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Conjugation of oligosaccharides by reductive amination to amine modified chondroitin oligomer and γ-cyclodextrin

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Abstract Carbohydrates present on cell surfaces participate in numerous biological recognition phenomena including cell–cell interactions, cancer metastasis and pathogen invasion. Therefore, synthetic carbohydrates have a potential to act as pharmaceutical substances for treatment of various pathological phenomena by inhibiting specifically the interaction between cell surface carbohydrates and their protein receptors (lectins). However, the inherently low affinity of carbohydrate-protein interactions has often been an obstacle for successful generation of carbohydrate based pharmaceuticals. Multivalent glycoconjugates, i.e. structures carrying several copies of the active carbohydrate sequence in a carrier molecule, have been constructed to overcome this problem. Here we present two novel types of multivalent carbohydrate conjugates based on chondroitin oligomer and cyclodextrin carriers. These carriers were modified to express primary amino groups, and oligosaccharides were then bound to carrier molecules by reductive amination. Multivalent conjugates were produced using the human milk type oligosaccharides LNDFH I (Lewis-b hexasaccharide), LNnT, and GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc.

Keywords Chondroitin sulphate A · γ-CD · Reductive amination · Multivalent oligosaccharides

Abbreviations

Ch14	chondroitin 14-mer
CMP-Neu5Ac	cytidine 5′-monophospho-5-N-acetyl neuraminic acid)
CS	chondroitin sulphate
DAP	1,3-diaminopropane
DAP-Ch14	1,3-diaminopropane amidated chondroitin 14-mer
DAP-ox-γ-CD	oxidized and 1,3-diaminopropane amidated γ-cyclodextrin
DIPEA	N-ethyldiisopropylamine
DMSO	dimethyl sulphoxide
γ-CD	γ-cyclodextrin
pNP-β-GlcA	para-nitrophenyl-β-glucuronide
GnLacNAcLac	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
HBTU	2-(1H-bentsotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphatel
LNDFH I	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc
LNnT	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
MALDI-TOF	matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
MS	
MES	morpholinoethane sulphonate
ox-γ-CD	oxidized γ-cyclodextrin
SA	sialyl
TEMPO	tetramethylpiperidine-1-oxy radical

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Introduction

Carbohydrate-protein interactions play an important role in various biological recognition phenomena, including cell–cell interactions, cancer metastasis, inflammation, immune surveillance, and pathogen invasion [1–5]. Synthetic carbohydrates have the potential to act as specific pharmaceutical substances for treatment of various pathological phenomena, as specific glycans can inhibit the interaction between cell surface glycans and their lectin receptors. Indeed, it has been shown that high doses of 3′-sialyllactose could cure *Helicobacter pylori* infection in Rhesus monkeys [6]. It has also been shown that conjugates bearing sialylated N-glycans increase the survival of mice experimentally infected with influenza viruses, probably by binding to the virus hemagglutinin and thus decreasing the virus infectivity [7].

The association constants between proteins and monomeric carbohydrate ligands are typically quite weak ($K_a = 10^3 - {}^4M^{-1}$), but in biological processes the interaction strength and specificity generally required for recognition is high [8–12]. Therefore, many carbohydrate-protein interactions rely on the amplification of low-affinity interactions by presenting binding epitopes in a multivalent fashion. For example, adhesion of the infecting organisms to host cell surfaces through multivalent carbohydrate-protein interaction is a prerequisite for many microbial infections. Multivalent glycoconjugates, i.e. structures carrying several copies of the active carbohydrate sequence in a carrier molecule, have been constructed to overcome this problem. Very high affinities have been reported for glycoconjugates carrying a low number of active ligands [13, 14]. Several types of scaffold molecules have been used as carriers in multivalent glycoconjugates and a wide variety of methods are available for the construction of multivalent structures based e.g. on dendrimers, polymers, proteins, and lipids [15–20]. Carbohydrate scaffolds may offer better biocompatibility, and multivalent conjugates have been constructed e.g. on hyaluronic acid [21], heparin [22], chitosan [22], and cyclodextrins [23–25].

The present study describes our initial goals: Synthesis and characterization of multivalent glycoconjugates based on chondroitin oligomer and γ-CD scaffolds. In the present study we have constructed two novel carbohydrate scaffolds that can be used to attach optimized carbohydrate analogs to form multivalent products. A linear scaffold was prepared from chondroitin sulphate A (CSA): First, a 14-mer chondroitin oligosaccharide (Ch14) preparation with low sulphation level was prepared by CSA desulphation and hydrolysis, followed by isolation with gel permeation chromatography. The glucuronic acid carboxyl groups of Ch14 were then amidated with 1,3-diaminopropane to form a chondroitin derivative carrying amino groups. A cyclic scaffold was prepared from γ-cyclodextrin (γ-CD): γ-CD was partially oxidized by TEMPO mediated process to introduce carboxyl groups in γ-CD, and these carboxyl units were further amidated with 1,3-diaminopropane. These scaffolds were then derivatized with human milk type oligosaccharides LNDFH I (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc Lewis-b hexasaccharide), LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), or GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc using reductive amination. The LNnT conjugate based on γ-CD was also shown to act as an acceptor for an α2,6-sialyltransferase.

Materials and methods

Carbohydrates

Lewis b hexasaccharide LNDFH I (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc) was purchased from IsoSep (Lund, Sweden). LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) and GnLacNAcLac (GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc) were from Kyowa Hakko (Japan). Chondroitin sulphate A (CSA) (bovine trachea), γ-CD, and *para*-nitrophenyl-β-glucuronide (pNP-β-GlcA) were from Calbiochem.

Desulphation and acid hydrolysis of chondroitin sulphate

Desulphation [26] and hydrolysis of chondroitin sulphate A (CSA) were carried out as follows: Pyridinium salt of CSA was desulphated in dimethyl sulphoxide (DMSO) containing 10% of methanol, incubated for 5 h at 80°C. Reaction mixture was diluted with water to DMSO concentration <5% (v/v) and pH was adjusted to 9.0–9.5 with NaOH. The mixture was dialyzed (CelluSep MWCO 6000–8000) against running tap-water for 5 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. Hydrolyzed chondroitin was fractionated with a column of Superdex 30 (5×95 cm) eluted with 200 mM NH_4HCO_3 and the eluent was monitored at 214 nm. Fractions were analyzed by mass spectrometry. Quantitation was performed by UV-absorbance comparison to external glucuronic acid and *N*-acetylglucosamine standards.

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 [27]. Briefly, 100 μmol of γ-CD, 20 μmol of TEMPO, and 640 μmol of NaBr were dissolved in 30 ml of 0.2 M Na-carbonate buffer, pH 10. The solution was cooled

on ice and 1.28 mmol of NaClO was added in several portions. The reaction was allowed to proceed for 10 min on ice and then terminated by neutralization 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 eluate was monitored at 214 nm and selected fractions were analyzed by mass spectrometry. Quantitation of products was performed by UV-absorbance comparison to external glucuronic acid standard.

Amidation with 1,3-diaminopropane

The glucuronic acid residues in the chondroitin 14-mer (Ch14) ([GlcAβ1-3GalNAcβ1-4]$_6$GlcAβ1-3GalNAc) were amidated with 1,3-diaminopropane as follows: 10 μmol of chondroitin 14-mer, 7 mmol of 1,3-diaminopropane (Aldrich), 350 μmol of HBTU (Novabiochem) and 350 μmol of DIPEA (*N*-ethyldiisopropylamine) (Fluka Chemika) were dissolved in 40 ml of pyridine containing 10% H_2O. This mixture was stirred in the dark at RT for 3 days, and then evaporated to dryness with rotary evaporator. The reaction mixture was subjected to gel filtration chromatography in a column of Superdex 30 (5× 95 cm) run in 200 mM NH_4HCO_3 and analyzed by MALDI-TOF mass spectrometry. The isolated product, amidated Ch14 (DAP-Ch14), was re-amidated with 1,3-diaminopropane due to moderate amidation level in the first reaction, and purified as described above.

Similarly, the oxidized γ-CD was amidated with 1,3-diaminopropane as follows: 20 μmol of ox-γ-CD, 600 μmol of HBTU, 600 μmol of DIPEA and 12 mmol of 1,3-diaminopropane were dissolved in 50 ml of pyridine containing 10% H_2O. Reaction was allowed to proceed for 3 days at RT in the dark under constant stirring. The reaction mixture was then evaporated to dryness with rotary evaporator. The amidated product (DAP-ox-γ-CD) was isolated by gel filtration and analyzed by MALDI-TOF mass spectrometry.

Due to the low amount of products, the quantitation of DAP-Ch14 as well as ox-γ-CD and DAP-ox-γ-CD products was only done by comparing the gel filtration UV-absorption to glucuronic acid and *N*-acetylglucosamine standards. It was assumed that each glucuronic acid unit that was generated by oxidation in the γ-CD molecule would have the absorptivity of a free glucuronic acid molecule. Similarly, the absorptivity of each amide bond between diaminopropane units and glucuronic acid units was considered equal to amide linkage in *N*-acetylglucosamine monosaccharide. This approach gives very rough estimates of compound yields, as the products are necessarily highly heterogeneous and their absorption properties may vary substantially.

Reductive amination

Three different oligosaccharides: LNDFH I, GnLacNAcLac, and LNnT were attached to 1,3-diaminopropane amidated chondroitin 14-mer (DAP-Ch14) by reductive amination. To 2 μmol of DAP-Ch14 35 μmol of LNDFH I or GnLacNAcLac and 0.5 mmol $NaCNBH_4$ were added, and each sample was dissolved in 500 μl of 0.1 M Na-borate pH 8.5. Similarly, to 1 μmol sample of DAP-Ch14 35 μmol LNnT and 1 mmol $NaCNBH_4$ (Aldrich) were added and sample was dissolved in 1 ml of 0.1 M Na-borate pH 8.5. All reactions were performed at room temperature for 6 days under constant magnetic stirring. Samples were purified using Superdex 30 chromatography. Fraction contents were analyzed using MALDI-TOF MS, multivalent products were pooled and finally products were subjected to MALDI-TOF MS and NMR spectroscopy.

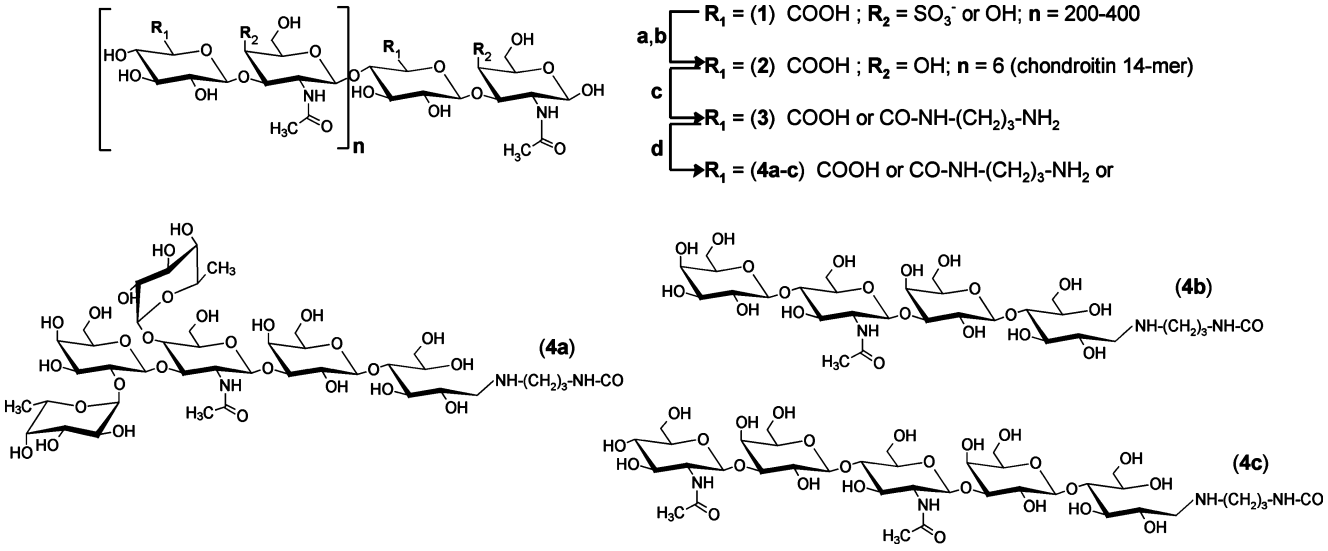
LNnT was attached to amidated ox-γ-CD (DAP-ox-γ-CD) by reductive amination as follows: 2.8 μmol of the DAP-ox-γ-CD, 50 μmol LNnT, and 1.5 mmol $NaCNBH_4$ were dissolved in 2.1 ml 0.1 M Na-borate pH 8.5. Reaction was performed at room temperature for 23 h under constant magnetic stirring and terminated by adding 100 μl 10% acetic acid (to pH 5). The mixture was purified using Superdex Peptide gel filtration and fraction contents were verified using MALDI-TOF MS. Fractions containing multivalent products were N-acetylated with acetic anhydride and purified as above. Fraction contents were verified using MALDI-TOF MS and multivalent products were pooled. Finally, the multivalent product (LNnT-DAP-ox-γ-CD) was analyzed using MALDI-TOF MS and NMR spectroscopy.

α2,6-sialylation

The LNnT-DAP-ox-γ-CD conjugate was sialylated using α2,6-sialyltransferase (rat; recombinant, *S. frugiperda*) (Calbiochem). 10 nmol of LNnT-DAP-ox-γ-CD conjugate containing on average 3 LNnT units per molecule was dissolved in 10 μl of 50 mM MES buffer (morpholinoethane sulphonate), pH 6.0, containing 640 nmol CMP-Neu5Ac (Kyowa Hakko), 5 μg bovine serum albumin (Sigma), 0.1% Triton X-100 and 0.02% NaN_3. 0.45 mU of α2,6-sialyltransferase was added and the reaction was allowed to proceed for 64 h at 37°C. The reaction was terminated by boiling for 3 min and purified using Superdex Peptide chromatography.

Chromatographic methods

Gel filtration 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 with Superdex 30



Scheme 1 **a** desulphation, **b** hydrolysis, **c** 1,3-diaminopropane amidation, **d** reductive amination

(Amersham Pharmacia Biotech, Sweden) (5×95 cm) with 200 mM NH_4HCO_3 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.

Mass spectrometry

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

Nuclear magnetic resonance spectroscopy

Prior to one dimensional ^{1}H NMR experiments, the samples were lyophilized twice from D_2O (99.9%) (Aldrich) and then dissolved in 38 µl D_2O. The ^{1}H 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 ^{1}H chemical shifts are presented by reference to internal acetone (δ=2.225 ppm).

Results

Generation of chondroitin 14-mer

To construct a linear multivalent molecule, chemically modified chondroitin sulphate A oligomer was created to act as a carrier. First, to produce a chondroitin oligomer mixture, chondroitin sulphate A was desulphated and hydrolyzed (Scheme 1a,b) as described in Materials and methods. The hydrolysate was fractionated by gel filtration. Mass spectrometry was used to verify fraction contents, and fractions containing 10-16-mers were pooled and re-fractionated as above. Fractions containing chondroitin 14-mer as the major compound were pooled and this fraction (Ch14, Compound **2**) was again subjected to MALDI-TOF MS analysis (Fig. 1a). The MS analysis revealed that the acid hydrolysis of chondroitin resulted mainly in even-numbered oligosaccharides, i.e. oligomers composed of the repeating disaccharide unit (-4GlcAβ1-3GalNAcβ1-). All major oligomers studied were found by ^{1}H-NMR studies to carry a GalNAc unit at the reducing end (data not shown), indicating that the GalNAcβ glycosidic linkage is more susceptible to acid hydrolysis than the GlcAβ linkage. The MS analysis also showed that the desulphation was not complete but some sulphate units were still observed in the oligomers. Higher level of desulphation was not attempted as sulphation was not expected to interfere with the oligosaccharide conjugation reactions. In addition, minor de-*N*-acetylated species were observed but due to their low amount they were not expected to participate in subsequent reactions.

1,3-diaminopropane amidation of chondroitin 14-mer

Primary amine groups were introduced to chondroitin 14-mer (Ch14, Compound **2**) by amidation of 1,3-diaminopropane (DAP) to glucuronic acid 6′-carboxyl groups (Scheme 1c). Reaction mixture was fractionated using gel filtration and the fractions were analyzed by mass spectrometry (data not shown). Fractions containing DAP-amidated Ch14 (Compound **3**) were combined and analyzed

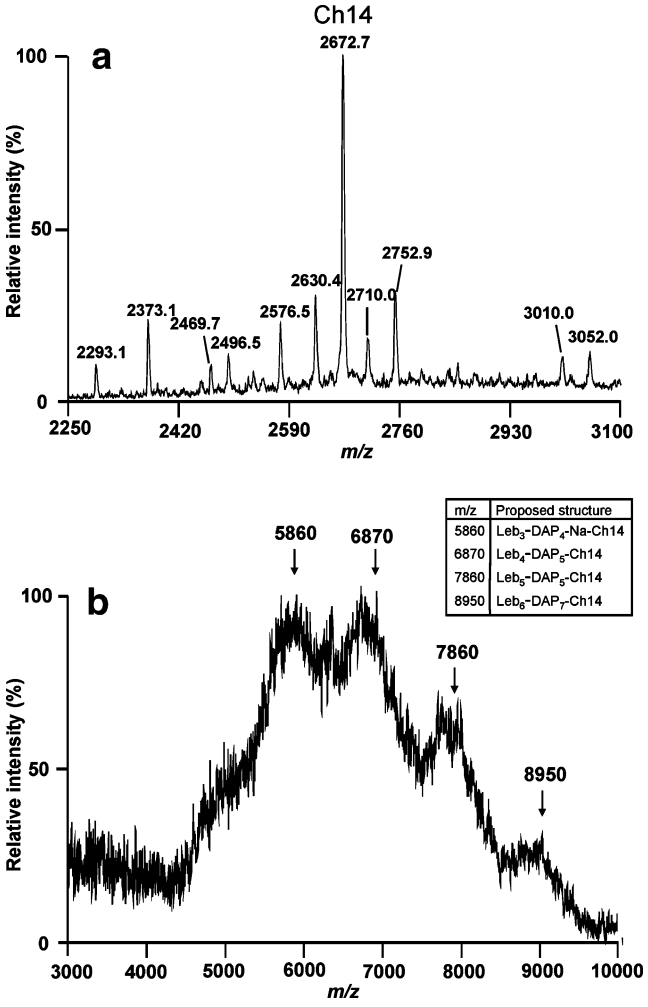


Fig. 1 **a** MALDI-TOF mass spectrum of chondroitin 14-mer fraction prepared by acid hydrolysis. The signals were identified as chondroitin 12-mer (*m/z* 2293.1 $[M-H]^-$, 2373.1 $[M-H+SO_3]^-$), chondroitin 14-mer (*m/z* 2672.7 $[M-H]^-$, 2752.9 $[M-H+SO_3]^-$, 2630.4 $[M-H-Ac]^-$, 2710.0 $[M-H+SO_3-Ac]^-$), and chondroitin 16-mer (*m/z* 3052.0 $[M-H]^-$, 3010.0 $[M-H-Ac]^-$. In addition, minor signals representing chondroitin 13-mer ($GalNAc_7GlcA_6$) *m/z* 2496.5 $[M-H]^-$, and 2576.5 $[M-H+SO_3]^-$ were observed. **b** MALDI-TOF mass spectrum of LNDFH I -DAP-Ch14 conjugate, obtained by reductive amination of LNDFH I (Lewis-b hexasaccharide) and diaminopropane modified chondroitin 14-mer fraction. Representative signals are indicated and the proposed structures are given in the inset (LNDFH I marked as Leb). The heterogeneity in the conjugate signals is due to chondroitin backbones of different sizes as well as variable level of amidation

by ^1H NMR (data not shown). The average DAP substitution level was 4.5 DAP-units per Ch14 molecule. This was calculated by comparing the integrated intensities of GalNAc βH1 and GlcA βH1 signals (4.491–4.557 ppm) to the intensity of innermost DAP methylene group signal ($NH_2CH_2CH_2CH_2NH_2$) (1.9 ppm). The reason for the relatively low DAP substitution level is not completely clear. Two successive reactions were performed, each containing 100-fold molar excess of 1,3-diaminopropane and 5-fold molar excess of HBTU per glucuronic acid unit. It is possible that higher amount of these reagents could have yielded higher amidation level. Alternatively, the use of other carboxylic acid activators, like DMTMM [28] or HBPyU [29] instead of HBTU could be beneficial.

Conjugation of LNDFH I, LNnT, and GnLacNAcLac to DAP-Ch14

LNDFH I, LNnT, or GnLacNAcLac were linked to DAP-Ch14 (Scheme 1d) by reductive amination. Reaction

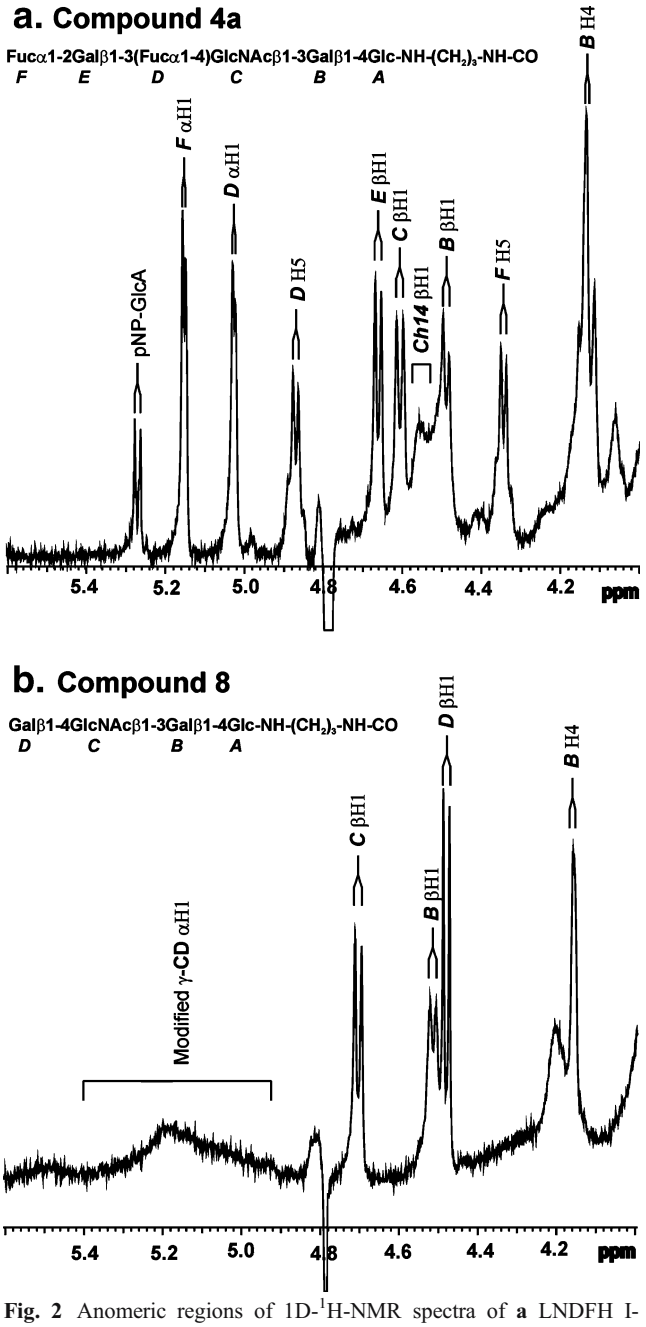
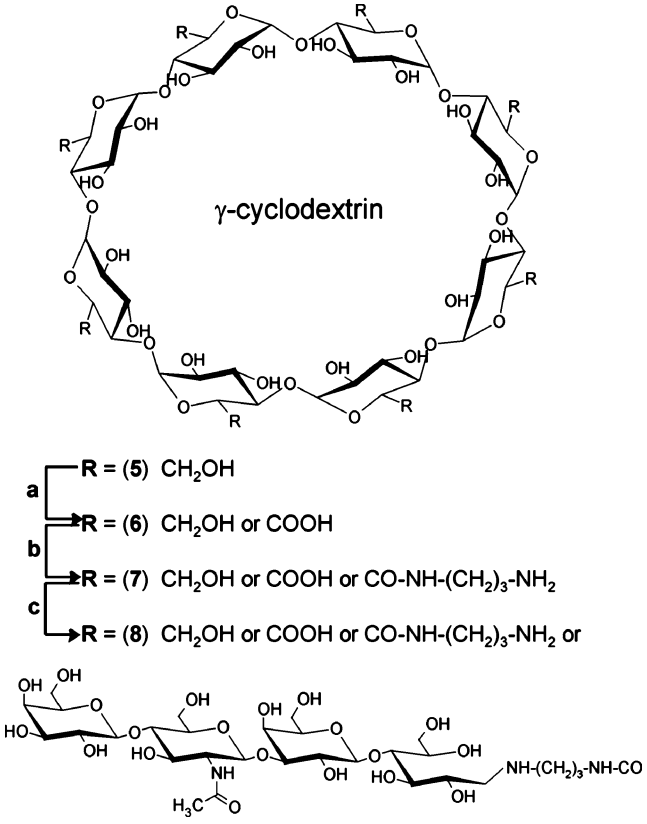


Fig. 2 Anomeric regions of 1D-^1H-NMR spectra of **a** LNDFH I-DAP-Ch14 (Compound 4a) with pNP-β-GlcA as internal quantification standard. **b** LNnT-DAP-ox-γ-CD (Compound 8). See Schemes 1 and 2 for more structural details



Scheme 2 **a** oxidation, **b** 1,3-diaminopropane amidation, **c** reductive amination

mixtures were fractionated using gel filtration, and fraction contents were verified using MALDI-TOF MS. MALDI-TOF mass spectrum of LNDFH I-DAP-Ch14 (Compound **4a**) showed that 2–6 oligosaccharides were attached to DAP-Ch14 backbone (Fig. 1b). Similarly, LNnT-DAP-Ch14 (Compound **4b**) and GnLacNAcLac-DAP-Ch14 (Compound **4c**) contained 2–6 oligosaccharides attached to DAP-Ch14 backbone, as analyzed by MALDI-TOF MS (data not shown).

The ^{1}H NMR spectrum of LNDFH I linked to DAP-Ch14 backbone (LNDFH I-DAP-Ch14, Compound **4a**) (Fig. 2a) (carrying 100 nmol pNP-β-GlcA as internal standard, see below), show in the anomeric region H-1 resonances αH1 of F—Fuc (5.153 ppm), αH1 of D—Fuc (5.027 ppm), βH1 of E—Gal (4.662 ppm), βH1 of C—GlcNAc (4.607 ppm) consistent with those reported for the free LNDFH I molecule. In addition, H4 of 3-substituted B—Gal at 4.134 ppm, H5 of D—Fuc at 4.871 ppm, H5 of F—Fuc at 4.344 ppm, and αCH_3 and βCH_3 of both D—and F—Fuc were consistent with the structure. The βH1 of B—Gal had shifted downfield, from 4.416 ppm to 4.490 ppm due to reductive amination of adjacent A—Glc. All βH1 signals that originate from the chondroitin oligomer monosaccharide units can be seen resonating approximately between 4.4–4.6 ppm. Importantly, the α/βH1 of A—Glc signals are missing indicating that no reducing LNDFH I was present in the sample.

In many cases, we have found it difficult to quantitate novel molecules that are obtained in nmol yields. UV-detection is often used but molecules carrying unknown absorptivity may be quite falsely quantitated. Adding an exact amount of an internal quantitation molecule (not overlapping with critical sample signals) to the NMR analysis yields a set of signals that can be integrated. These areas are easily compared to those of selected sample signals and thus reliable quantitation can be accomplished. Here, pNP-β-GlcA was added as a quantitation standard to a sample of multivalent product. pNP-β-GlcA yields signals at 5.271 ppm, 7.255 ppm, and 8.270 ppm, which do not interfere with the product signals (data not shown). The average substitution level was 4.6 LNDFH I oligosaccharides per DAP-Ch14 molecule, as calculated by comparing the integrated intensities of GalNAc *N*-acetyl proton and LNDFH I C—GlcNAc *N*-acetyl proton signals. This implies that the reductive amination reaction was essentially complete as the average DAP substitution level was 4.5 (see above).

Correspondingly the ^{1}H NMR spectra of LNnT-DAP-Ch14 and GnLacNAcLac-DAP-Ch14 (Compound **4b** and **4c**, respectively) showed that βH1 B—Gal signal had shifted downfield due to reductive amination of adjacent A—Glc and no signals were present for α/βH1 A—Glc (data not shown). This indicated that no reducing oligosaccharides remained in the sample.

Oxidation and 1,3-diaminopropane amidation of γ-cyclodextrin

Carboxylic acid groups were introduced to γ-CD by TEMPO catalyzed oxidation [27] (Scheme 2a). A mixture of mono- to heptacarboxy-γ-CD was obtained and fractionated using gel filtration. Fraction contents were verified using MALDI-TOF MS and fractions containing penta- to heptacarboxy γ-CD were combined yielding a product (ox-γ-CD, Compound **6**) with an average of six carboxylate groups as analyzed using MALDI-TOF MS (data not shown).

Primary amine groups were introduced to oxidized γ-CD (ox-γ-CD, Compound **6**) by amidation of 1,3-diaminopropane (DAP) to 6′-position carboxyl-groups (Scheme 2b) in a reaction containing DIPEA and HBTU. Reaction mixture was fractionated using gel filtration. Fraction contents were analyzed by mass spectrometry and fractions containing 1–5 DAP units were combined. The average DAP substitution level in this fraction (DAP-ox-γ-CD) was 2.5–3 (data not shown).

Conjugation of LNnT to DAP-ox-γ-CD

LNnT was reductively aminated to DAP amidated ox-γ-CD (DAP-ox-γ-CD, Compound **7**, Scheme 2c) in a buffered

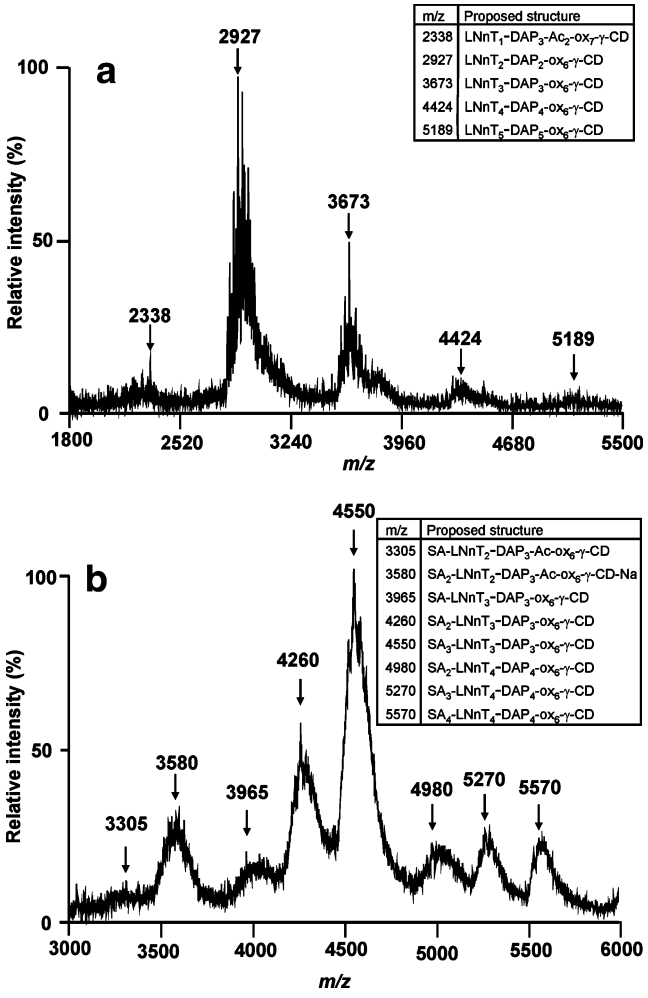


Fig. 3 **a** MALDI-TOF mass spectrum of LNnT-DAP-ox-γ-CD conjugate. **b** MALDI-TOF mass spectrum of sialylated LNnT-DAP-ox-γ-CD conjugate. Representative signals are indicated and the proposed structures are given in the inset. The heterogeneity in the conjugate signals is due to variations in γ-CD scaffold oxidation, amidation and N-acetylation

system. Reaction mixture was fractionated using gel filtration. Fraction contents were verified by MALDI-TOF MS. The isolated multivalent product was *N*-acetylated to eliminate remaining amino groups and the end product was purified again using gel filtration. Fraction contents were analyzed by MALDI-TOF MS and multivalent products were pooled. The multivalent product (LNnT-DAP-ox-γ-CD, Compound **8**) was subjected to MALDI-TOF MS (Fig. 3a).

To further verify and elucidate the structure of the synthesized multivalent product, ^{1}H-NMR analysis was performed. The resonances of the structural reporter groups observed LNnT-DAP-ox-γ-CD (Compound **8**) (Fig. 2b) show in the anomeric region αH1 resonances of the modified γ-CD around 5.166 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-DAP-ox-γ-CD is very heterogenous due to the complex nature of the molecule. The βH1 signals for Galβ1-4GlcNAcβ1-3Galβ1-4Glc (LNnT) C—GlcNAc at 4.703 ppm and D—Gal at 4.479 ppm at the β-anomeric region were found to be consistent with those reported for the free molecule as was H4 of 3-substituted B—Gal at 4.157 ppm. The βH1 signal for B—Gal when compared to free molecule had shifted downfield from 4.435 ppm to 4.513 ppm due to reductive amination of adjacent A—Glc. The α/βH-1 signals of A—Glc are missing indicating that no free reducing LNnT remains in the sample. In addition, the methylene signals of 1,3-diaminopropane, NAc-group signals of both C—GlcNAc and *N*-acetylated DAP were observed at the expected ppm-values (data not shown).

α2,6-sialylation of LNnT-DAP-ox-γ-CD

Many bacteria (eg. *H. pylori*), their toxins (eg. *Cholera* toxin), and viruses (eg. influenza virus) attach to host cell surface carbohydrates containing sialic acid. Therefore, it was interesting to test whether the multivalent molecules could act as an acceptor to sialyltransferase to yield sialylated multivalent conjugates. This was tested by performing α2,6-sialyltransferase reaction with $(LNnT)_{2-4}$-DAP-ox-γ-CD, which contains terminal β1-4 linked galactose residues serving as possible acceptors. $(LNnT)_{2-4}$-DAP-ox-γ-CD was incubated with CMP-Neu5Ac and α2,6-sialyltransferase as described in Materials and methods. The sialylated product (SA-LNnT-DAP-ox-γ-CD) was isolated by gel filtration and analyzed using MALDI-TOF mass spectrometry (Fig. 3b). The major product was found to be the fully sialylated SA_3-$LNnT_3$-DAP-ox-γ-CD.

Discussion

The development of carbohydrate-based anti-adhesives presents a promising approach for the prevention of microbial infections, even more so given the increasing incidence of bacterial resistance to traditional antibiotics. Natural carbohydrate ligands are in many cases presented as clusters [30, 31], which increases the functional affinity (avidity) of monomeric carbohydrate ligands usually expressing very low affinities to their protein receptors. Therefore, artificial carbohydrate pharmaceuticals should be constructed as multivalent carbohydrates or glycoclusters [32, 33].

In the present study, we have conjugated by reductive amination unmodified reducing oligosaccharides (tetra-, penta- and hexasaccharides) to scaffold molecules containing free amino groups. Reductive amination is an established method in neoglycoconjugate synthesis and the reactions can be performed in the absence of protective groups on the sugar units and under aqueous conditions.

Two different scaffold molecules were used in the present study: (1) a chondroitin 14-mer fraction modified to express primary amino groups and (2) γ-cyclodextrin modified to express primary amino groups. The chondroitin 14-mer fraction used in these experiments was isolated from a desulphated chondroitin sulphate acid hydrolysate by gel filtration chromatography, and primary amine groups were added by amidation of 1,3-diaminopropane to carboxyl groups. To prepare the γ-cyclodextrin scaffold, glucuronic acid units were first introduced by oxidizing the primary hydroxyl groups by TEMPO oxidation to carboxyl groups, followed by diaminopropane amidation. The amine modified scaffolds described here are versatile and effective as these can be modified by sugar ligands to create multivalent conjugates of different specificities.

GAGs are excellent scaffold candidates for constructing multivalent glycoconjugates because their carboxyl-groups can be functionalized for subsequent attachment of carbohydrate units. However, only a few studies showing GAG based oligosaccharide conjugates have been published. These include sialyl-Lewis x-heparin conjugates [22] and conversion of hyaluronan to its β-cyclodextrin derivate [21]. Here we prepared a chondroitin 14-mer fraction, which was used as a scaffold to which tetra-, penta-, or hexasaccharides were attached. The chondroitin oligomer based conjugates present their oligosaccharide ligands on a linear scaffold, which may mimic e.g. natural mucins and polylactosaminoglycans. Polyvalent sialyl-Lewis x conjugates based on mucin type or polylactosamine scaffolds have been shown to bind selectins with high affinity [34, 35].

Substituted cyclodextrins may present their ligands in a relatively rigid fashion, and these can be useful binders to bacterial toxins and influenza virus hemagglutinin type proteins [13, 36]. The multivalency effect of CD-carbohydrate conjugates has been previously demonstrated in several studies, e.g. [37–39]. Here, we constructed a novel LNnT conjugate based on γ-CD scaffold. A similar linker build by coupling ox-β-CD and carbohydrate glycosides with primary amino groups has been described previously [38]. The method of the present study, however, has the advantage of using unmodified reducing sugars, and thus it is not necessary to synthesize a glycoside of each oligosaccharide ligand.

A human milk tetrasaccharide γ-CD conjugate (LNnT-DAP-ox-γ-CD) synthesized in the present study was also effectively sialylated by a α2,6-sialyltransferase. The fact that all LNnT units could be sialylated shows that they are well available for biological recognition. Based on the structural data from influenza hemagglutinin, a chemoenzymatic approach has previously been used to construct cyclic peptide scaffolds presenting three sialotrisaccharide units and these conjugates were shown to exhibit scaffold-dependant binding affinities against hemagglutinin [36]. On the same concept, it would be of great interest to study sialyloligosaccharide conjugates based on cyclic carbohydrate based scaffolds (α-, β-, or γ-CD).

All oligosaccharides used for conjugation in the present study are established *H. pylori* binding epitopes [40, 41]. It has previously been shown that high doses of antiadhesive carbohydrates could cure *H. pylori* in Rhesus monkeys [6]. However, monovalent carbohydrate molecules are generally weak binders, and therefore it will be of great interest to assess the *H. pylori* binding activity of the present conjugates.

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