (35.8 mg) was added, and the reaction mixture was refluxed at 50 °C for 3 d. The product was then dialyzed using a dialysis tube (MWCO 3500) and was obtained by freeze-drying from water with a yield of 60 mg. 6AC-100Mal with low DS was synthesized in the same way by reducing the amount of maltose.

Gel Permeation Chromatography Analysis of 6AC-100Lac and 6AC-100Mal. The weight-average molecular weights of 6AC-100Lac and 6AC-100Mal were determined by gel permeation chromatography (GPC) using a Waters HPLC instrument (Waters Co., Milford, MA, USA) with a 7.8 × 300 mm column (Waters Co., Milford, MA, USA) eluted with acetic acid (0.2 M)/sodium acetate (0.1 M) buffer. The flow rate is 0.8 mL min^{-1}, and the temperature is 40 °C.

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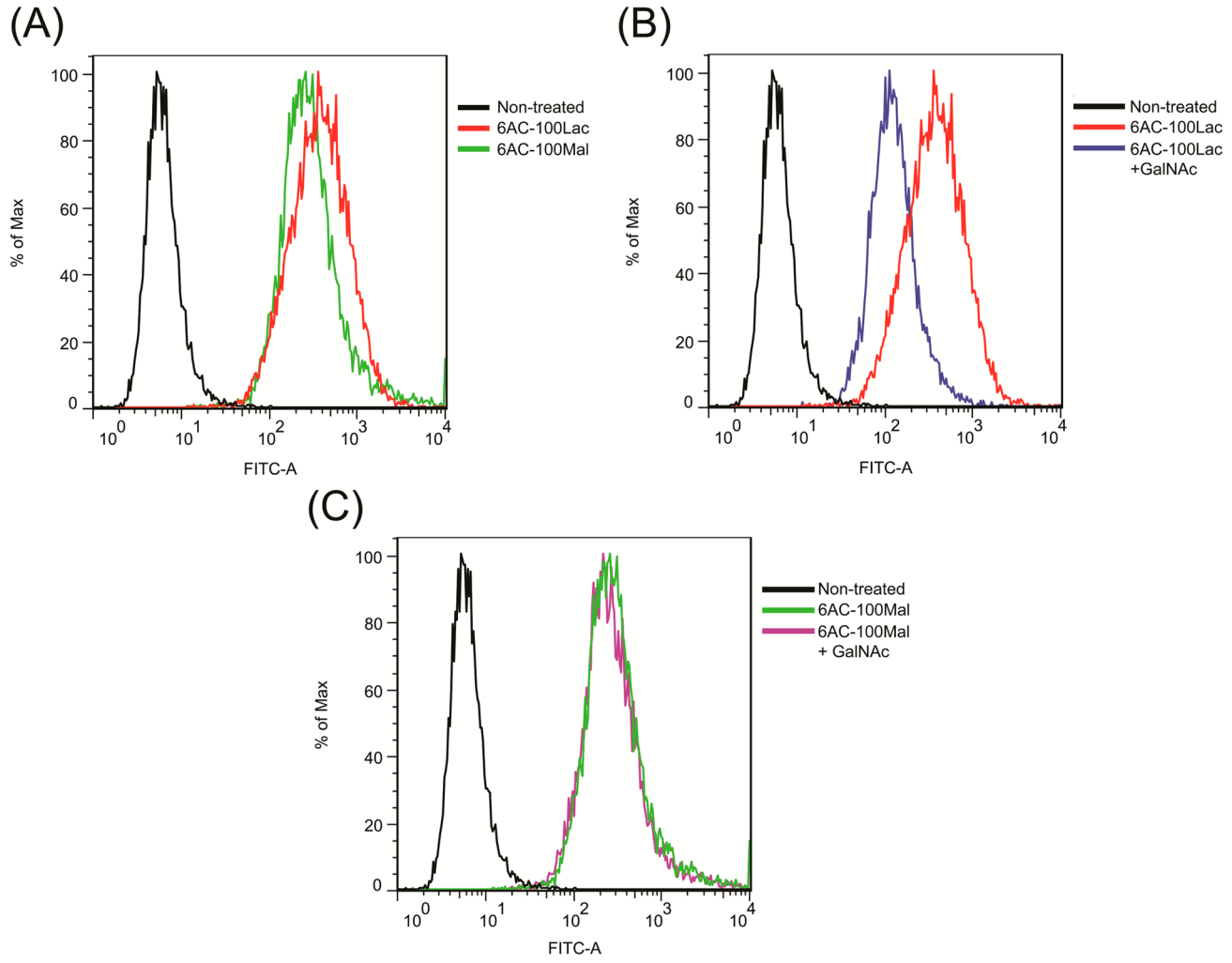

Figure 6. (a) Flow cytometric analyses of HepG2 cells treated with FAM-siRNA/6AC-100Lac for 4 h at 37 °C. (b) Flow cytometric analyses of HepG2 cells treated with FAM-siRNA/6AC-100Lac in the absence or presence of 60 mM *N*-acetyl-D-galactosamine. (c) Flow cytometric analyses of HepG2 cells treated with FAM-siRNA/6AC-100Mal in the absence or presence of 60 mM *N*-acetyl-D-galactosamine. Doses of FAM-siRNA were 100 nM.

Buffering Capacity. The buffering capacity of 6AC-100Lac and 6AC-100Mal was determined by acid−base titration. Either 6AC-100Lac (10 mg) or 6AC-100Mal (10 mg) was dissolved into 10 mL of H_2O, and the pH of the solution was adjusted to 9.5 using 0.1 M NaOH. Then the solution was titrated by adding 10 μL of 0.1 M HCl until the pH of the solution reached to 2.0. The pH values were measured using OHAUS Starter 300C pH meter (Parsippany, NJ).

Electrophoretic Mobility Shift Assay. The sequence of siRNA was as same as the sequence of siRNA that was used in the siRNA transfection experiments. The amine-to-phosphate ratio (N/P) of mixtures composed of 6AC-100Lac or 6AC-100Mal and siRNA ranged from 0 to 16. Mixtures were incubated for 20 min at room temperature and then analyzed on a 2% agarose gel at 120 V for 40 min in 1X TBE running buffer. The gel was stained using 0.5 μg/mL ethidium bromide and captured using a Gel Logic 212 PRO imaging system (Carestream, Toronto, Canada).

Measurement of Particle Size and Zeta Potential. siRNA/6AC-100Lac and siRNA/6AC-100Mal complexes were prepared at different weight ratio, and the complexes were incubated for 20 min at room temperature. The particle size and zeta potential of siRNA/6AC-100Lac and siRNA/6AC-100Mal complexes were measured on a Zetasizer Nano S instrument (Malvern Instrument, U.K.).

Cell Culture, Cytotoxicity Measurement. HepG2 cells were cultured in DMEM/HIGH GLUCOSE with 10% FBS, 100 IU/ml penicillin, 100 μg/mL streptomycin. A549 cells were cultured in DMEM/F12 with 10% FBS, 100 IU/ml penicillin, 100 μg/mL streptomycin. Cells were grown at 37 °C under an atmosphere of 5% CO_2 in air. The cytotoxicity of 6AC-100Lac and 6AC-100Mal was evaluated using MTT assay. Cells were planted at a density of 1.0×10^4 cells per well in 96-well plates and cultured for 24 h. Then the culture medium was replaced with 100 μL of medium containing different concentrations of 6AC-100Lac or 6AC-100Mal ranging from 0.2 to 5.0 μL of stock solution (2.0 mg/mL). Twenty-four hours later, cell viability was assayed using the MTT test.

Cellular Uptake. The cellular uptake ability is assessed by fluorescence microscopy and flow cytometry (FCM). HepG2 cells (2.5×10^5 cells per well) were plated into 12-well plates. Silencer FAM-labeled GAPDH siRNA (Life Technologies, Carlsbad, CA, USA) was used to observe cellular uptake. Twenty-four hours later, cells were treated with either FAM-siRNA/6AC-100Lac or FAM-siRNA/6AC-100Mal at FAM-siRNA dose of 100 nM. For microscopic observation, cells were washed five times with ice-cold PBS (pH 7.4) after 4 h of incubation and then observed with fluorescence microscopy (Axio Vert.A1, ZEISS, Germany). For FCM, after 4 h of incubation, cells were trypsinized, washed with ice-cold phosphate-buffered saline (PBS; pH 7.4), and fixed with 70% ice-cold ethanol for 3 h. Then cells were resuspended in 1 mL of PBS and assessed on a BD LSRFortessaTM Cell Analyzer (BD Bioscience, Bedford, MA). For the competition assay, *N*-acetyl-D-galactosamine (60 mM) was preadded into the cells 1 h before the transfection.

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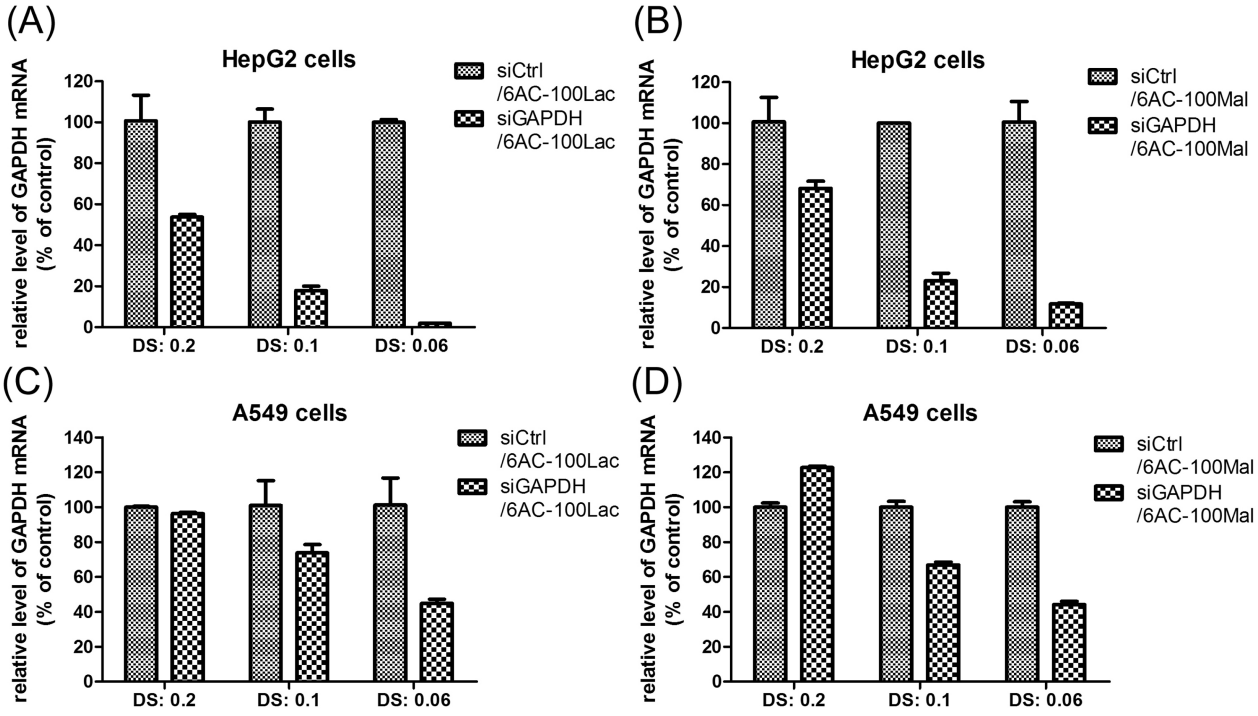

Figure 7. In vitro silencing of endogenous GAPDH in HepG2 cells by (a) siRNA/6AC-100Lac or (b) siRNA/6AC-100Mal nanoparticles. Transfection of ASGPR-negative A549 cells with (c) siRNA/6AC-100Lac or (d) siRNA/6AC-100Mal nanoparticles were shown as control. Cells were treated for 4 h with 6AC polymers containing nontargeting siRNA (siCtrl) or siRNA targeting human GAPDH. mRNA levels are expressed as percent of control.

In Vitro siRNA Transfection. For in vitro siRNA delivery experiments, we used siGenome Non-Targeting siRNA Control and siGenome Human GAPDH siRNA (Dharmacon, Lafayette, CO, USA). HepG2 and A549 cells were seeded in 12-well plates at 2.5×10^5 cells per well and incubated for 24 h. Then, the culture medium was replaced with 1 mL of Opti-MEM containing 4 μL of stock solution (2.0 mg/mL) of 6AC-100Lac or 6AC-100Mal complexed with siRNA (final concentration: 50 nM). After 4 h of incubation, the Opti-MEM was removed, and 1 mL of original medium was added to each well. Twenty-four hours later, the total RNA of cells was extracted by Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The expression of mRNA was measured using iScriptTM Reverse Transcription Supermix for RT-qPCR and iTaqTM Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) for quantitative PCR. The sequences of primers used for RT-qPCR were as follows: human β-actin, forward: 5′-ccaaccgcgagaagatga-3′, reverse: 5′-ccagaggcgta-cagggatag-3′, human GAPDH, forward: 5′-agccacatcgctcagacac-3′, reverse: 5′-gcccaatacgaccaaatcc-3′.

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Notes

The authors declare no competing financial interest.

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