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Efficient and widely applicable method of constructing neo-proteoglycan utilizing copper(I) catalyzed 1,3-dipolar cycloaddition

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Abstract—Efficient constructions of two types of neo-proteoglycan are described. Enzymatically prepared alkyne containing chondroitin 6-sulfate chains and chemically synthesized azido group having compounds are linked by utilizing the copper(I) catalyzed 1,3-dipolar cycloaddition.
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Proteoglycans exist in tissues of many mammalian species, both at the cell surface and in the extracellular matrix. These consist of a protein core, which has one or more glycosaminoglycan side chains bound covalently to it. Chondroitin sulfate (ChS) is a constituent of proteoglycan and has various biological activities.[1] This paper describes the effective introduction of chondroitin 6-sulfate (Ch6S) saccharide chains to artificial material **3** and protein **4**. In the previous study, we achieved neo-proteoglycan synthesis by exploiting the transglycosylation activity of *endo*-β-xylosidase from Patnopecten Mid-gut Gland.[2] This method can directly transfer the intact ChS chain which contains linkage-region tetrasaccharide, to the biologically active peptides of the serine residue.[2,3] However, the method of synthesis gives a low yield (>5%) due to amino acids bearing a sterically hindered sugar moiety, and has a limited range of possible substrates. To overcome these problems, we have established a more efficient and widely applicable method for constructing neo-proteoglycan utilizing copper(I) catalyzed 1,3-dipolar cycloaddition between organic azides and alkyne, which was recently reported by Meldal and Sharpless[4,5] (Scheme 1). To utilize this ligation reaction in neo-proteoglycan synthesis, we synthesized alkyne containing Ch6S **2** and azido containing compounds **3** and **4** as substitutes for core proteins.

First, in order to obtain the alkyne containing Ch6S **2**, we employed peptide chondroitin 6-sulfate (Peptide Ch6S) **1** as a donor, which was prepared from proteoglycan derived from salmon nose cartilage.[6] **1** was treated with *endo*-β-xylosidase in the presence of propargyl alcohol. The enzyme cleaved the β-xyloside linkage between the reducing end xylose and the hydroxyl group of the side chain of Ser, and at the same time introduced the alkyne into the xylose in accordance with its transglycosylation activity[7] (Scheme 1).

Second, we prepared two compounds **3**[8] and **4**,[9] for uniting with Ch6S **2**. We chose polyethylene glycol (PEG) as an artificial material instead of core proteins. It is advantageous to use PEG as a synthetic neo-proteoglycan, because its safety for medical treatment has been confirmed.[10] The azido group containing PEG **3** which can be readily prepared by chemically introduced 4-azido benzoic acid (Tokyo Kasei Kogyo Co., Japan) to PEG methyl ether (Aldrich, average MW ca 550) (Scheme 1). Bovine serum albumin (BSA) was used as the model protein for conjugation of Ch6S. 4-Azido benzoic acid was introduced into BSA to afford **4**. The degree of average incorporation of the acid in the BSA

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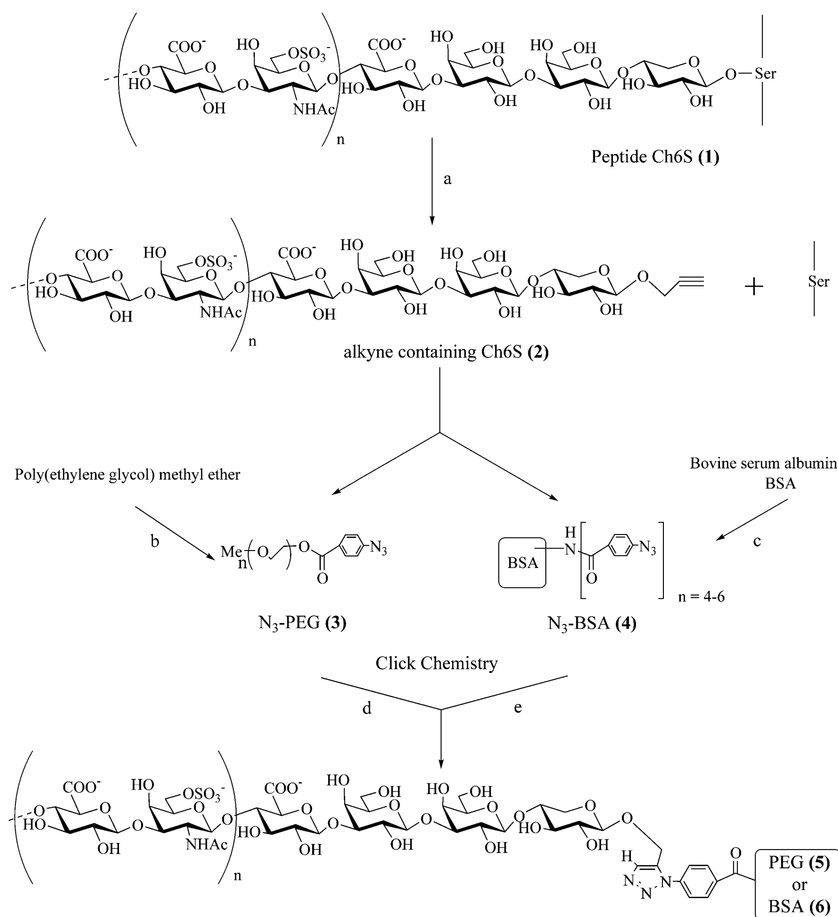

Scheme 1. Reagents and conditions: (a) *endo*-β-Xylosidase, propargyl alcohol, sodium acetate buffer (0.1 M); (b) 4-azidobenzoic acid, DCC, DMAP, CH_2Cl_2, rt, 12 h, quant.; (c) 4-azidobenzoic acid, EDC, DMF/H_2O, 4 °C, 12 h; (d) 10 mM $CuSO_4$, 10 mM DIPEA, 37 °C, 12 h; (e) 10 mM $CuSO_4$, 10 mM DIPEA, 37 °C, 24 h.

($n = 4$–6) was determined using an ESI-MS spectrometer (Scheme 1).

Synthesis and analysis of neo-proteoglycan (**5**): the reagents were added in the following sequence: **2** (40 μL; 20 mg mL^{-1}), **3** (15 μL; 90 μM in DMSO), $CuSO_4$ (15 μL; 10 mM) and DIPEA (15 μL; 10 mM). The reaction mixture was vortexed and allowed to react at 37 °C for 12 h under a N_2 atmosphere, followed by repetitive evaporation to remove the DMSO. Column chromatography (H_2O/EtOH = 9:1) of the residue on Sephadex G-25 gave the crude target molecule, and this was purified using a C18-Sep-Pac cartridge (MeOH/H_2O = 0:100–50:50) to afford the neo-proteoglycan **5**.[11] The yield of **5** based on **3** was 54%. In our previous method,[3] Ch6S could not be introduced into PEG. Electrophoresis of **5** was carried out on a cellulose acetate membrane and stained with Alcian Blue which detects glycosaminoglycans. Compound **5** was a stained single band and its migration was near Ch6S standard (Fig. 1).

Furthermore, we analyzed **5** using MALDI-TOF mass DHBA as a matrix. The average molecular weight of N_3-PEG was m/z 695 and the distribution of the observed mass spectrum was about m/z 400 (Fig. 2B). Over the click chemical reaction, the average molecular

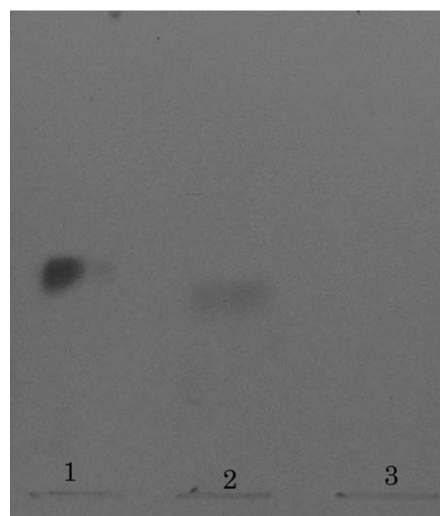

Figure 1. Electrophoretogram of compound **5** on cellulose acetate membrane. Conditions: 0.1 M pyridine/0.47 M formic acid buffer (pH = 3.0) at 1 mA/cm for 10 min and staining of Ch6S on the cellulose acetate membrane was carried out with 0.05% Alcian Blue in 70% ethanol. Lane 1; chondroitin 6-sulfate from shark cartilage was purchased from Seikagaku Kogyo Co. (Tokyo Japan). Lane 2; compound **5**. Lane 3; compound **3**.

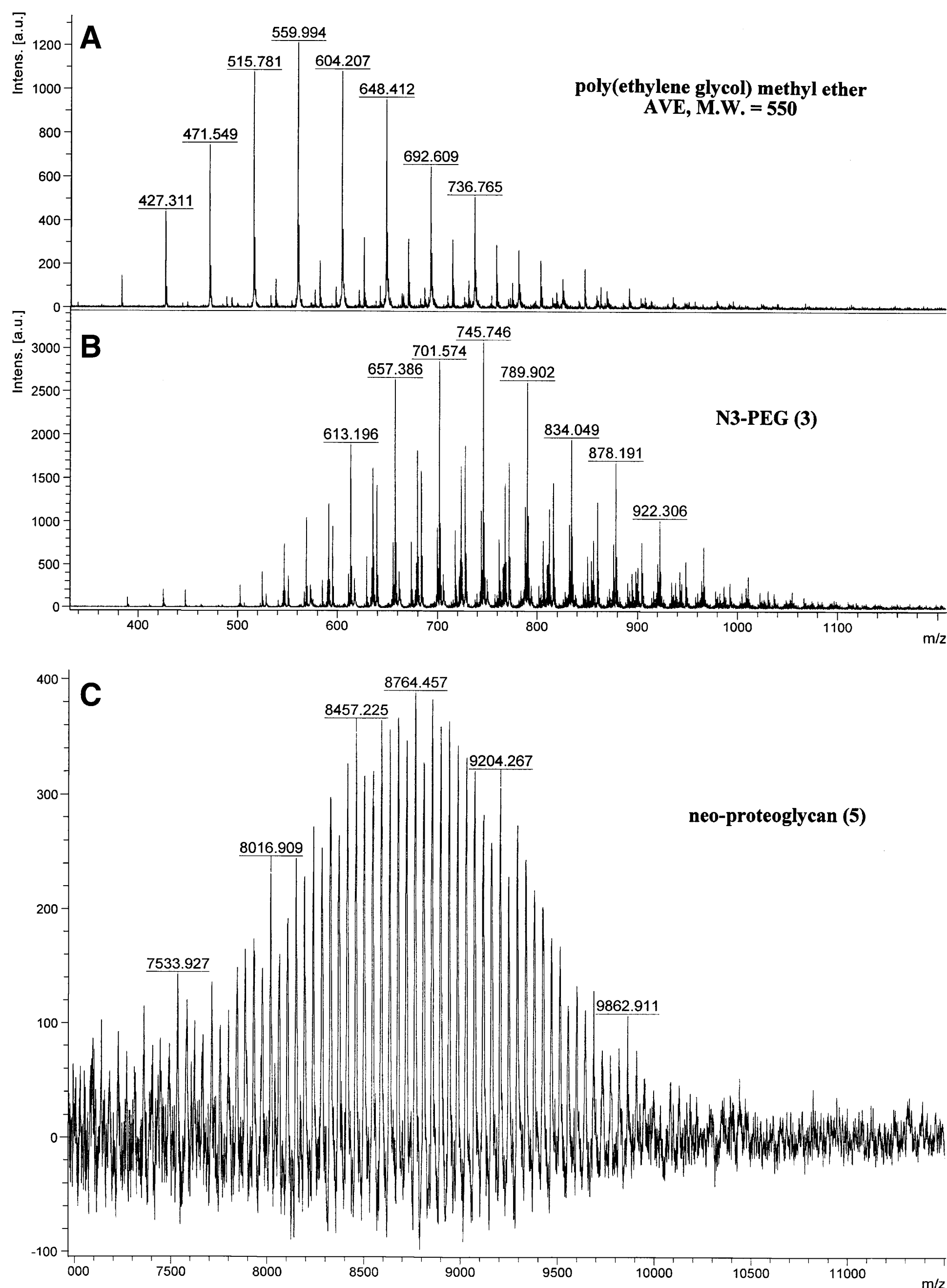

Figure 2. MALDI-TOF mass spectrum of poly(ethyleneglycol) methyl ether (A), compound **3** (B), compound **5** (C).

weight increased to *m*/*z* 8764 and the distribution increased to about *m*/*z* 2000, resulting from the binding of Ch6S to PEG (Fig. 2C).

Synthesis and analysis of neo-proteoglycan (**6**): the reagents were added in the following sequence: **2** (20 μL; 20 mg mL^{-1}), **4** (20 μL; 15 nM), $CuSO_4$ (15 μL; 10 mM) and DIPEA (15 μL; 10 mM). The conditions of click chemistry were similar to those for the neo-proteoglycan **5**, and the synthesized **6** was analyzed by HPLC. The yield of **6** based on **4** was almost quantitative. In our previous method,[3] we could not generate the Ch6S bounded BSA.

First analysis: the reaction product solution was subjected to gel-filtration HPLC (Shodex OH pack SB-803 HQ). The reaction product appeared at an earlier elution time than **4** (data not shown). Second analysis: the reaction mixture was subjected to HPLC (TSK-gel octyl-80TS; RP-HPLC column 250 × 4.6 mm, TOSOH Co., Japan). The peak of **4** (peak I) disappeared completely and a new peak appeared at early time (peak II), resulting from the high polar Ch6S chains bound to the BSA (Fig. 3; lane a and b). Third analysis: the reaction product was digested with chondroitinase ABC (protease-free; Seikagaku Kogyo Co.), which acts on the GlcUA β (1–4) GalNAc 6-sulfate linkage and converts Ch6S to 4,5 unsaturated disaccharide 6-sulfate, except for the reducing end, hexasaccharide i.e., unsaturated-GlcUA-GalNAc-GlcUA-Gal-Gal-Xyl.[12] After enough digestion, the reaction mixture was subjected to

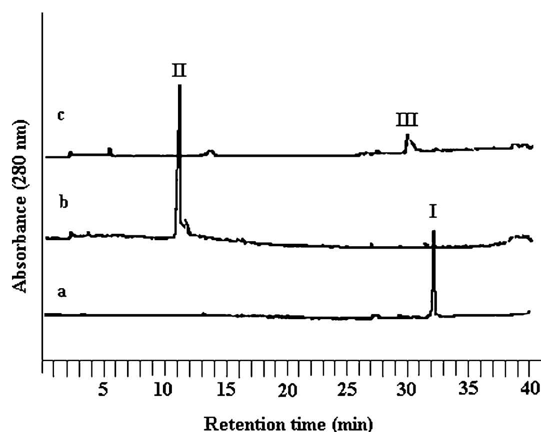

Figure 3. HPLC elution profiles of synthetic compounds. lane a; compound **4**, lane b; compound **6**, lane c; Chondroitinase ABC digested compound **6**. Elution condition; flow rate: 1 mL/min, elution buffer: (A) H_2O containing 0.14% TFA, (B) MeCN containing 0.14% TFA. The linear gradient being with 100:0 A:B to 60:40 A:B after 40 min.

HPLC. Peak II disappeared completely, and a new peak (peak III) appeared about 2 min earlier than that of **4** (Fig. 3; lane c). This data indicated that the digested product had slightly higher polarity than that of **4**, resulting from the residual hexasaccharide on the BSA that could not be digested by the enzyme. These results of HPLC indicated that Ch6S **2** bound to **4** through the click chemical reaction.

In conclusion, we have performed the preparation of an alkyne containing Ch6S **2** using an enzymatic method and synthesized an azido group containing PEG **3** and BSA **4**. These obtained compounds were utilized for click chemistry, and we succeeded in constructing neo-proteoglycans **5** and **6**. It is impossible to construct these neo-proteoglycans by our previous method.[3] So, we think this method is widely applicable for attachment of intact long ChS chain to various compounds, such as artificial materials, proteins and therapeutic reagents amenable to introduction of an azido group. Biological assays employing these neo-proteoglycans are now under investigation.

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7. Alkyne containing Ch6S (**2**). Peptide Ch6S **1**, (70 μL; 2 wt. % H_2O solution) as a donor and propargyl alcohol (65 μL) as an acceptor were incubated at 37 °C for 24 h with *endo*-β-xylosidase in sodium acetate buffer (0.1 M, PH 5.0). After lypophilization to remove the excess acceptor, the residue was dissolved in H_2O (100 μL), the solution was passed trough a C18-Sep-Pac cartridge and eluted with H_2O (2 mL) to remove the hydrophobic components resulting from the enzyme reaction. The resulting elute subsequently applied to Sephadex G-25 column gave the alkyne containing Ch6S **2**. FT-IR (KBr) 3294, 2933, 2093, 1578, 1412, 1356, 1151, 1077, 1026, 931, 704 cm^{-1}.
8. N_3-PEG (**3**). ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.05$ (d, 2H, *J* 8.6 Hz, Ph), 7.07 (d, 1H, *J* 8.6 Hz, Ph), 4.46 (m, 2H), 3.82 (m, 2H), 3.69–3.64 (m, PEG-OCH_2, 42H), 3.54 (m, 2H), 3.37 (s, 3H, PEGO*Me*).
9. N_3-BSA (**4**). To a solution of 4-azido benzoic acid (5 mg, 0.03 mmol) in DMF (200 μL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (6 mg 0.03 mmol). The mixture was incubated for 30 min at room temperature, and then the solution was injected into the BSA solution (10 mg/1 mL) slowly. The reaction mixture was left for 12 h at 4 °C. The mixture was centrifuged 50×100 rpm for 15 min at 4 °C and the clear layer applied to Sephadex G-25 column gave the N_3-BSA **4** (9.4 mg) as a white soild. ESI-MS positive ion pode (m/z) = 6782.5 broad peak.
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11. Neo-proteoglycan (**5**). ^1H NMR (400 MHz, D_2O): $\delta = 8.71$ (s), 8.50 (s), 8.38 (s), 8.24 (d, *J* 6.8 Hz), 8.03 (d, *J* 8.6 Hz, Ph), 7.94 (dd, *J* 6.8, *J* 12.0 Hz), 7.17 (d, *J* 6.8 Hz, Ph), 4.49 (br-d), 4.44 (m), 3.88 (m), 3.86 (m), 3.71–3.69 (m), 3.63 (s, PEG-OCH_2), 3.61–3.53 (m, sugar parts), 3.31 (s, PEGO*Me*), 3.30–3.29 (m), 1.83 (s, AcN).
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