Study on Synthesis of Multivalent Neoglycoproteins and Their Binding Properties to Hepatic Stellate Cells†

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Neoglycoproteins of human serum albumin (HSA) modified with some kind of carbohydrates were synthesized. Their molecular weight and purification were determined by HPSEC and SDS-PAGE, respectively. The binding properties of these conjugates to hepatic stellate cells (HSCs) were evaluated by confocal fluorescence microscopy. The bioactivity revealed that compound 4a (HSA modified with glucose) showed high binding affinity.

Keywords neoglycoprotein, carbohydrate, HSC, binding property

Introduction

Liver fibrosis is characterized by an increased deposition of extracellular matrix constituents. The major cell type responsible for hepatic fibrogenesis is the hepatic stellate cells (HSCs).[1-5] So this cell type is an important target for antifibrotic therapies.[6,7] However, antifibrotic drugs may not be efficiently taken up by HSCs or may produce unwanted side-effects outside the liver. Drug targeting may be exploited to elicit cell-specific uptake of drugs.[8] To attain HSCs-specific uptake, drug can be coupled to carrier that are designed for selective uptake by target cells. So the carrier molecule is important in drug targeting. Recently, it has been reported that some neoglycoproteins have binding specificity to hepatocytes, kupffer cells and sinusoidal endothelial cells.[8-10] The studies have showed that the binding affinity of a carbohydrate ligand to a certain class of cell membrane receptors is highly influenced by the number and orientation of the sugar residues, while the aglycon part plays a minor role in ligand recognition.[11,12] A dramatic increase in binding affinity was observed when mono- and multivalent carbohydrate were introduced.[13,14] Now, we synthesized neoglycoproteins of human serum albumin (HSA) modified with some kind of carbohydrates, which are appropriate tools for carbohydrate-mediated cellular uptake. The binding property of these conjugates to HSCs were evaluated by confocal fluorescence microscopy.

Experimental

General methods

^{1}H NMR spectra were recorded on a Varian 300 spectrometer (300 MHz). Elemental analysis were performed on a Carlo Erba 1106. High performance size-exclusion chromatography (HPSEC) analysis was performed on a Biosep-SEC-S400 column with 0.1 mol/L phosphate buffer (pH = 7.4) as mobile phase, at a flow rate of 1 mL/min. Thin-layer chromatography (TLC): E. Merck slica Gel60 F254, compounds were detected under UV or by spraying 5% concd. H_2SO_4 in MeOH and then by heating the plates. Sephadex G-25 was purchased from Ammershan Pharmacia Biotech. HSA was purchased from Sigma. The solvents used were of analytical grade.

The *p*-nitrophenyl glycosides **1a**—**1g** were synthesized according to the literatures.[15-20]

Preparation of **2a**—**2g**

To a solution of **1** (0.66 mmol) in dried MeOH (20 mL) was added 10% Pd/C (20 mg). The suspension was stirred under H_2(1×10^5 Pa) for 3 h. After filtration, the solvent was evaporated under reduced pressure. The residue was recrystallized from MeOH to give **2** (Scheme 1).

p-Nitrophenyl α-D-glucopyranoside (**2a**) White solid, yield 97.2%; ^{1}H NMR (D_2O) δ: 6.83 (d, J = 8.4 Hz, 2H), 6.63 (d, J = 8.4 Hz, 2H), 5.27 (d, J = 3.6 Hz, 1H), 3.15—3.69 (m, 6H). Anal. calcd for $C_{12}H_{17}NO_6$: C 53.13, H 6.32, N 5.16; found C 52.91, H 6.47, N 5.02.

p-Nitrophenyl α-D-galactopyranoside (**2b**) White solid, yield 91.8%; ^{1}H NMR (D_2O) δ: 6.95 (d, J = 8.7 Hz, 2H), 6.75 (d, J = 8.7 Hz, 2H), 5.40 (d,

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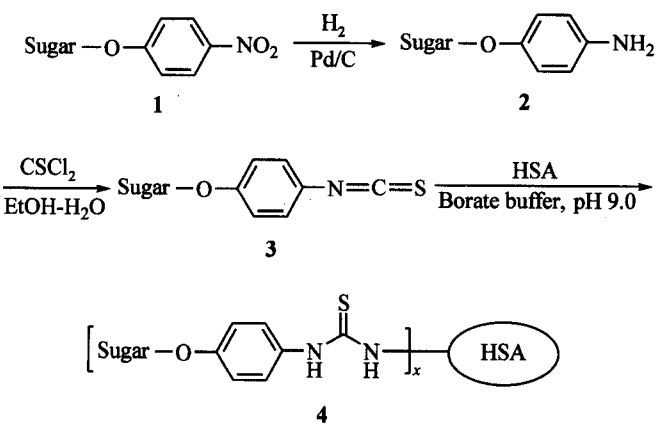
J = 3.6 Hz, 1H), 3.88—4.07 (m, 6H). Anal. calcd for $C_{12}H_{17}NO_6$: C 53.13, H 6.32, N 5.16; found C 52.86, H 6.30, N 5.11.

Scheme 1



p-Nitrophenyl α-D-mannopyranoside (**2c**) White solid, yield 64.2%; ^{1}H NMR (D_2O) δ: 6.87 (d, J = 8.7 Hz, 2H), 6.69 (d, J = 8.7 Hz, 2H), 5.29 (d, J = 3.6 Hz, 1H), 3.36—4.99 (m, 6H). Anal. calcd for $C_{12}H_{17}NO_6$: C 53.13, H 6.32, N 5.16; found C 52.90, H 6.34, N 4.93.

p-Nitrophenyl α-L-rhamnopyranoside (**2d**) White solid, yield 97.8%; ^{1}H NMR (D_2O) δ: 6.94 (d, J = 8.4 Hz, 2H), 6.78 (d, J = 8.1 Hz, 2H), 5.33 (s, 1H), 4.10 (t, J = 1.8 Hz, 1H), 3.78—3.92 (m, 2H), 3.45 (t, J = 9.6 Hz, 1H), 1.18 (d, J = 6.6 Hz, 3H). Anal. calcd for $C_{12}H_{17}NO_5 \cdot 0.5H_2O$: C 54.54, H 7.08, N 5.30; found C 55.07, H 7.08, N 5.29.

p-Nitrophenyl α-D-fucopyranoside (**2e**) White solid, yield 90.8%; ^{1}H NMR (D_2O) δ: 6.89 (d, J = 8.7 Hz, 2H), 6.73 (d, J = 8.7 Hz, 2H), 5.33 (d, J = 3.6 Hz, 1H), 4.18 (q, J = 6.6, 7.2, 6.3 Hz, 1H), 3.94 (ddd, J = 3.0, 7.2, 3.3 Hz, 1H), 3.77—3.83 (m, 2H), 1.07 (d, J = 6.6 Hz, 3H). Anal. calcd for $C_{12}H_{17}NO_5 \cdot 0.5H_2O$: C 54.54, H 7.08, N 5.30; found C 55.34, H 6.92, N 5.26.

p-Nitrophenyl β-D-fucopyranoside (**2f**) White solid, yield 96.5%; ^{1}H NMR (D_2O) δ: 6.79 (d, J = 8.7 Hz, 2H), 6.63 (d, J = 8.7 Hz, 2H), 4.70 (d, J = 6.3 Hz, 1H), 3.70 (q, J = 6.6 Hz, 1H), 3.50—3.59 (m, 4H), 1.06 (d, J = 6.0 Hz, 3H). Anal. calcd for $C_{12}H_{17}NO_5 \cdot 0.5H_2O$: C 54.54, H 7.08, N 5.30; found C 55.21, H 6.92, N 5.20.

p-Nitrophenyl β-lactoside (**2g**) White solid, yield 85.7%; ^{1}H NMR (D_2O) δ: 6.80 (d, J = 9.3 Hz, 2H), 6.63 (d, J = 9.3 Hz, 2H), 4.79 (d, J = 7.8 Hz, 1H), 4.26 (d, J = 7.8 Hz, 1H), 3.31—3.79 (m, 12H). Anal. calcd for $C_{18}H_{27}NO_{11} \cdot 1.5H_2O$: C 46.96, H 6.57, N 3.04; found C 47.17, H 6.65, N 2.96.

Preparation of neoglycoproteins **4a—4g**

Thiophosgen (27 μL, 0.35 mmol) was added with stirring to the solution containing **2** (0.062 mmol) in 80% EtOH (5 mL). The solution was stirred for 1.5 h at room temperature. Nitrogen was bubbled through the solution until most of the odor was removed. The pH was adjusted to 6.0 with 0.1 N NaOH and the solution was evaporated under reduced pressure to give **3**, IR ν: ~2125 cm^{-1}. The crude **3** was used for the next step without further purification. Distilled water (5 mL) was added to dissolve the solid. The aqueous solution of sugar derivative was then added slowly to a solution of the HSA (14.5 mg, 0.22 μmol) in 0.01 mol/L borate buffer (7.5 mL), pH = 9.0. The reaction mixture was stirred at room temperature for 18 h, during which the pH was kept at 9.0. This was then dialyzed against 0.15 mol/L NaCl solution (2 × 500 mL), distilled water (4 × 500 mL, 1000 MW cutoff) respectively, lyophilized to afford white solid. The crude product was purified with Sephadex G-25 gel chromatography, eluted with 0.15 mol/L NaCl to give neoglycoproteins **4a—4g**.

Cell isolation and culture

HSCs were isolated from rat liver as described by Hu[21] with slight modification. The HSCs showed a typical stellate-like shape containing fat droplet in cytoplasm. After two weeks, HSCs looked like myofibroblast. Cells were sealed on 24-well culture plates at a density of 1 × 10^5 cell/mL cultured media. Two days later, the neoglycoproteins **4a—4g** were added into wells at a final concentration of 0.1 mg/mL, respectively, and incubated for 4 h. After washed with 0.1 mol/L PBS (pH = 7.4) for three times, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were washed with PBS for three times, and incubated with FITC-labeled monoclonal antibody rabbit anti-human albumin (DaKo, 1:40 dilution) for 30 min at 37 ℃. At the end, the cells were washed thoroughly with PBS for three times (10 min once), mounted in an antifade medium, and sealed.

For all studies, the cells were imaged on a Zeiss Axioplan 2 microscope (40 × plan-apo objective, FITC, excitation = 488 nm, emission = 505 nm long-pass filter).

Result and discussion

Chemical synthesis

Isothiocyanate method[22] was used in the preparation of neoglycoproteins **4a**—**4g**. After the nitro group of compound **1** was reduced with Pd/C under hydrogen, the aminophenyl glycoside **2** then reacted with thiophosgene giving compound **3** as shown by TLC, and infrared spectroscopy on the basis of the characteristic broad band centered at about 2125 cm^{-1}. Without further purification, compound **3** were reacted with HSA (free lysyl ε-amino groups) to give neoglycoproteins **4a**—**4g** (Scheme 1).

Sugar/protein ratio in neoglycoprotein was assessed by sugar determination according to the phenol-H_2SO_4 method.[23] The molecular weight and purification was determined by HPSEC (Fig. 1) and SDS-PAGE (Fig. 2), respectively. All data are shown in Table 1.

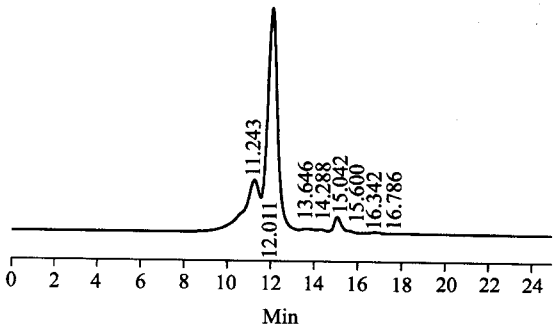


Fig. 1 HPSEC of compound **4a** on a Bio-Sep-SEC-S4000 column (300 × 7.80 mm) in 0.1 mol/L phosphate buffer, pH = 7.4 at a flow rate of 1 mL/min.

HSCs *binding property*

Compared with the control group (Fig. 3, a), only cells incubated with compound **4a** was observed diffuse fluorescence at the cytoplasm and intensive nucleolar accumulation (Fig. 3, b). The low binding affinity of compounds **4b** and **4g** (Fig. 3, c) demonstrated that the terminal galactose can recognize the receptor on the parenchymal, *i.e.*, hepatocytes,[24] but not the receptor on HSCs. Although the 6-deoxy carbohydrates **4d**—**4f** can efficiently bind to Kupffer cells in the liver,[25] they also showed low binding affinity to HSCs in our experiment (Fig. 3, d). So compound **4a** can be a suitable candidate for delivery drugs to hepatic stellate cells (HSCs).

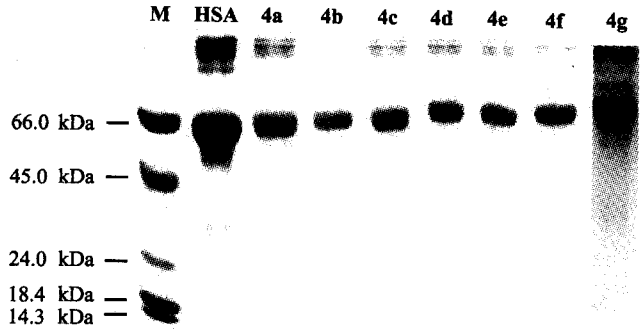


Fig. 2 SDS-PAGE analysis of HSA and compound **4a**—**4g**, line M is molecular marker: 14.3, 18.4, 24.0, 45.0, 66.0 kDa.

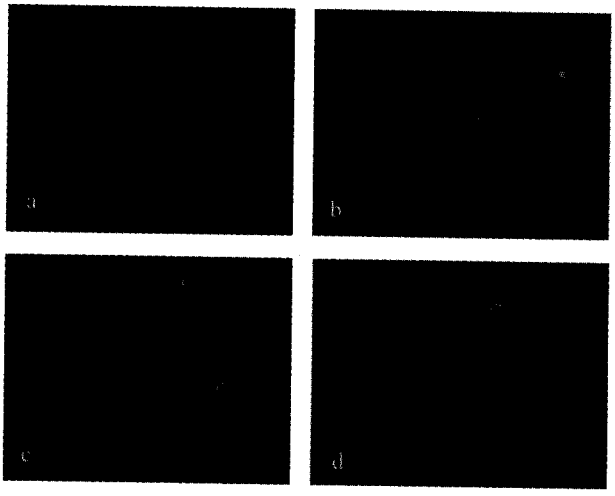


Fig. 3 Fluorescence images of cells incubated with (a) control group, (b) compounds **4a**, (c) compound **4g** and (d) compound **4f** (original magnification 40 ×).

Table 1 Characterization of the different preparation of neoglycoproteins

Neoglycoprotein	Sugar/protein ratio (x) (mol/mol)	Yield (%)	HPSEC		SDS-PAGE
			t_R(min)	Molecular weight (kDa)[a]	R_f
HSA	0	—	12.197	61.1	0.333
4a	26.7	64.5	12.011	74.7	0.300
4b	27.4	54.3	12.000	75.6	0.295
4c	25.5	64.2	12.109	67.2	0.299
4d	15.9	49.5	12.150	64.3	0.301
4e	15.8	42.9	12.162	63.5	0.302
4f	15.8	56.1	12.089	68.7	0.300
4g	31.5	59.4	12.007	75.0	0.252

[a] Molecular weight was calculated according to the standard curve equation: $\lg M_w = 10.49424 - 0.46797 t_R$, $\gamma = 0.99951$, which was obtained from HPSEC.

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