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Conjugation of oligosaccharides to chondroitin oligomer and γ-cyclodextrin

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Abstract

Carbohydrate epitopes on cell surfaces are involved in various biological processes including cell–cell interactions, cell differentiation, cancer metastasis, and pathogen invasion. By blocking clinically relevant sugar receptors, synthetic carbohydrates have potential to become specific pharmaceuticals in various diseases. To achieve the necessary avidity, multivalent glycoconjugates have been constructed thus mimicking multivalent binding often adopted by cells and tissues. Here, we present methodology to create multivalent carbohydrate conjugates based on chondroitin and cyclodextrin carriers. Human milk tetrasaccharide LNnT was attached through a β-glycosylamide linkage to a chondroitin oligomer or an oxidized γ-cyclodextrin. In addition, a facile method was created to attach oligosaccharides to a cyclodextrin carrier using a linker including an oxime-linkage.

Keywords: Chondroitin sulphate A; γ-CD; β-Glycosylamine; Oxime-linkage; Neoglycoconjugate

1. Introduction

Carbohydrates are widely expressed on cell surfaces where they form an important class of biological recognition molecules. The multilateral importance of glycosylated structures ranges from beneficial biological events, such as tissue development, cell division processes, and immune response to detrimental disease processes, such as pathogen homing on their target tissues, cancer metastasis, and inflammation (Davis, 2000; Dwek, 1996; Gabius, 1997; Lis & Sharon, 1998; Varki, 1993). The role of carbohydrates as cell surface receptors enabling adherence of bacteria, viruses, and parasites in the early stages of infection has in recent years gained growing therapeutic interest. Inhibition of pathogen–host recognition/interaction using carbohydrate-based pharmaceuticals is under intensive development and presents a promising approach for the prevention of susceptible microbial infections.

The relatively weak affinity between carbohydrates and their receptors is often overcome by adopting multivalent binding, in which carbohydrate recognition domains in receptors (lectins) are clustered, as was recently illustrated and summarized for mammalian lectins (Gabius, Andre, Kaltner, & Siebert, 2002). This generates challenges for successful development of carbohydrate-based pharmaceuticals. To mimic the natural multivalent presentation, a number of scaffolds have been used. Among the carriers employed for constructing multivalent conjugates, the most common are dendrimers (Röckendorf & Lindhorst, 2001), cyclodextrins (Roy, Hernandez-Mateo, & Santoyo-Gonzalez, 2000), calixarenes (Dondoni et al., 1997), and neoglycoconjugates (Roy, 1996). As an example, it has been shown that single intranasal inoculations with polyacrylamide-based conjugates bearing sialylated *N*-glycans increase the survival of mice experimentally infected with influenza viruses, probably by binding to the virus

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hemagglutinin and thus decreasing the virus infectivity (Gambaryan et al., 2002). In addition, adhesion of *Helicobacter pylori* was inhibited to gastrointestinal epithelial cells by monomeric 3′-sialyllactose at millimolar concentrations whereas multivalent neoglycoproteins bearing 3′-sialyllactose were 1000-fold more potent (Simon, Goode, Mobasseri, & Zopf, 1997).

Details of multivalent protein–carbohydrate interactions are not well understood making an empirical approach to the development of multivalent ligands necessary. There is therefore a growing need for development of new linking chemistry on scaffolds already in use as well as for new types of structural scaffolds. As part of our ongoing project on the development of multivalent carbohydrate analogues we have focused on synthesis of multivalent glycoconjugates based on carbohydrate scaffolds. Carbohydrate scaffolds in general may offer better biocompatibility, as they exhibit excellent solubility in water and low antigenicity. Carbohydrate-based multivalent conjugates previously described include cyclodextrins (Fulton & Stoddart, 2001; Houseman & Mrksich, 2002; Matsuda et al., 1997; Ortiz Mellet, Defaye, & Fernández, 2002), hyaluronic acid (Soltés et al., 1999), chitosan (Sakagami, Horie, Nakamoto, Kawaguchi, & Hamana, 2000), and heparin (Sakagami et al., 2000).

The present study describes our initial goals: Synthesis and characterization of novel glycoconjugates based on chondroitin oligomer and γ-CD scaffolds. Two different methods were created to attach the human milk type oligosaccharide LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), an analogue of a recently published binding epitope of the human *H. pylori* receptor (Miller-Podraza et al., 2005), to these carriers. In the first method two different scaffolds were used: (1) A linear 14-mer scaffold, prepared from chondroitin sulphate A (CSA) by desulphation and hydrolysis, followed by isolation by gel filtration chromatography and (2) a partially oxidized γ-CD. The glucuronic acid carboxyl groups of both scaffolds were then amidated by LNnT converted to its glycosylamine form. Partially desulphated chondroitin oligomer instead of polymeric chondroitin sulphate was used in the present experiments as the reaction products were more easily analyzed by mass spectrometry. It is possible that the presence of sulphate groups in the multivalent drug candidate would be beneficial (higher negative charge and hydrodynamic radius), and this has to be assessed in each case. The glycosylamine amidation method is obviously suitable for other glycosaminoglycans as well (e.g. hyaluronic acid), but their market prices are substantially higher than that of chondroitin sulphate.

The second method was used to attach oligosaccharides to γ-CD scaffold: Aminooxyacetic acid linker was esterified to 6′-position alcohol groups of γ-CD after which LNnT was attached to the conjugate using an oxime linkage. In addition, the stability of the oxime-bond was analyzed under highly acidic conditions thus mimicking the surroundings experienced by orally administered therapeutic agents in the stomach.

2. Materials and methods

2.1. Carbohydrates

LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) was from Kyowa Hakko (Japan). Chondroitin sulphate A (CSA) (bovine trachea) and γ-CD were from Calbiochem.

2.2. Desulphation and acid hydrolysis of chondroitin sulphate

Desulphation of chondroitin sulphate A (CSA) was carried out essentially as described previously (Nagasawa, Inoue, & Tokuyasu, 1979). Pyridinium salt of CSA was prepared by passing the sample in water through a cation exchange column (AG50W-X8, 200–400 mesh, hydrogen form) (Bio-Rad) at room temperature. The eluate was neutralized with pyridine and dried by rotary evaporator. The obtained pyridinium salt of CSA was dissolved in DMSO containing 10% of methanol and incubated for 5 h at 80 °C. Reaction was terminated by cooling and the content was diluted with water to dimethyl sulphoxide (DMSO) concentration <5% (v/v). The solution was then adjusted to pH 9.0–9.5 with NaOH and dialyzed in a regenerated cellulose tubing (MWCO 6000–8000) against running tap-water for 5 h and then against distilled water overnight. The dialyzed desulphated CS was dried by rotary evaporator.

Desulphated CS was partially hydrolyzed in 0.5 M TFA for 20 h at 60 °C. Reaction was terminated by cooling, concentrated to 20 ml and then adjusted to pH 8 with 1 M NH_4HCO_3. Hydrolyzed chondroitin was fractionated with a column of Superdex 30 (5 × 95 cm) eluted with 200 mM NH_4HCO_3 and the eluate was monitored at 214 nm. Fractions were analyzed by mass spectrometry. Quantification was performed by UV-absorbance comparison to external glucuronic acid and *N*-acetylglucosamine standards.

2.3. Oxidation

Selective oxidation of primary alcohol groups of γ-CD with TEMPO (2,2,6,6-tetramethylpiperidine-1-oxy radical) (Aldrich) catalysis was carried out essentially as described previously (Fraschini & Vignon, 2000). Briefly, 300 μmol of γ-CD, 62.4 μmol of TEMPO, and 1920 μmol of NaBr were dissolved in 90 ml of 0.2 M Na-carbonate buffer, pH 10. The solution was cooled on ice and 3.36 mmol of NaClO was added in several portions. The reaction was allowed to proceed for 10 min on ice. Remaining aldehyde groups were reduced by adding 2.4 mmol $NaBH_4$ and incubating for 1 h. The sample was neutralized to pH 7 with 4 M HCl. The oxidized γ-CD species (ox-γ-CD) were isolated by gel filtration chromatography on a column of Superdex 30 (5 × 95 cm) eluted with 200 mM NH_4HCO_3. The eluent was monitored at 214 nm and selected fractions were analyzed by mass spectrometry. Quantification of products was performed by UV-absorbance comparison to external glucuronic acid standard.

2.4. Formation of LNnT-glycosylamine

LNnT was converted to glycosylamine form (LNnT-NH_2) essentially as described previously (Tamura, Wadhwa, & Rice, 1994) by incubating LNnT in 1 μmol aliquots in saturated NH_4HCO_3 and incubating samples at 50 °C for 24 h. LNnT-NH_2 was recovered by repeated lyophilization from 10 μl H_2O until no NH_4HCO_3 was visualized.

2.5. Amidation of LNnT-glycosylamine to chondroitin oligomer and ox-γ-CD

Desulphated chondroitin 14-mer (Ch14) was amidated using LNnT-NH_2 as follows: Chondroitin 14-mer (150 nmol), LNnT-NH_2 (10 μmol), HBTU (10 μmol) (Novabiochem), and DIPEA (*N*-ethyldiisopropylamine) (10 μmol) (Fluka Chemika) were dissolved in dry pyridine (2.35 ml). Reaction was performed at room temperature, in the dark and in constant magnetic stirring for four days. Reaction mixture was then dried in a rotary evaporator, followed by addition of 5 ml of methanol and evaporation repeated three times. Sample was purified in three experiments using Superdex Peptide, and fraction contents were verified using MALDI-TOF MS, then pooled. Similarly, the oxidized γ-CD was amidated using LNnT-NH_2 as follows: ox-γ-CD (200 nmol), LNnT-NH_2 (10 μmol), HBTU (10 μmol), and DIPEA (10 μmol) were dissolved in dry pyridine (3 ml). Reaction was performed, purified and verified as described above for Ch14.

2.6. Esterification

Boc-aminooxyacetic acid (Boc-Aoa) (Novabiochem) was ester-linked to γ-cyclodextrin (γ-CD) by dissolving 20 μmol γ-CD, 1.6 mmol Boc-Aoa, 1.6 mmol HBTU, and 1.6 mmol DIPEA in pyridine (40 ml). Reaction was performed at room temperature, in the dark, and in constant magnetic stirring for two days. Reaction mixture was then dried in a rotary evaporator, followed by addition of 10 ml of methanol and evaporation repeated three times. Sample was dissolved in 10% ethanol and centrifuged 4000 rpm for 10 min at room temperature. Supernatant was transferred into a cellophane tube (MWCO 500), dialyzed against running tap-water for 4 h, then against 20% ethanol/10 mM NH_4Ac, pH 5, for two days with one change of solution, and finally dried by a rotary evaporator.

Boc was removed from aliquots of sample just prior to oxime reaction by dissolving 10 μmol Boc-Aoa-γ-CD in 10 ml of TFA (Aldrich) and incubating for 10 min at room temperature. Solution was dried by a rotary evaporator, followed by addition of 10 ml of methanol and evaporation repeated three times.

2.7. Oxime formation

LNnT was linked to γ-CD in an ester linked oxime-bridge. Ten micromoles of Aoa-γ-CD and 1630 μmol LNnT (Kyowa Hakko, Japan) were dissolved in 12 ml 0.2 M Na-acetate pH 4 and pH was adjusted to pH 4 by adding 700 μl of 0.5 M Na-acetate, pH 5.5. The reaction was allowed to proceed at room temperature, under constant magnetic stirring for 15 h. The reaction mixture was fractionated in three runs using Superdex 30 (5 × 95 cm, Amersham Pharmacia Biotech, Sweden) in 10 mM NH_4Ac, pH 5.0.

2.8. Stability analysis of the oxime-linkage

To analyze the stability of oxime-bond we generated a mixture of LNnT modified using aminooxyacetic acid (LNnT-Aoa) and LNnT: 50 μmol of LNnT and 100 μmol Aoa (Sigma) were dissolved in 1.2 ml of 0.2 M Na-acetate buffer, pH 4.0 and the reaction was allowed to proceed at room temperature for 48 h. This reaction resulted in a mixture containing LNnT-Aoa and LNnT in a molar ratio of 60/40. The sample was desalted using gel filtration chromatography and aliquots of 100 nmol were incubated in 1.0 M and 0.1 M HCl (pH 0 or pH 1, respectively) at room temperature and at +37 °C. Aliquots were removed at selected time points and analyzed by MALDI-TOF MS.

2.9. Chromatographic methods

Gel permeation chromatography was performed with Superdex™ Peptide HR 10/30 (10 × 300 mm) (Amersham Pharmacia Biotech, Sweden) with 200 mM NH_4HCO_3 as eluent, at a flow rate of 1 ml/min or Superdex 30 (5 × 95 cm) (Amersham Pharmacia Biotech, Sweden) with 200 mM NH_4HCO_3 or 10 mM NH_4Ac pH 5.0 as eluent, at a flow rate of 5 ml/min. All experiments were monitored at 214 nm. Fractions of 10 ml were collected in Superdex 30 runs.

2.10. Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were recorded on a Voyager-DE™ STR BioSpectrometry™ (PerSeptive Biosystems) time-of-flight instrument. Samples were analyzed in either positive ion delayed extraction mode using 2,5-dihydroxybenzoic acid (DHB) (Aldrich) matrix (10 mg/ml in H_2O) or in negative ion linear delayed extraction mode using 2,4,6-trihydroxyacetophenone (Fluka) (3 mg/ml in acetonitrile/20 mM aqueous diammonium citrate, 1:1, by volume).

2.11. Nuclear magnetic resonance spectroscopy

Prior to one-dimensional ^1H NMR experiments, the samples were lyophilized twice from D_2O (99.9%) (Aldrich) and then dissolved in 38 μl D_2O. The ^1H NMR spectra were recorded with a Varian Unity 500 spectrometer (Varian Inc., CA, USA) at 23 °C using a gHX nano-NMR probe (Varian Inc., CA, USA). The ^1H chemical shifts are presented by reference to internal acetone ($\delta = 2.225$ ppm).

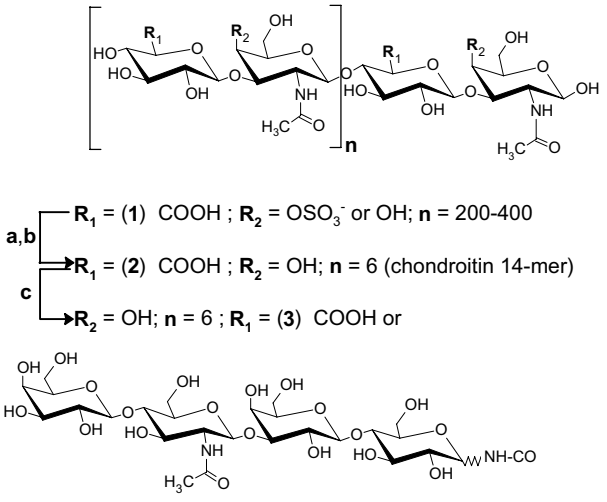
3. Results

3.1. Generation of chondroitin oligomer

To construct a linear multivalent molecule, chondroitin sulphate A oligomer was prepared to act as a carrier. Acid hydrolysate of desulphated chondroitin sulphate A (from bovine trachea) (Scheme 1) (see Section 2 for details) was fractionated by gel filtration. Mass spectrometry was used to verify fraction peak contents and fractions containing 10–16-mers were pooled and re-fractionated as above. Fractions containing chondroitin 14-mer (Ch14, compound **2**) as the major compound were pooled and analyzed using MALDI-TOF MS in the linear negative mode (Fig. 1a). The signals were identified as chondroitin 12-mer (*m/z* 2292.5 $[M-H]^-$, 2372.7 $[M-H+SO_3]^-$), chondroitin 14-mer (*m/z* 2672.1 $[M-H]^-$, 2752.9 $[M-H+SO_3]^-$, 2630.4 $[M-H\text{-}Ac]^-$, 2709.7 $[M-H+SO_3\text{-}Ac]^-$), and chondroitin 16-mer (*m/z* 3052.2 $[M-H]^-$, 3009.3 $[M-H\text{-}Ac]^-$. In addition, minor signals representing chondroitin 13-mer ($GalNAc_7GlcA_6$) *m/z* 2496.0 $[M-H]^-$, and 2576.2 $[M-H+SO_3]^-$ were observed.

3.2. Conjugation of LNnT-NH_2 to chondroitin oligomer

Amine function was introduced to LNnT by converting reducing end of the oligosaccharide to the glycosylamine. Resulting LNnT-NH_2 was then conjugated to Ch14 by amidation to GlcA carboxyl-groups (Scheme 1) in a reaction containing DIPEA as a catalyst and HBTU to create an oxoammonium ion. Reaction mixture was purified and fractionated by gel filtration. Fraction contents were verified using MALDI-TOF MS and multivalent products were pooled. The multivalent product (LNnT-NH-Ch14, compound **3**) was analyzed using MALDI-TOF MS in the linear negative ion mode (Fig. 1b). The indicated signals were identified as Ch14 (*m/z* 2672 $[M-H]^-$), $(LNnT\text{-}NH)_1$-Ch14 (*m/z* 3360 $[M-H]^-$), $(LNnT\text{-}NH)_2$-Ch14 (*m/z* 4048 $[M-H]^-$), $(LNnT\text{-}NH)_3$-Ch14 (*m/z* 4735 $[M-H]^-$), all proposed structures. The heterogeneity in the conjugate signals is due to chondroitin backbones of different sizes.



Scheme 1. (a) desulphation: 90% DMSO-10% MeOH, 80 °C, 5 h; (b) hydrolysis: 0.5 M TFA, 60 °C, 20 h; (c) amidation: LNnT-NH_2, HBTU, DIPEA, pyridine, room temperature, 4 days.

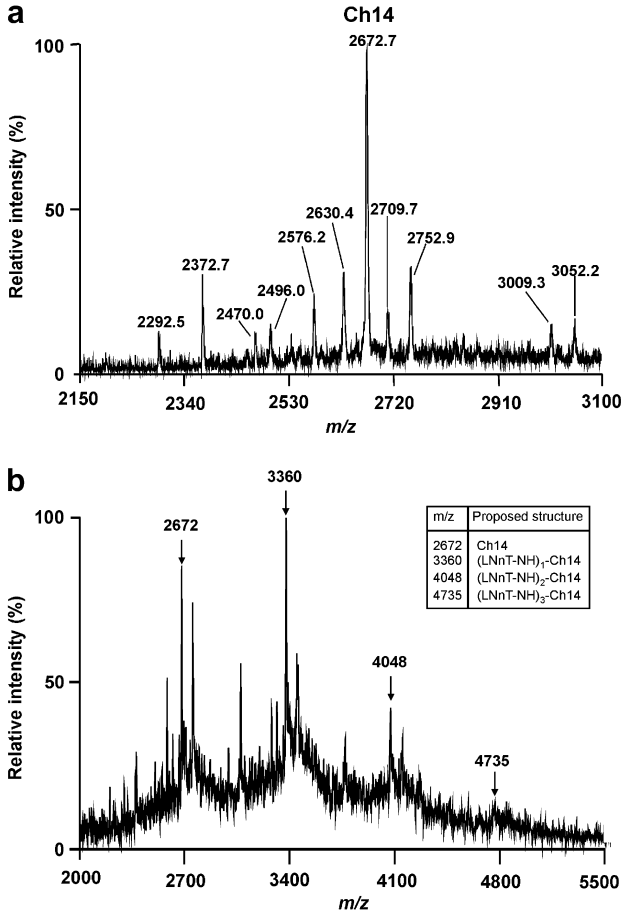


Fig. 1. (a) MALDI TOF mass spectrum of a chondroitin 14-mer (compound **2**) isolated by gel filtration chromatography from CSA hydrolysate. See text for peak assignements. (b) MALDI-TOF mass spectrum of LNnT glycosylamine derivatized chondroitin 14-mer (compound **3**). Representative signals are indicated and the proposed structures are given in the inset.

The ^1H NMR spectrum of LNnT-NH_2 linked to Ch14 backbone (LNnT-NH-Ch14, compound **3**) (Fig. 2a), shows in the anomeric region H1 resonances βH1 of *D*-Gal and βH1 of *C*-GlcNAc, and H4 of 3-substituted *B*-Gal (4.159 ppm), consistent with those reported for the free LNnT molecule. Compared to the free LNnT the βH1 of *B*-Gal had shifted downfield, from 4.436 to 4.49 ppm (overlapping with βH1 of *D*-Gal and βH1 of Ch14 GlcA) due to amidation of the *A*-Glc unit. All βH1 signals that originate from the chondroitin oligomer monosaccharide units resonate between 4.4 and 4.6 ppm. In addition, H4 signals of GalNAc and H2 signals of GlcA from the chondroitin oligomer are also seen. Importantly, the α/βH1 of *A*-Glc signals are missing indicating that no reducing LNnT is present in the sample. The average substitution level was 1.6 LNnT oligosaccharides per Ch14 molecule as calculated comparing the integrated intensities of LNnT *N*-acetyl proton signals and GlcA H2 signals of Ch14.

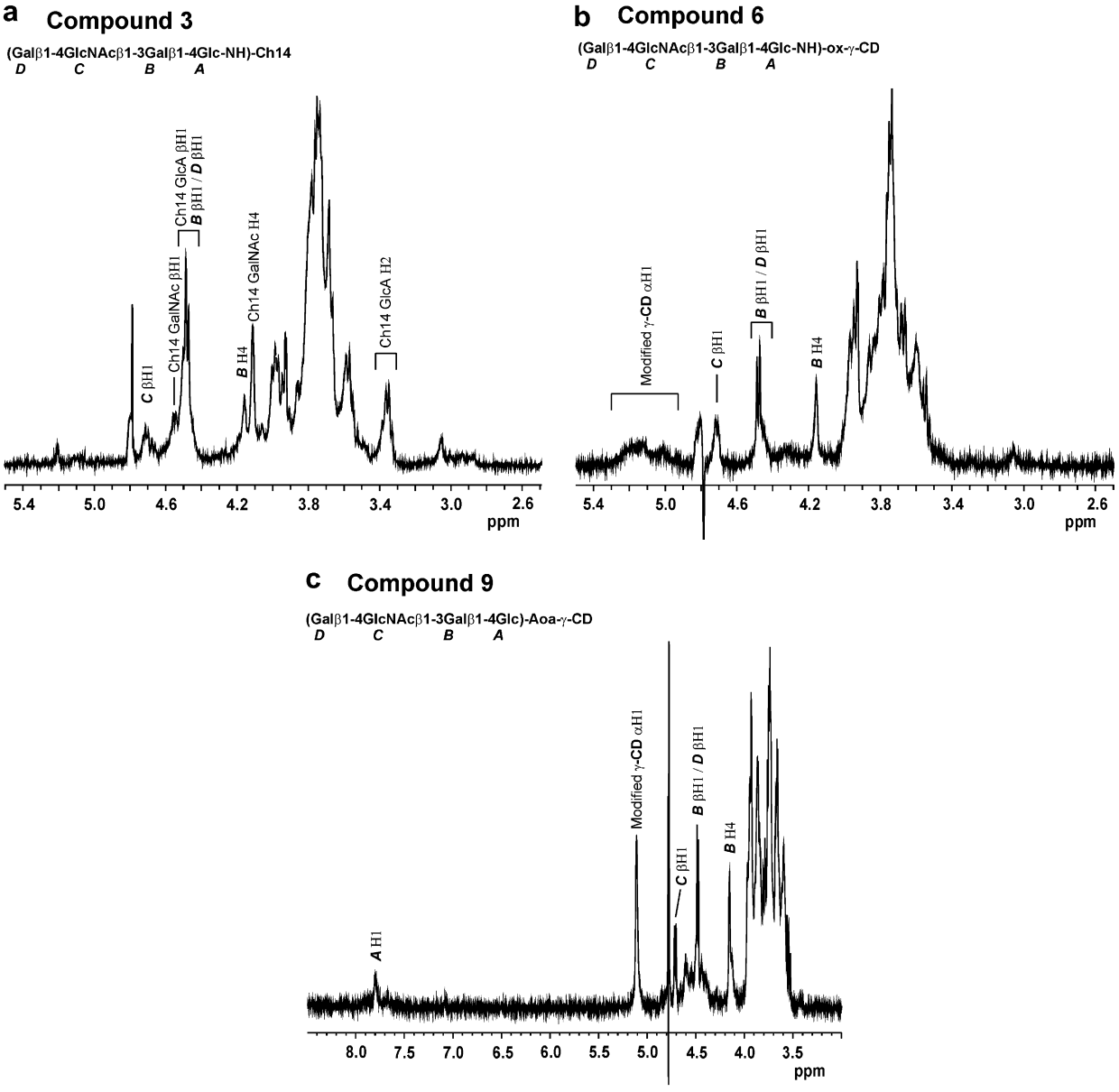


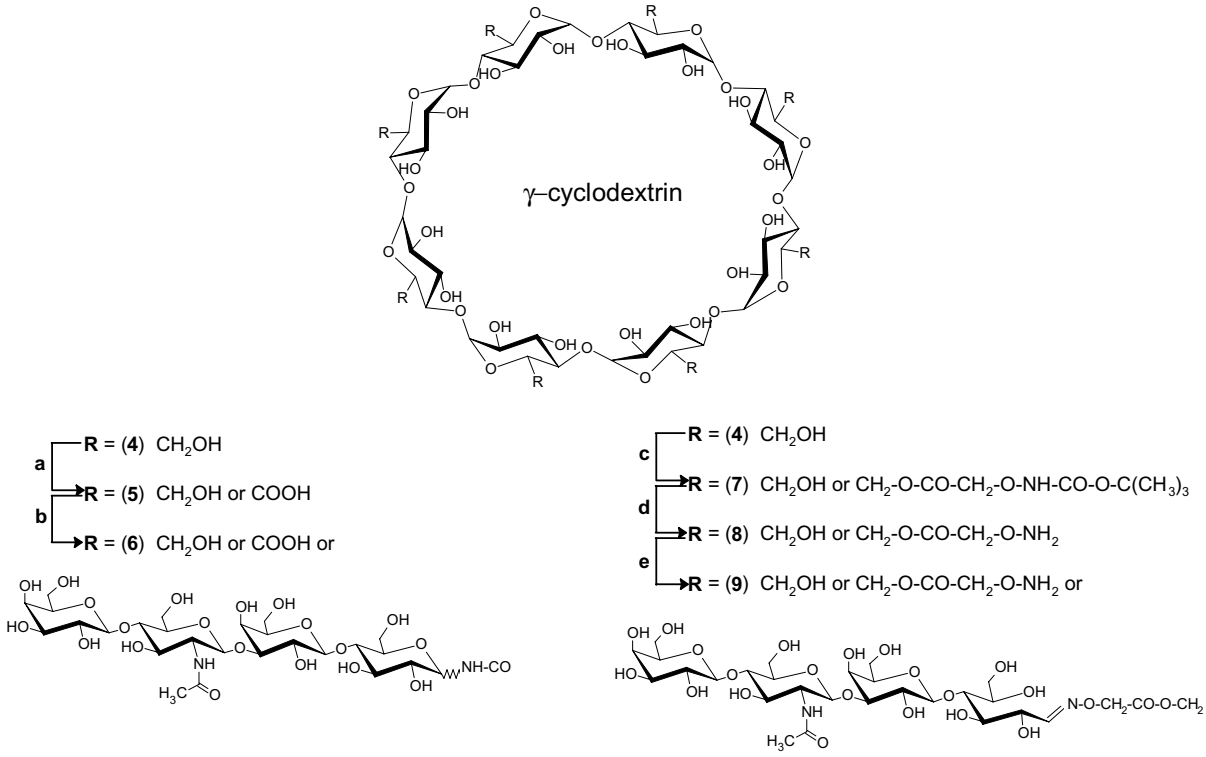
Fig. 2. 1D-^{1}H-NMR spectra of (a) LNnT-NH-Ch14 (compound **3**). (b) LNnT-NH-ox-γ-CD (compound **6**). (c) LNnT-Aoa-γ-CD (compound **9**). See Scheme 1and 2 for more structural details.

3.3. Oxidation

Carboxylic acid groups were introduced to γ-CD by TEMPO catalyzed oxidation (Fraschini & Vignon, 2000) (Scheme 2). The conversion of alcohol groups to carboxylates proceeds via reactive aldehyde-intermediates, which are present at low concentration throughout the oxidation. Consequently, the remaining aldehyde groups were reduced at the end of oxidation reaction using $NaBH_4$. A mixture of mono- to heptacarboxy-γ-CD was obtained and fractionated using gel filtration (data not shown). Fraction contents were verified using MALDI-TOF MS and fractions containing tetra- to heptacarboxy γ-CD were combined. The average oxidation level of γ-CD was five carboxylate groups as determined by MALDI-TOF MS analysis (data not shown).

3.4. Conjugation of LNnT-NH_2 to ox-γ-CD

LNnT-NH_2 was conjugated to oxidized γ-CD (ox-γ-CD, compound **5**) by amidation to 6′-position carboxyl-groups (Scheme 2) in a reaction containing DIPEA and HBTU. Reaction mixture was fractionated by gel filtration. Fraction contents were verified using MALDI-TOF MS and multivalent products were pooled. The multivalent product (LNnT-NH-ox-γ-CD, compound **6**) was analyzed using MALDI-TOF MS in the reflector positive ion mode (Fig. 3a). The indicated signals were tentatively identified as $(LNnT\text{-}NH)_1\text{-}ox_7\text{-}\gamma\text{-}CD$ (*m*/*z* 2107 $[M+Na]^+$), $(LNnT\text{-}NH)_2\text{-}ox_5\text{-}\gamma\text{-}CD$ (*m*/*z* 2766 $[M+Na]^+$), $(LNnT\text{-}NH)_3\text{-}ox_5\text{-}\gamma\text{-}CD$ (*m*/*z* 3455 $[M+Na]^+$), $(LNnT\text{-}NH)_4\text{-}ox_5\text{-}\gamma\text{-}CD$ (*m*/*z* 4146 $[M+Na]^+$). The heterogeneity in the spectrum is due to variable levels of γ-CD oxidation.



Scheme 2. (a) oxidation: TEMPO, NaBr, NaClO, 0.2 M Na-carbonate buffer, pH 10, on ice, (remaining aldehyde groups reduced by $NaBH_4$, on ice, 1 h); (b) amidation: LNnT-NH_2, HBTU, DIPEA, pyridine, room temperature, 4 days; (c) esterification: Boc-Amoc-HAc, HBTU, DIPEA, pyridine, room temperature, 2 days; (d) Boc removal: TFA, room temperature, 10 min; (e) oxime ligation: LNnT, 0.2 M Na-acetate, pH 4, room temperature, 15 h.

The ^{1}H NMR spectrum of LNnT-NH_2 linked to oxidized γ-CD backbone (LNnT-NH-ox-γ-CD, compound **6**) (Fig. 2b), show in the anomeric region H1 resonances βH1 of *D*-Gal and βH1 of *C*-GlcNAc, and H4 of 3-substituted *B*-Gal (4.157 ppm) consistent with those reported for the free LNnT molecule. αH1 resonances of the modified γ-CD are seen around 5.126 ppm. When compared to the spectrum of unmodified γ-CD where αH1 signals (Glcα1-4) resonate at the same frequency (5.09 ppm), the αH-1 signal area of LNnT-NH-ox-γ-CD is very heterogenous due to the complex nature of the molecule. Compared to the free tetrasaccharide the βH1 of *B*-Gal had shifted downfield from 4.436 to 4.48 ppm (overlapping with βH1 of *D*-Gal) due to amidation of the *A*-Glc unit as observed for LNnT-NH-Ch14. The α/βH-1 signals of *A*-Glc are missing indicating that no free reducing LNnT remains in the sample. The average substitution level could not be established from the spectrum because the heterogenous nature of the αH1 signals of the modified γ-CD resulted in unreliable integration of this area.

3.5. Esterification and oxime-ligation

Boc-Aoa was ester-linked to 6′-position hydroxyl groups of γ-CD (Scheme 2) in dry pyridine. Reaction mixture was purified using dialysis. The average substitution level of Boc-Aoa was 3.5 as determined by MALDI-TOF MS analysis (data not shown).

To attach carbohydrate groups protecting Boc groups were removed from Boc-Aoa-γ-CD (compound **7**) using dry TFA after which chemoselective oxime ligation of LNnT to unprotected Aoa-γ-CD (Scheme 2, compound **8**) was performed in weakly acidic aqueous solution. The reaction mixture was fractionated using gel filtration chromatography and the multivalent product (compound **9**) was analyzed using MALDI-TOF MS in the linear positive ion mode (Fig. 3b). The indicated signals were tentatively identified as $LNnT_2$-Aoa_2-γ-CD (*m/z* 2845.4 $[M+Na]^+$), $LNnT_3$-Aoa_3-γ-CD (*m/z* 3607.8 $[M+Na]^+$), $LNnT_4$-Aoa_4-γ-CD (*m/z* 4370.0 $[M+Na]^+$), and $LNnT_5$-Aoa_5-γ-CD (*m/z* 5132.6 $[M+Na]^+$). The heterogeneity in the conjugate signals is due to variable level of aminooxyacetic acid modification in LNnT-Aoa-γ-CD. In addition, molecular species were observed where the amine groups have probably been lost from the aminooxy units revealing hydroxyl groups ($O\text{-}NH_2$ converted to OH = *m/z* −15).

The ^{1}H NMR spectrum of LNnT linked to Aoa-γ-CD backbone (LNnT-Aoa-γ-CD, compound **9**) (Fig. 2c), show in the anomeric region H-1 resonances βH1 of *D*-Gal and βH1 of *C*-GlcNAc, and H4 of 3-substituted *B*-Gal at (4.152 ppm) consistent with those reported for the free LNnT molecule. αH1 resonances of the modified γ-CD are seen at 5.109 ppm. The βH1 signal for *B*-Gal when compared to free LNnT had shifted downfield from 4.436 to 4.48 ppm due to the modification of *A*-Glc unit. Signal representing oxime proton *A*-Glc H1 is seen at 7.796 ppm. The α/βH-1 signals of *A*-Glc are missing indicating that no free reducing LNnT remains in the sample. The average substitution level was 3.1 LNnT oligosaccharides per modified γ-CD molecule as

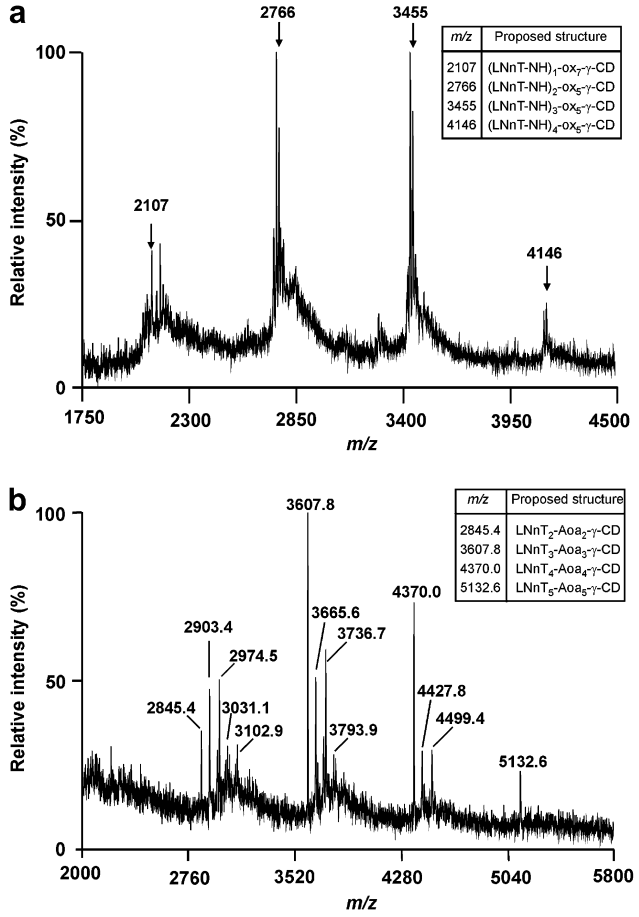


Fig. 3. (a) MALDI-TOF mass spectrum of LNnT glycosylamine derivatized ox-γ-CD (compound **6**). (b) MALDI-TOF mass spectrum of γ-CD derivatized with LNnT through an oxime-linkage (compound **9**). Representative signals are indicated and the proposed structures are given in the inset.

calculated by comparing the integrated intensities of LNnT βH1 of *C*-GlcNAc and αH1 signals of the modified γ-CD.

3.6. Stability analysis of the oxime-linkage under highly acidic conditions

Stability of sugar oxime conjugates under highly acidic conditions was studied. Samples containing approximately 40% LNnT and 60% LNnT-Aoa were incubated in 1.0 M or 0.1 M HCl (at pH 0 or pH 1, respectively) at room temperature or at +37 °C. At selected time points aliquots were removed and analyzed using MALDI-TOF MS. The relative amounts of LNnT and LNnT-Aoa were deduced from spectra (Fig. 4). Typically, orally administered molecules probably experience conditions in the stomach similar to +37 °C, pH ~1, studied here. At these conditions we found the half-life of approximately 3 h for LNnT-Aoa. Even at +37 °C, pH 0, half-life of about 1 h was observed.

4. Discussion

The majority of infectious diseases are initiated by adhesion of pathogenic organisms to cells and tissues of the host

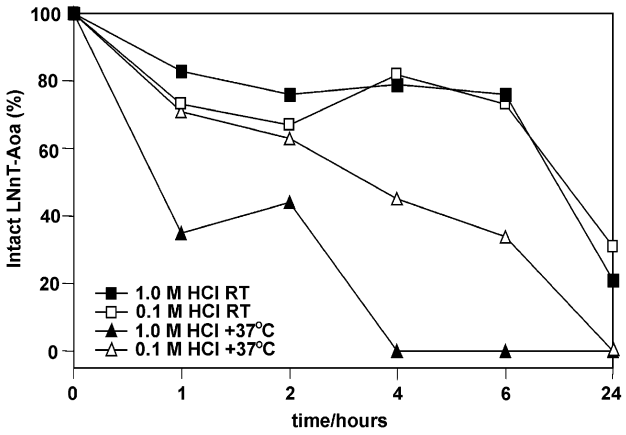


Fig. 4. Analysis of oxime-bond stability under acidic conditions. LNnT-Aoa was incubated under acidic conditions at room temperature and at +37 °C. The relative amounts of LNnT-Aoa and the breakdown product LNnT were analyzed at different time points by MALDI-TOF MS.

(Ofek & Doyle, 1994). This adhesion is often mediated by lectins that bind to their complementary carbohydrate epitopes of glycoproteins or glycolipids on the surface of the host tissue. Therefore, prevention of adhesion or attachment of bacteria, viruses, or fungi to their host tissues, or detaching them from the tissue at the early stages of infection using carbohydrate-based anti-adhesion therapy presents a highly promising approach. In addition, the alarming increase in antibiotic resistant bacterial pathogens makes it even more necessary to intensify the search for new means of combating bacteria.

In general, to design effective multivalent ligands, detailed information about the native structure of the binding proteins is needed. If this information is not available a number of scaffold systems presenting the carbohydrate ligand must be prepared. In addition, manipulation of the linker chemistry used to attach the carbohydrate epitope to its scaffold can have significant impact on the multivalent ligand affinity. We have in our studies focused on carbohydrate-based architectures, where the conformational, constitutional, and configurational diversity of carbohydrates is used to control the presentation of carbohydrate ligands.

The present study presents conjugation of human milk tetrasaccharide LNnT through a β-glycosylamide linkage to scaffold molecules containing 6′-position carboxyl-groups. Two different scaffold molecules were used: A chondroitin 14-mer fraction and γ-cyclodextrin oxidized to express carboxyl groups. The chondroitin 14-mer fraction was isolated from an acid hydrolysate of desulphated chondroitin sulphate by gel filtration chromatography. Carboxyl groups were introduced to γ-cyclodextrin by oxidizing the primary hydroxyl groups by TEMPO oxidation (Fraschini & Vignon, 2000). In addition, we describe here a novel type of oxime-linked sugar-sugar conjugate that has not, to our knowledge, been previously described.

Many pharmaceutical products based on glycosaminoglycans (GAGs) have previously been prepared, the most well known being heparin, which is utilized therapeutically

as an anticoagulant and antithrombotic drug. GAGs are excellent scaffold candidates for creating multivalent glycoconjugates because their carboxyl-groups can be either directly substituted by sugar moieties or functionalized for subsequent attachment of carbohydrate units. However, few studies showing GAG-based oligosaccharide conjugates have been published. These include conversion of hyaluronan to its β-cyclodextrin derivate (Soltés et al., 1999) and sialyl-Lewis x-heparin conjugates (Sakagami et al., 2000). In the present study a chondroitin 14-mer fraction was prepared, and subsequently substituted by the human milk tetrasaccharide LNnT. The tetrasaccharide was first converted to a glycosylamine form by incubation in saturated ammonium bicarbonate (Manger, Rademacher, & Dwek, 1992), and the crude glycosylamine was amidated with the chondroitin oligomer carboxyl groups. Oligosaccharide derivatization through a β-glycosylamide linkage is an established method in glycobiology (Chiu, Thomas, Stubbs, & Rice, 1995; Wong, Manger, Guile, Rademacher, & Dwek, 1993). The present conjugates are to our knowledge the first where oligosaccharide glycosylamines have been conjugated directly to glycosaminoglycan chains by amidation, without including spacers. These conjugates have the advantage that their degradation products are devoid of any additional linker structures.

The amidation of oligosaccharide glycosylamine to the chondroitin oligomer proceeds with the current reaction conditions relatively slowly. The main product carried one oligosaccharide chain; di- and trisubstituted products were also present (about 15% and 5%, respectively, as deduced from the mass spectrum). Oxidized γ-cyclodextrin expressing 6′-carboxyl groups was however amidated more efficiently, yielding as the major products di- and trisubstituted species. With a similar methodology, synthesis of glycosylated calixarene through the formation of amide bonds using calix[4]arene diacid and galactosamine has been attempted. It is noteworthy that in this study steric effects prevented the coupling when using simple aminoglycosides and longer spacers were needed for successful reactions (Schädel, Sansone, Casnati, & Ungaro, 2005). Other carboxyl activators may yield even higher derivatization levels with the present scaffold types. Carbodiimides are reportedly poor activators in uronic acid amidations (Pumphrey, Theus, Li, Parrish, & Sanderson, 2002), but the activity of e.g. DMTMM (Sekiya, Wada, & Tanaka, 2005) and HBPyU (Baisch & Ohrlein, 1998) remains to be established.

The chondroitin oligomer-based conjugates present their oligosaccharide ligands on a linear scaffold, which may mimic e.g. natural mucins and polylactosaminoglycans. These may find preferential use in e.g. selectin inhibitor area: Polyvalent sialyl-Lewis x conjugates based on polylactosamine (Renkonen et al., 1997) or mucin type (Satomaa et al., 2000) scaffolds have been shown to bind selectins with high affinity. The method described here can also be used to create multivalent molecules on other GAG structures. Although GAG materials can be obtained in good quantities from animal sources, biotechnologically produced GAGs would be preferred. Indeed, GAG type polysaccharides are available biotechnologically from *Escherichia coli* K4 (Volpi, 2003) and K5 (Lindahl et al., 2005) capsular polysaccharides.

Synthesis of several neoglycoconjugates based on cyclodextrin scaffold having one or many carbohydrates attached with varying chemical linker length and specificity have been described previously (Fulton & Stoddart, 2001; Houseman & Mrksich, 2002; Ortiz Mellet et al., 2002). The present study introduces two new types of oligosaccharide-CD conjugates: (1) the tetrasaccharide LNnT was linked through a β-glycosylamide bond to oxidized γ-CD carboxyl-groups; and (2) nonmodified reducing LNnT was linked by oxime linkage to γ-CD which carries esterified aminooxyacetic acid units.

Aminooxy nucleophiles reacting with aldehydes or ketones result in formation of oxime bond (Rose, 1994). Synthesis of several glycopeptide analogues containing this non-natural sugar-peptide oxime-linkage has been reported previously (Marcaurelle, Rodriguez, & Bertozzi, 1998; Marcaurelle, Shin, Goon, & Bertozzi, 2001; Peri, Cipolla, La Ferla, Dumy, & Nicotra, 1999; Peri, Dumy, & Mutter, 1998; Renaudet & Dumy, 2001; Rodriguez, Marcaurelle, & Bertozzi, 1998; Singh, Renaudet, Defrancq, & Dumy, 2005). Most of these conjugates were prepared by conjugating aminooxy sugar analogues (sugar-α or β-ONH_2) (Cao, Tropper, & Roy, 1995; Marcaurelle et al., 1998; Renaudet & Dumy, 2001; Rodriguez et al., 1998; Rodriguez, Winans, King, & Bertozzi, 1997) to modified peptides presenting ketone/aldehyde groups. Alternatively, keto function present on *C*-glycosyl carbohydrate analogue was coupled to aminooxy-functionalized peptide backbone (Peri et al., 1999) or reducing carbohydrates were coupled to a peptide substrate containing an N,O-disubstituted hydroxylamine group (Peri et al., 1998). In addition, Boc-aminooxyacetic acid (Boc-Aoa) can be used to introduce hydroxylamine functionality to various carriers (Brask & Jensen, 2000). The present study shows that γ-CD was effectively esterified with Boc-Aoa and, after Boc removal, unprotected reducing LNnT was bound by oxime linkage in good yield to the modified γ-CD. This approach was not employed with the chondroitin oligomer, because we have been interested in attaching ligands to chondroitin through glucuronic acid –COOH groups, which may yield multivalent structures with regular spacing. The esterification of the chondroitin oligomer with Boc-Aoa was presumed to substitute not only GalNAc 6′-OH but other OHs as well (Brask & Jensen, 2000).

Both methods employed here to bind oligosaccharide groups to the γ-CD scaffold were moderately efficient, yielding products carrying on average 2–3 oligosaccharide units with the glycosylamide method and 3–4 units with the oxime linkage method. This substitution level may actually be quite sufficient in many applications. For example, it has been reported that certain cyclic peptides carrying two or three sialylated oligosaccharide ligands bind efficiently influenza hemagglutinin (Ohta et al., 2003). In addition,

other studies have shown that a higher number of ligands in multivalent conjugates does not necessarily lead to increased affinity (Kalovidouris et al., 2003; Thoma, Duthaler, Magnani, & Patton, 2001). These studies show that the mode of ligand presentation is crucial for generation of efficient inhibitor molecules. Therefore, it may be necessary to test several scaffold types as well as various linking methodologies for optimal multivalent product.

It has been reported that peptide-oximes, while stable under mildly acidic and neutral conditions, are unstable at high pH (Rose, 1994; Shao & Tam, 1995). If orally administered, oxime linked molecules experience highly acidic conditions in the stomach (pH ~ 1). At this pH, we found the half-life of approximately 3 h for LNnT-Aoa. Even at +37 °C, pH 0, half-life of about 1 h was observed. The residence time of compounds in the stomach has been reported to be as low as 0.5 h (Sakkinen et al., 2006). Thus, the stability of the oxime bond in general is expected to be sufficient for therapeutic gastric applications. The oxime linked conjugate prepared in the present study however contains an ester linkage and is probably degraded by intestinal esterases.

LNnT used for conjugation in the present study is an established *H. pylori* binding epitope (Miller-Podraza et al., 2005). *Helicobacter pylori* persistently infects the gastric mucosa of a majority of the global human population. It is implicated in several diseases of the gastrointestinal tract including chronic gastritis, gastric and duodenal ulcers, and gastric adenocarcinoma (Israel & Peek, 2001; Peek & Blaser, 2002). We have here described synthesis of multivalent molecules based on linear (chondroitin 14-mer) and cyclic (γ-CD) scaffolds both presenting this established *Helicobacter* receptor. It will be of great interest to assess the *H. pylori* binding activity of these neoglycoconjugates.

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