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Chemoenzymatic syntheses of sialyl Lewis X–chitosan conjugate as potential anti-inflammatory agent

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ABSTRACT

This paper describes the facile synthesis and bioevaluations of a novel conjugated polymer having tetrasaccharide SLe^X branch and chitosan backbone (SLe^X–chitosan conjugate). The synthesis was achieved by a convenient chemoenzymatic approach using an aldehyde-functionalized *N*-acetylglucosamine to branch it onto chitosan amino groups followed by three enzymatic reactions to further append galactose, sialic acid, and fucose residue to the branch. Surface plasmon resonance (SPR) study of SLe^X–chitosan conjugate revealed a high affinity binding with E-selectin (K_d = 920 nM) and a potent inhibitory effect on the binding of E-selectin with SLe^X–BSA (IC_{50} = 240 μM). By using biocompatible chitosan as the scaffold for presenting SLe^X ligands, as well as the concise route tailored for the conjugate syntheses, the present study provides a practical method to explore safe and efficient anti-inflammatory agents.

1. Introduction

Selectins (E-, P- and L-selectin), a family of cell adhesion proteins expressed on the surface of leukocytes and activated vascular endothelial cells, mediates leukocyte tethering and rolling along the blood vessel wall. This initial cell–cell binding event eventually leads to emigration of leukocytes from blood to the site of injury (Lasky, 1992; Springer, 1994). Excessive or misdirected recruitment of leukocyte is harmful to the tissue and results in a number of inflammatory diseases such as reperfusion injuries, psoriasis, or rheumatoid arthritis (Mousa & Cheresh, 1997). A promising therapy is to use agents that inhibit the interaction of selectins with their counterpart ligand. The tetrasaccharide sialyl Lewis X (SLe^X, Fig. 1) has been identified from leukocyte and endothelial cell glycoproteins as the minimum element required by the selectins recognition (Phillips et al., 1990; Springer & Lasky, 1991). During the past decade, numerous studies have been conducted to create SLe^X mimics as competitive inhibitors of the selectin-induced leukocyte binding toward the discovery of new anti-inflammatory therapeutics (e.g. Baumann et al., 2009; Davenpeck, Berens, Dixon, Dupre, & Bochner, 2000; Eniola & Hammer, 2005; Kaila et al., 2005). In some cases, potent inhibitory effect can be detected by simple derivatizations of SLe^X (Ernst et al., 2001) or the coupling of monovalent SLe^X with oligopeptides (Filser et al., 2007). However, progress in these small molecule-based strategies is greatly hampered by the essentially weak affinity of SLe^X with selectins (dissociation constant K_d = 0.1–1.0 mM) (Beauharnois et al., 2005; Jacob et al., 1995; Wild, Huang, Horsel, Merwe, & Vestweber, 2001) and the predictable in vivo short circulating half-time. Multivalent presentation of glycan ligands on an appropriate macromolecular scaffold can amplify the affinity of carbohydrate-mediated binding (multivalency effect) (Lee & Lee, 1995), providing a unique opportunity for design and synthesis of SLe^X-incorporated polymers as potent anti-inflammatory agents. In this regard, natural polysaccharide chitosan is a desirable scaffold material for in vivo blockage of selectins due to its biocompatibility, biodegradability, low-antigenicity (Degim et al., 2002; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Shahidi, Arachchi, & Jeon, 1999; Suh & Matthew, 2000), anti-inflammatory activity (Yang et al., 2010; Yoon, Moon, Park, Im, & Kim, 2007), low-cost, and availability of reactive amino groups. An appealing combination of diverse chitosan bioactivities with SLe^X ligand would generate safe and practical anti-inflammatory drug candidates. Several studies have previously demonstrated that incorporation of sialic acid or sialyloligosaccharides including sialyllactose, free sialyl glycan (FSG), and complex disialo-oligosaccharide (CDO) onto chitosan backbone was an effective approach to generate influenza virus hemagglutinin (HA) inhibitors (e.g. Makimura et al., 2006; Sashiwa, Makimura, Shigemasa, & Roy, 2000; Umemura et al., 2008; Umemura et al., 2010). Herein, we describe a facile synthesis of a new sialyloligosaccharide-conjugated chitosan derivative, SLe^X–chitosan conjugate **1** (Scheme 1), as

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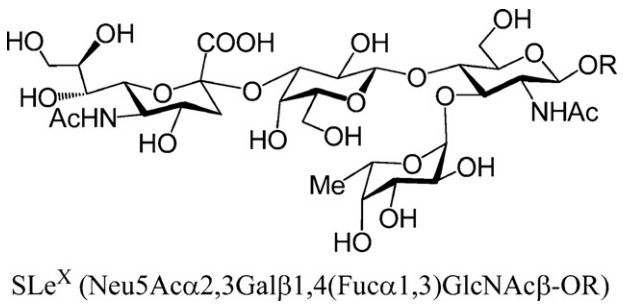
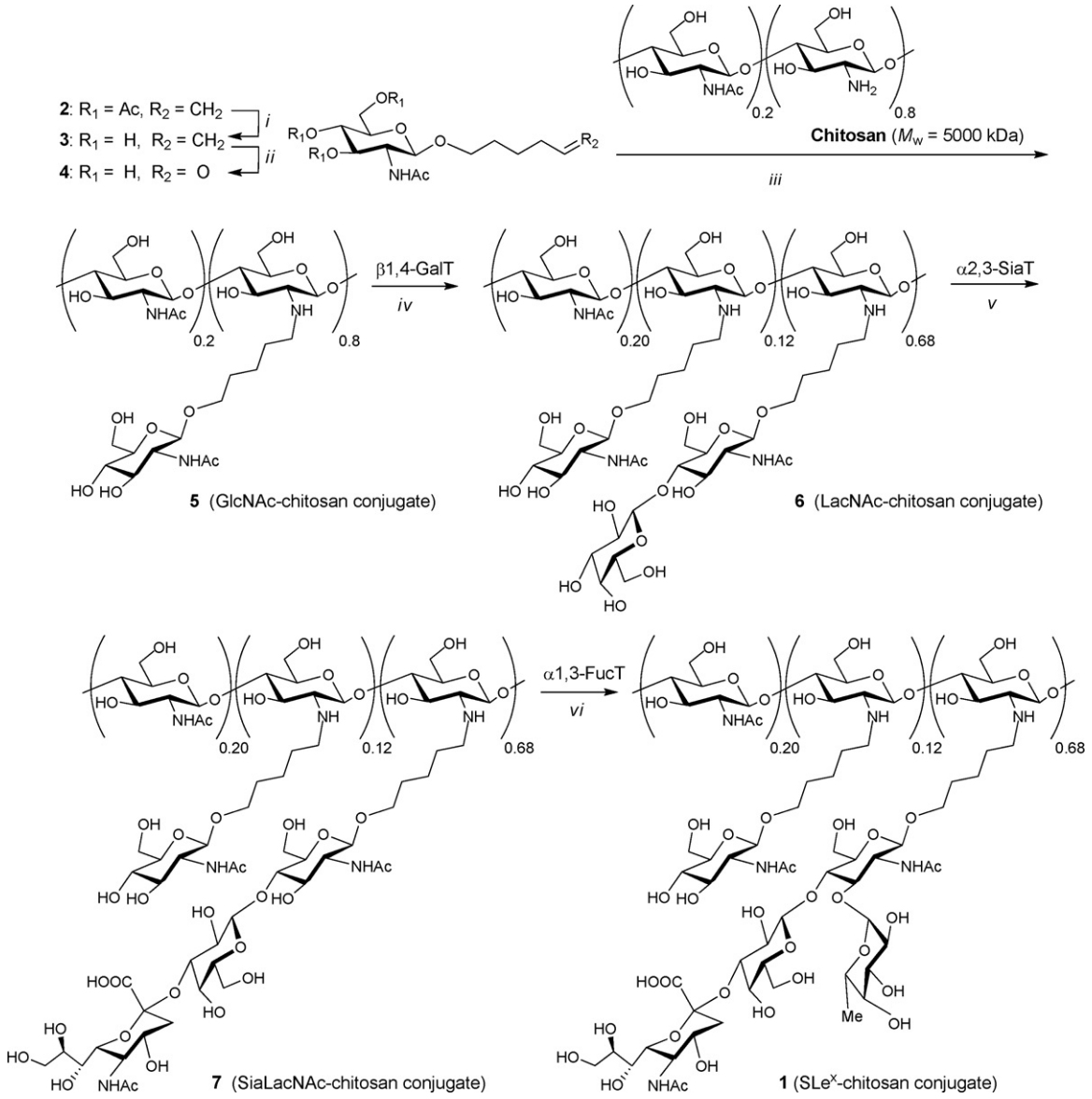


Fig. 1. Chemical structure of SLe^X.

a potential selectin blocker. So far, promising results have been reported using multivalent conjugate inhibitors of SLe^X or its mimics with a variety of polymer scaffold such as carboxymethyl–chitosan and carboxymethyl–pullulan (Sakagami, Kazutoshi, Nakamoto, Kawaguchi, & Hamana, 2000), polyacrylamide (Enders, Bernhard, Zakrzewicz, & Tauber, 2007), polylysine (Thoma, Duthaler, Magnani, & Patton, 2001), polyethyleneglycol (Zeisig, Stahnb, Wenzeld, Behrensc, & Fichtner, 2004), poly(ethylene oxide) (Rele et al., 2005), cyclodextrin (Furuike et al., 2005), and *N*-(2-hydroxypropyl)-methacrylamide copolymer (Shamay, Paulin, Ashkenasy, & David, 2009). However, the complicated chemical synthetic procedure involving multi-step assemblies of SLe^X unit and modifications of polymer backbone might limit their practical applications that must meet the drug safety requirement and also produce an abundant supply. Our synthesis was achieved by using an *N*-acetylglucosamine (GlcNAc)-derived aldehyde **4** for coupling the sugar branch onto chitosan backbone and three glycosyltransferases for further appending galactose (Gal), *N*-acetylneuraminic acid (Neu5Ac), and fucose (Fuc) residue to the GlcNAc branch. Here we demonstrate the high efficiency of this chemoenzymatic approach (Simanek, McGarvey, Jablonowski, & Wong, 1998) in the synthesis of SLe^X–chitosan conjugate **1** with a 75% overall yield (4 steps). Furthermore, surface plasmon resonance (SPR) analyses of the conjugate revealed the high affinity for E-selectin (K_d = 920 nM) as well as the potent inhibitory effect on the binding of E-selectin with SLe^X–BSA



Scheme 1. Chemoenzymatic syntheses of SLe^X–chitosan conjugate. Regents and conditions: (i) NaOMe, MeOH, 89%; (ii) O_3, Me_2S, quantitatively; (iii) $NaCNBH_3$, AcOH (aqueous wt. 5% solution), 88%; (iv) UDP-Gal, HEPES buffer (pH 6.5), 92%; (v) CMP-Neu5Ac, sodium cacodylate buffer (pH 7.3), 98%; (vi) GDP-Fuc, HEPES buffer (pH 7.5), 94%.

(IC_{50} = 240 μM), highlighting its potential as an active E-selectin blocker in explorations of safe and effective anti-inflammatory agents.

2. Materials and methods

2.1. Materials

Chitosan (M_w 5000 kDa) was obtained from Yaizu Suisankagaku Industry Co., Ltd. The deacetylation degree of chitosan was determined to be 80% (80% GlcN, 20% GlcNAc) by the ^{1}H NMR spectrum. Peracetylated alkenyl GlcNAc (compound **2**) was synthesized according to a published method (Fairweather, Stick, & Tilbrook, 1998). β1,4-Galactosyltransferase (from bovine milk, M_w 4.4 kDa) and SLe^X was purchased from Sigma Co. α2,3-Sialyltransferase (rat recombinant, *Spodoptera frugiperda*, M_w 3.9 kDa), α1,3-fucosyltransferase V (human recombinant, *S. frugiperda*, M_w 3.9 kDa), SLe^X–BSA, and E-selectin (human recombinant, CHO cell line) were purchased from Calbiochem Co.

2.2. Measurement

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX 600 spectrometer at 600 MHz and 150 MHz, respectively. Elemental analysis was performed with Flash EA 1112 equipment of ThermoQuest Co., Ltd. Molecular weight (M_w) of chitosan and conjugates were measured by a laser light scattering detector (BI-MwA, Brookhaven Instruments Corp.) in an aqueous solution of 1% acetic acid. FAB-mass spectra were obtained with a JMS-HX 110 spectrometer (Jeol Ltd.). Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Dialysis was conducted using a cellulose ester (CE) membrane tube with the MWCO of 300 kDa (Thermo Fisher Scientific Inc.). SPR analysis was performed by a BIAcore biosensor system (BIAcore 3000, BIAcore Co., Ltd.).

2.3. Syntheses

2.3.1. Pent-5-enyl 2-acetamido-2-deoxy-β-D-gluctopyranoside (**3**)

Peracetylated alkenyl GlcNAc **2** (1 g, 2.33 mmol) was dissolved in a solution of sodium methoxide in methanol (0.05 M, 30 mL). The mixture was stirred for 20 h at room temperature followed by neutralization with Dowex-50 (50 W, X-8, H^+). The resin was filtered off and the filtrate was evaporated. Chromatography (9:1 chloroform/methanol) of the residue gave **3** as white crystals (634 mg, 89%). $[\alpha]_D$ = +20.3° (*c* 0.21, H_2O). ^{1}H NMR (D_2O): δ 5.82 (1H, m, –C*H*=), 4.96 (1H, d, *J* = 17.3 Hz, =CH_2), 4.90 (1H, d, *J* = 10.2 Hz, =CH_2), 4.40 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 3.83–3.79 (2H, m, OCH_2 and H-6_a), 3.63 (H, m, H-6_b), 3.58 (1H, t, *J* = 10.0 Hz, *J* = 8.4 Hz, H-2), 3.50 (1H, m, OCH_2), 3.42 (1H, m, H-3), 3.33 (2H, m, H-4 and H-5), 1.97 (1H, m, $OCH_2CH_2CH_2CH_2CHCH_2$), 1.93 (3H, s, COCH_3), 1.47 (2H, m, $OCH_2CH_2CH_2CH_2CHCH_2$), 1.32 (2H, m, $OCH_2CH_2CH_2CH_2CHCH_2$). ^{13}C NMR (D_2O): δ 174.46 (*C*OCH_3), 139.63 (–CH=), 114.38 (=CH_2), 101.10 (C-1), 75.84 (C-5), 73.82 (C-3), 70.26 (OCH_2), 69.92 (C-4), 60.75 (C-6), 55.60 (C-2), 32.57, 28.05, 24.38 (3×CH_2), 22.21 (CO*C*H_3). FAB-MASS (*m*/*z*) calcd. for $C_{14}H_{25}NO_6$: 303.17. Found 304 $[M+H]^+$. Anal. calcd. for $C_{14}H_{25}NO_6$: C, 55.43; H, 8.31; N, 4.62. Found: C, 55.39; H, 8.30; N, 4.66.

2.3.2. GlcNAc–chitosan conjugate (**5**)

To a stirred solution of compound **3** (285 mg, 0.94 mmol) in methanol (20 mL) at −78 °C was successively introduced ozone until TLC (9:4:2 ethyl acetate/2-propanol/water) indicated that no starting material was left. Nitrogen was introduced for 10 min to remove the remained ozone. After addition of dimethyl sulfide (830 μL, 4.71 mmol), the reaction mixture was stirred at room temperature for 2 h followed by evaporation to give aldehyde **4** as a white solid, which was directly subjected to the next coupling reaction with chitosan. Thus, to a stirred aqueous wt. 5% acetic acid solution (10 mL) of chitosan (100 mg, 0.59 mmol, 0.47 mmol of GlcN) was added dropwise a solution of aldehyde **4** in methanol (6 mL). The mixture was stirred for 1 h at room temperature. Sodium cyanotrihydroborate (118 mg, 1.88 mmol) was added. After stirring the mixture overnight, the acidic solution was neutralized with the 1N aqueous solution of sodium hydroxide. The mixture was subjected to dialysis. After filtration to remove the precipitate, lyophilization of the filtrate gave 208 mg of GlcNAc–chitosan conjugate **5** as a white amorphous solid. The degree of GlcNAc loading was 0.80 determined by the ^{1}H NMR spectrum. The yield was 88% on the bases of the 0.8 degree of GlcNAc loading. The M_w was approximately 12,000 kDa. ^{1}H NMR (D_2O, 27 °C): δ 4.38 (1.00H, d, *J* = 7.8 Hz, GlcNAc branch H-1), 4.05–3.27 (13.25H, m, protons of sugar ring and linker OCH_2), 3.16–2.79 (4.25H, brm, NHC*H* and NHCH_2), 2.0 (3.75H, brs, COCH_3), 1.69 (2.00H, brs, linker CH_2), 1.54 (2.00H, brs, linker CH_2), 1.38 (2.00H, brs, linker CH_2). Anal. calcd. for $(C_8H_{13}NO_5)_{0.20}(C_{19}H_{34}N_2O_{10})_{0.80}\cdot(H_2O)_{0.08}$: C, 50.14; H, 7.50; N, 6.26. Found: C, 49.98; H, 7.39; N, 6.26.

2.3.3. LacNAc–chitosan conjugate (**6**)

GlcNAc–chitosan conjugate **5** (45 mg, 110 μmol, 90 μmol of GlcNAc residue), UDP-Gal (82 mg, 134 μmol), β1,4-GalT (1 unit) and α-lactalbumin (700 μg) in 50 mM HEPES buffer (pH 6.5, 2 mL) containing 15 mM manganese chloride and 0.25 mg/mL bovine serum albumin (BSA) were incubated at 37 °C for 2 days. The mixture was subjected to dialysis through a CE membrane tube (300 kDa MWCO) to remove the proteins and small-molecule compounds. After filtration to remove the precipitate, lyophilization gave 52 mg of **6** as a white amorphous solid. The degree of Gal loading was 0.68 determined by the ^{1}H NMR spectrum, and 0.69 estimated from the elemental analysis. The yield was 92% on the base of 0.68 degree of Gal loading. The M_w was approximately 15,000 kDa. ^{1}H NMR (D_2O, 27 °C): δ 4.38 (0.85H, brs, Gal H-1), δ 4.31 (1.00H, d, *J* = 7.8 Hz, GlcNAc H-1 in the branch), 4.06–3.26 (17.68H, m, protons of sugar ring and linker OCH_2), 3.14–2.92 (4.25H, brm, NHC*H* and NHCH_2), 1.95 (3.75H, brs, COCH_3), 1.71 (2.00H, brs, linker CH_2), 1.52 (2.00H, brs, linker CH_2), 1.32 (2.00H, brs, linker CH_2). Anal. calcd. for $(C_8H_{13}NO_5)_{0.20}(C_{19}H_{34}N_2O_{10})_{0.11}(C_{25}H_{44}N_2O_{15})_{0.69}\cdot(H_2O)_{0.61}$: C, 48.01; H, 7.30; N, 4.81. Found: C, 48.14; H, 7.19; N, 4.80.

2.3.4. SiaLacNAc–chitosan conjugate (**7**)

LacNAc–chitosan conjugate **6** (20 mg, 39 μmol, 27 μmol of LacNAc residue), manganese chloride tetrahydrate (1.7 mg), BSA (10 mg), CMP-Neu5Ac (30 mg, 45 μmol), α2,3-SiaT (50 mU), triton (100 μL), calf intestine alkaline phosphatase (20 U), in 50 mM sodium cacodylate buffer (pH 7.3, 5 mL) was incubated at 37 °C for 2 days. Purification as carried out for conjugate **6** afforded **7** as a white amorphous solid (27 mg, 0.68 Neu5Ac loading degree, 21,000 kDa M_w, 98% Yield). ^{1}H NMR (D_2O, 27 °C): δ 4.41 (1.00H, brm, Gal H-1 and GlcNAc H-1 in the branch), 4.02–3.33 (12.31H, m, protons of sugar ring and linker OCH_2), 3.33–2.85 (2.76H, brm, NHC*H* and NHCH_2), 2.64 (0.46H, brd, *J* = 7.8 Hz, H-3_{eq} of Neu5Ac), 1.98 (3.41H, brs, COCH_3), 1.74–1.55 (2.62H, brm, H-3_{ax} of Neu5Ac and 2×CH_2), 1.35 (1.08H, brs, CH_2). Anal. calcd. for $(C_8H_{13}NO_5)_{0.20}(C_{19}H_{34}N_2O_{10})_{0.12}(C_{36}H_{61}N_3O_{23})_{0.68}\cdot(H_2O)_{0.15}$: C, 47.84; H, 6.86; N, 4.88. Found: C, 47.78; H, 6.91; N, 4.86.

2.3.5. SLe^X–chitosan conjugate (**1**)

Conjugate **7** (18 mg, 25 μmol, 17 μmol of SiaLacNAc residue), GDP-Fuc (16 mg, 25 μmol), calf intestine alkaline phosphatase (20 U) and α1,3-FucT V (100 mU) in 50 mM HEPES buffer (pH 7.5,

1 mL) containing 10 mM manganese chloride were incubated at 37 °C for 36 h. Purification by dialysis, filtration, and lyophilization gave 19 mg of **1** as a white amorphous solid (0.68 Fuc loading degree, 24,000 kDa M_w, 94% yield). ^{1}H NMR (D_2O, 27 °C): δ 4.99 (0.46H, brs, Fuc H-1), 4.41 (1.00H, brm, Gal H-1 and GlcNAc H-1 in the branch), 4.40–3.33 (14.15H, m, protons of sugar ring and linker OC$\underline{H}_2$), 3.21–2.58 (3.22H, brm, NHC$\underline{H}$ and NHC$\underline{H}_2$ and H-3$_{eq}$ of Neu5Ac), 1.98 (3.41H, brs, COC$\underline{H}_3$), 1.75–1.49 (2.62H, brm, H-3$_{ax}$ of Neu5Ac and 2×C$\underline{H}_2$), 1.35 (1.08H, brs, C$\underline{H}_2$), 1.14 (1.38H, brd, CH_3 of Fuc). Anal. calcd. for $(C_8H_{13}NO_5)_{0.20}(C_{19}H_{34}N_2O_{10})_{0.12}(C_{42}H_{71}N_3O_{27})_{0.68}\cdot(H_2O)_{0.04}$: C, 48.14; H, 6.85; N, 4.29. Found: C, 48.10; H, 6.66; N, 4.28.

2.4. SPR analyses

2.4.1. Binding assay

Binding and binding inhibition assays were carried out by using a BIAcore biosensor system based on the SPR technique. E-selectin was covalently immobilized on a sensor chip (CM-5, research grade) coated with carboxymethyl dextran. Bindings of the conjugates or SLe^X in a running buffer to the immobilized E-selectin were evaluated by the assay system. Briefly, E-selectin (50 μg/mL) in acetate buffer (pH 5.5) was injected over the activated chip surface for 6 min at the flow rate of 20 μL/min. The remaining activated residues (succinimide ester) were blocked with ethanolamine (1 M, pH 8.0). The serial dilutions of conjugate **1** (1–0.03 mg/mL), SLe^X (1–0.03 mg/mL), or a solution (1 mg/mL) of conjugate **7** in running buffer (HBS-N, pH 7.4) was injected over the sensor chip surface at a flow rate of 2 μL/min for 5 min respectively. During the dissociation phase, the sensor surface was exposed to running buffer at the flow rate of 2 μL/min. The kinetic constants were determined by using the standard BIAevaluation software (version 3.1).

2.4.2. Binding inhibition assay

For inhibition assay, SLe^X–BSA was immobilized on a CM-5 sensor chip by a similar procedure described above. Injection of E-selectin (1 μM) in HEPES buffer (10 mM, pH 7.4) containing 150 mM NaCl at the flow rate of 10 μL/min gave approximately 320 relative resonance unit (RU). Preincubated E-selectins (37 °C, 5 min) with various concentrations of conjugate **1**, **7** and SLe^X (0–1 mg/mL) were injected over the sensor chip surface at a same condition. The inhibitions on the E-selectin–SLe^X–BSA binding were recorded as the decrease of resonance unit.

3. Results and discussion

3.1. Syntheses and characterizations

A spacer arm linking the sugar branch and polymer backbone are often used in current glycoconjugation techniques in order to endow the branch with more flexibility from the steric hindrance of polymer backbone. This is particularly important for the sugar branch to perform its bioactivity as well as the chemical reactivity. We designed an aliphatic aglycone linker with a terminal aldehyde functionality to graft GlcNAc residue to chitosan backbone. Aldehyde **4** was synthesized by saponification (NaOMe/MeOH) of a peracetylated alkenyl GlcNAc **2** followed by quantitative ozonolysis of the CC double bound. Reductive *N*-alkylation of chitosan with **4** (1.5 equiv.) was conducted smoothly in an aqueous solution of acetic acid in the presence of $NaCNBH_3$, affording the fully *N*-substituted GlcNAc–chitosan conjugate **5** in 88% yield. Three enzymatic reactions were stepwise carried out for elongations of GlcNAc branch with Gal, Neu5Ac, and Fuc residues, in the presence of corresponding sugar nucleotide as donor substrates. Treatment of **5** with β1,4-GalT and UDP-Gal (1.5 equiv.) afforded 68% Gal-loaded LacNAc–chitosan conjugate **6**. Large excess use of the donor and enzyme has been tested no effect on further increasing Gal loading likely due to the increased steric hindrance of the conjugate. In contrast, sialylation (α2,3-SiaT, CMP-Neu5Ac) and subsequent fucosylation (α1,3-FucT V, GDP-Fuc) completely proceeded, providing the fully Neu5Ac- and Fuc-loaded products, SiaLacNAc–chitosan conjugate **7** and the target SLe^X–chitosan conjugate **1**, respectively.

All products of enzymatic reactions were purified by exhaustive dialysis against water using a cellulose ester membrane tube with MWCO of 300 kDa, which allowed differentiation of the conjugate (M_w > 5000 kDa) from the contamination of proteins and small molecule by-products (the M_w of proteins used in the reactions including the enzymes were lower than 160 kDa). Yields of the enzymatic reaction were generally higher than 90%, indicating that multivalent presentation of sugar substrates on an appropriate scaffold can enhance the affinity for enzymes (Nagahori & Nishimura, 2006) and result in the high efficiencies (high yields). All conjugates showed good aqueous solubility (≥1 mg/mL, PBS, pH 7.2) in contrast to water-insoluble chitosan, due to the highly incorporated hydrophilic sugar branch. Characterizations were carried out by ^{1}H NMR spectroscopy, CHN elemental analysis and laser light scattering. Structural composition of the conjugates was verified by ^{1}H NMR integrations of several baseline-separated signals of each sugar unit in the branch and the Ac group in chitosan main chain, as illustrated in Fig. 2. The loading degrees (80% for GlcNAc, 68% for Gal, Neu5Ac and Fuc) estimated from the NMR spectra were in good agreement with the CHN elemental analyses. Moreover, the successive increase in M_w of chitosan, conjugate **5**–**7** and **1** measured by laser light scattering provided additional evidence of the incorporated sugar unit. However, attempt for the further characterization by ^{13}C NMR spectroscopy has failed because of the rigid chitosan backbone (Rinaudo, 2006) and increased macromolecule size of the conjugates.

3.2. Bioevaluations

Binding property of E-selectin with SLe^X–chitosan conjugate **1** was characterized by SPR analysis. To mimic in vivo interaction of E-selectin on the blood vascular surface with the leukocytes SLe^X ligand in the blood stream, E-selectin was covalently immobilized on the SPR sensor chip using a standard amine-coupling method, followed by injections of conjugate **1** (or conjugate **7** and SLe^X as control) in flowing buffer over the chip surface. Simultaneously, the interactions were monitored. A typical SPR sensorgram (in resonance units, RU) along with the affinity and kinetic data calculated by a standard BIAevaluation software is shown in Fig. 3. The significant binding of conjugate **1** to immobilized E-selectin was detected with much higher affinity compared to monovalent SLe^X, indicating that multivalent presentation of SLe^X ligands on chitosan backbone is an effective way to enhance its binding ability (multivalency effect). Like previous reported selectin–ligand interactions (Jacob et al., 1995; Beauharnois et al., 2005; Wild et al., 2001), association and dissociation of the binding of conjugate **1** with E-selectin is a rapid process occurring within seconds. The K_d of E-selectin binding with SLe^X (530 μM) is at the same level with the published data, but K_d of the binding with conjugate **1** (0.92 μM) is lower by a factor of 576 than that of SLe^X. This greatly improved binding ability is even higher (61-fold) than the naturally occurring E-selectin ligand-1 (ESL-1, K_d = 56 μM) (Wild et al., 2001). In addition, no response was observed for conjugate **7**, proving that the specific binding was derived from multivalent SLe^X branches of the conjugate.

The above confirmation of high affinity binding with E-selectin set a stage for the detection of inhibitory effect of conjugate **1**. A commercially available multivalent E-selectin ligand SLe^X–BSA was used as a model for the inhibition test due to its usefulness

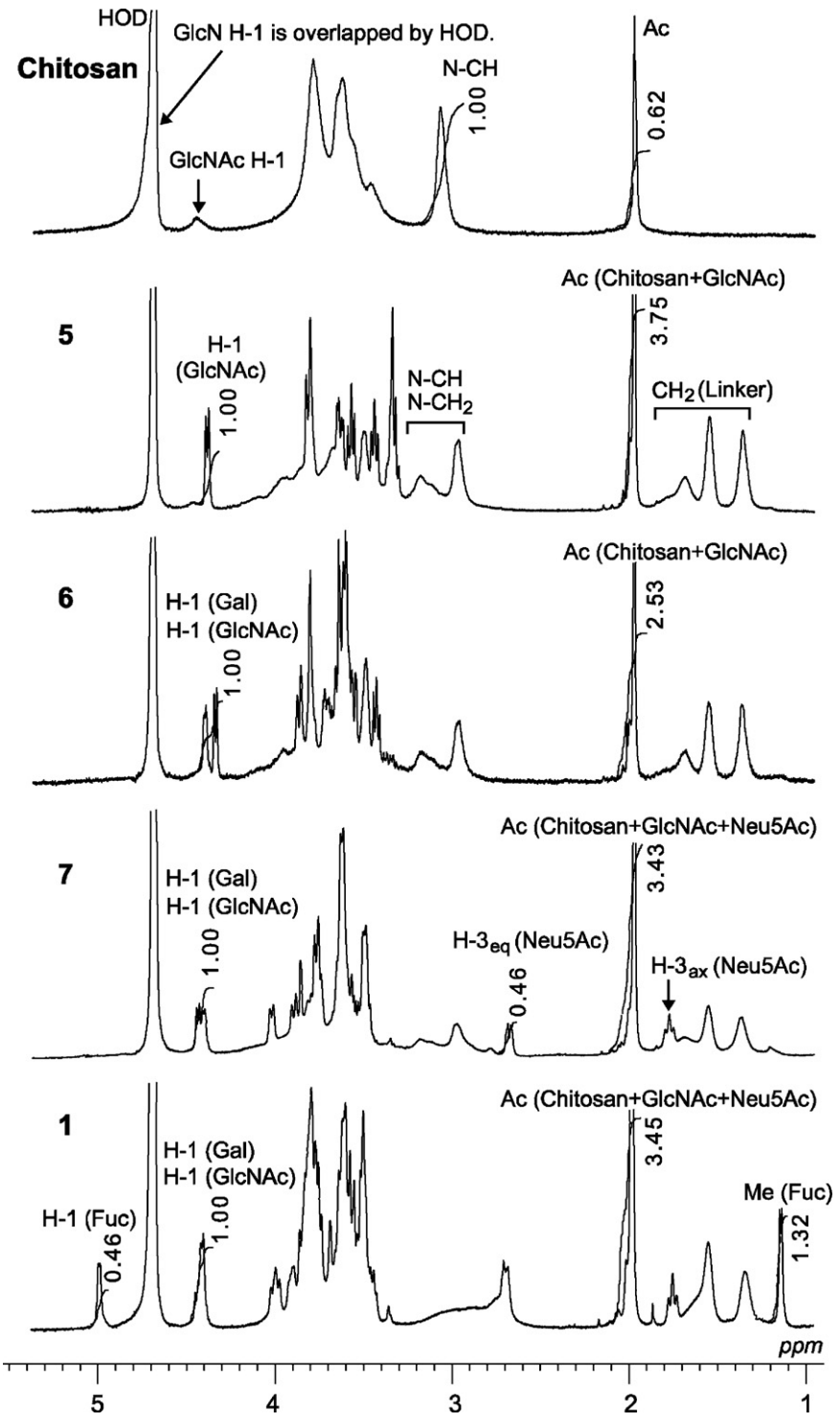
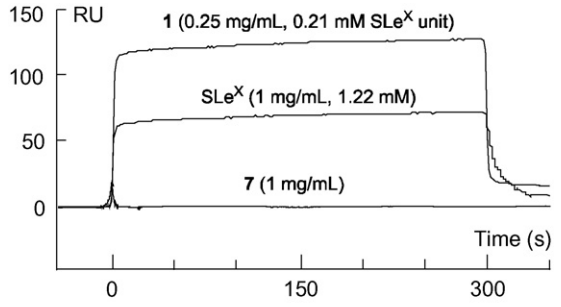


Fig. 2. ^{1}H NMR spectra (600 MHz, 27 °C) of chitosan in DCl/D_2O and conjugates **5**–**7**, and **1** in D_2O.

to inhibit E-selectin-mediated cell adhesion and collect selectin-like peptides and antibody (e.g. Hyun, Kim, Kwon, & Yu, 2007; Mao et al., 1999; Welply et al., 1994). SLe^X–BSA was immobilized on the SPR sensor chip and solutions of E-selection in flowing buffer in the absence or presence of conjugate **1** (or conjugate **7** and SLe^X as the control) were injected over the chip surface. As illustrated in Fig. 4, the binding of E-selectin with immobilized SLe^X–BSA was observed with the maximum signal at approximately 320 RU, and significantly inhibited by conjugate **1** in a dose-dependent manner (IC_{50} = 0.28 mg/mL, 240 μM of SLe^X unit). In contrast, no obvious inhibition was detected for conjugate **7** and monovalent SLe^X at the concentration of 1 mg/mL or less, demonstrating the enhanced inhibitory effect of conjugate **1**. It is notable that comparisons of the binding ability and inhibitory effect of conjugate **1** with the previously reported multivalent SLe^X–conjugates is difficult duo to the different assay systems and models for binding and inhibition studies. However, the above preliminary data highlighted a great potential of conjugate **1** as an active E-selectin blocker in explorations of new anti-inflammatory therapies.



Affinities and kinetics data.

Binding	K_d (μM)	k_{on} ($M^{-1}\cdot s^{-1}$)	k_{off} (s^{-1})
Conjugate **1**-E-selectin	0.92	7.2×10^6	6.6
SLe^X-E-selectin	530	3.8×10^5	203

Fig. 3. Representative sensorgrams for bindings of conjugate **1**, **7** and SLe^X to immobilized E-selectin.

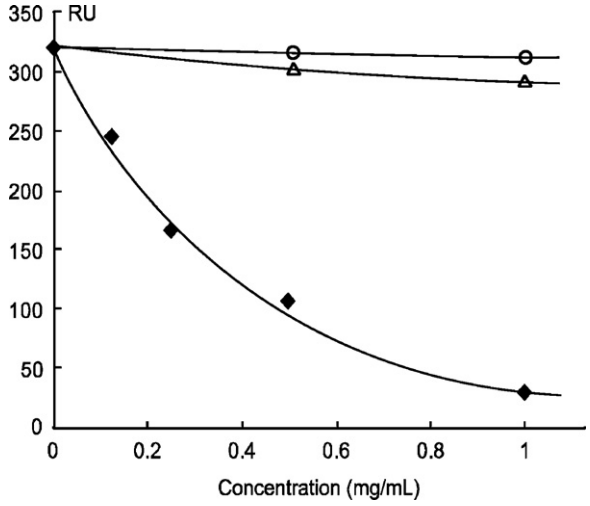


Fig. 4. Inhibition of conjugate **1** (♦), conjugate **7** (○) and SLe^X (Δ) on the binding of E-selectin with SLe^X–BSA. Immobilized ligand: SLe^X–BSA on the SPR sensor chip; injected analyte: E-selectin (1 μM, in HEPES buffer) in the absence or presence of the inhibitors.

4. Conclusions

A novel SLe^X-incorporated chitosan conjugate has been synthesized as a potential E-selectin blocker by a chemoenzymatic approach. The synthetic process including one step of *N*-alkylation and three steps of enzymatic reactions, as well as the simple product purifications procedure, is easily accessible. Bioevaluations of the conjugate revealed the high affinity for E-selectin and potent inhibitory effect on the binding of E-selectin with SLe^X–BSA. The key advantages of the present SLe^X–chitosan conjugate compared to previously reported polymer inhibitors are the concise synthetic process as well as the use of biocompatible and cheaply available chitosan as the scaffold that enables not only the large-scale preparation but also the improved drug safety. Since E-selectin has been recently focused as the target for not only drug but also drug delivery (Ehrhardt, Kneuerb, & Bakowsky, 2004), in addition to the existing broad interest in chitosan-based drug delivery systems (Agnihotri, Mallikarjuna, & Aminabhavi, 2004), we believe that this work is of considerable value in the exploration of new anti-inflammatory therapies. Currently, more extensive bioevaluations and syntheses of further SLe^X–chitosan conjugates with different ligand loading and M_w are in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.07.031.

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