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Chemo-enzymatic synthesis of poly-*N*-acetyllactosamine (poly-LacNAc) structures and their characterization for CGL2-galectin-mediated binding of ECM glycoproteins to biomaterial surfaces

Birgit Sauerzapfe · Karel Křenek · Judith Schmiedel · Warren W. Wakarchuk · Helena Pelantová · Vladimir Křen · Lothar Elling

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Abstract Poly-*N*-acetyllactosamine (poly-LacNAc) structures have been identified as important ligands for galectin-mediated cell adhesion to extra-cellular matrix (ECM) proteins. We here present the biofunctionalization of surfaces with poly-LacNAc structures and subsequent binding of ECM glycoproteins. First, we synthesized β-GlcNAc glycosides carrying a linker for controlled coupling onto chemically functionalized surfaces. Then we produced poly-LacNAc structures with defined lengths using human β1,4-galactosyltransferase-1 and β1,3-*N*-acetylglucosaminyltransferase from *Helicobacter pylori*. These compounds were also used for kinetic characterization of glycosyltransferases and lectin binding assays. A mixture of poly-LacNAc-structures covalently coupled to functionalized microtiter plates were identified for best binding to our model galectin His_6CGL2. We further demonstrate for the first time that these poly-LacNAc surfaces are suitable for further galectin-mediated binding of the ECM glycoproteins laminin and fibronectin. This new technology should facilitate cell adhesion to biofunctionalized surfaces by imitating the natural ECM microenvironment.

Keywords Poly-LacNAc · Chemo-enzymatic synthesis · Galectin binding · ECM glycoproteins · Biomaterials

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B. Sauerzapfe · J. Schmiedel · L. Elling (✉)
Laboratory for Biomaterials, Institute of Biotechnology and Helmholtz-Institute for Biomedical Engineering, RWTH Aachen University, 52074 Aachen, Germany
e-mail: l.elling@biotec.rwth-aachen.de

K. Křenek · H. Pelantová · V. Křen
Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic

W. W. Wakarchuk
Institute for Biological Sciences, National Research Council of Canada, Ottawa, ON K1A 0R6, Canada

Introduction

Glycan chains on cell-surface bound glycoproteins or glycolipids play a major role in biological recognition events like cell–cell interactions and cell–matrix adhesion [1–4]. The most common and important sugar structure of membrane glycoconjugates is poly-*N*-acetyllactosamine (poly-LacNAc) [5, 6]. Poly-LacNAc is a carrier of terminal ligand structures like Lewis blood groups or ABH-blood groups and a binding partner of galectins, a diverse group of galactose-binding lectins [7–11]. Both, type 2 (Galβ1-4GlcNAc) and type 1 LacNAc (Galβ1-3GlcNAc) structures, were found to interact with galectins in the context of complex N-glycans [12]. The binding of galectins to poly-LacNAc structures on cell adhesion molecules and extracellular matrix (ECM) glycoproteins confirm their function as modulators of cell adhesion [13, 14]. It has been hypothesized that galectins are essential for normal differentiation and growth of all multi-cellular animals by controlling complex functions like cell proliferation and cell cycle arrest, as well as cell–cell and cell–matrix interactions [15].

The targeting of cells to surfaces can be achieved by biofunctionalization of surfaces with poly-LacNAc glycan

structures and the formation of an artificial extracellular matrix. We describe here a suitable strategy for the chemo-enzymatic synthesis of defined poly-LacNAc structures and their immobilization onto surfaces with an appropriate orthogonal linker. The chemo-enzymatic synthesis of poly-LacNAc structures has been reported using modified and natural acceptor substrates in combination with recombinant and commercial glycosyltransferases as well as endo-β-galactosidases [8, 16–19]. In order to make functionalized carbohydrate derivatives we employed chemical synthesis for the linker which is still time-consuming with several protection and deprotection steps [20]. For the targeting of surfaces, the most challenging synthetic task is the selection of orthogonal protection group and their selective manipulation during the synthesis [21]. Despite of all these disadvantages many strategies have been successfully developed, and in fact a dodecasaccharide made by solid-phase synthesis has been reported [22–24]. With enzymatic methods higher yields and absolute selectivity are achieved along with recycling protocols for biocatalysts and donor substrates during the synthesis of complex saccharide structures [25–27]. This was impressively demonstrated by Blixt *et al.* with a large-scale enzymatic synthesis of poly-LacNAc based glycan structures using bacterial enzyme systems including LgtA (β1-4galactosyltransferase, β4GalT) and LgtB from *Neisseria meningitidis* (β1-3*N*-acetyl-glucosaminyltransferase, β3GlcNAcT) [17, 28, 29]. Other groups utilized enzymes from bovine milk (β4GalT) and human serum (β3GlcNAcT) [8, 9, 30]. However, detailed kinetic data of bacterial enzymes in comparison to human enzymes, especially with respect to distinct acceptor substrates with multiple repeats of LacNAc units have not been shown so far.

In this manuscript, we describe the chemo-enzymatic synthesis of poly-LacNAc structures by a combination of a fusion protein of human β4GalT-1 [31] and β3GlcNAcT from *Helicobacter pylori* [32]. Defined poly-LacNAc structures with a *t*Boc protected linker-amino-group and with easily reductable azido group were synthesized and used as acceptor structures for the kinetic characterization of the enzymes. *In situ* deprotection of the linker-amino-group at the reducing end of the oligosaccharides facilitates immobilization onto amino-reactive microtiter plates. Along with the established characterization by binding experiments with lectins we introduce an innovative labeling method of the immobilized glycans by an enzymatic method in this study. Finally, comprehensive studies were performed with poly-LacNAc structures and the galectin CGL2 from *Coprinus cinereus* [33, 34] as a model lectin. Our results reveal the dependency of binding with the length of immobilized poly-LacNAc structures and that a time-dependent one-pot synthesis of poly-LacNAc yields a size-distribution of poly-LacNAc structures promoting optimal binding of the galectins.

Finally, we demonstrate that the ECM glycoproteins fibronectin and laminin can be bound onto immobilized poly-LacNAc *via* cross-linking by CGL2. Immobilization of ECM components by this layer-by-layer technology should imitate the cell's natural microenvironment [35, 36] and facilitate dynamic control of cell development onto biomaterial surfaces.

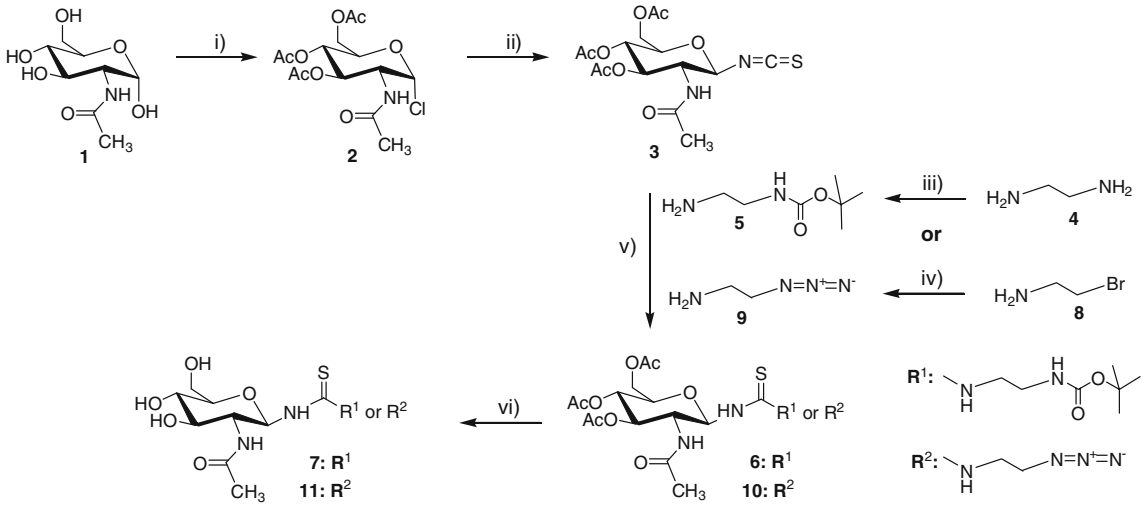
Results and discussion

The strategy to obtain defined poly-LacNAc structures suitable for immobilization onto surfaces comprises four steps. First, the chemical synthesis provides two different GlcNAc derivatives carrying functionalized linkers for orthogonal coupling onto functionalized surfaces (Scheme 1). Secondly, the biocatalytic step involves the selection of the best GlcNAc derivative with respect to the first enzymatic step, the synthesis of LacNAc (Scheme 2), as well as the synthesis of defined poly-LacNAc structures (Scheme 3) with kinetic characterization of each subsequent enzymatic conversion. Thirdly, binding studies with the galectin CGL2 should reveal the best poly-LacNAc structure with respect to the design of glycan structures for optimal galectin binding onto surfaces. Finally, the concept of an artificial ECM by immobilization of ECM glycoproteins onto poly-LacNAc functionalized surfaces using a layer-by-layer technology is presented.

Chemical synthesis of functionalized β-*N*-acetylglucosaminides

The chemical synthesis of *t*Boc-amino-linker and azido-linker modified GlcNAc was done in gram scale (Scheme 1). The synthesis followed the strategy to synthesize isothiocyanate β-glycoside **3** and to couple it with the bi-functional amino-linkers **5** and **9**.

Glycosylthioureido linker proved to be biocompatible and stable in the bio-systems. It was also demonstrated that this linker does not interfere with the protein–sugar recognition processes [37]. The critical step of this procedure was the synthesis of compound **3**. The reaction produced three by-products which had to be removed by column chromatography. Therefore, the yield of this step was only 30%. Further reaction to the final products **7** and **11** could be done with high yields. The structures of the products were determined by NMR and MS and characterized as acceptor substrates of β4GalT enzyme constructs. Both sugar derivatives provide a linker at their reducing end for immobilization onto surfaces. The use of the azido-group for the immobilization to surfaces is well described [17]. However, GlcNAc-linker-NH_2-*t*Boc **7** has some advantages as acceptor for the enzymatic synthesis. The linker and the *t*Boc protection group sugar provide a UV-



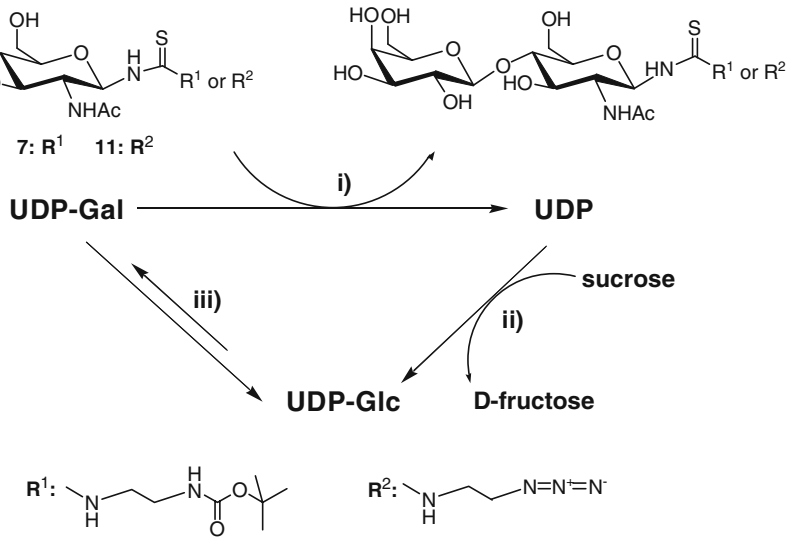
Scheme 1 Synthesis of β-*N*-acetylglucosaminides **7** and **11** with *i*) CH_3COCl, CH_2Cl_2, RT, 36 h; *ii*) KSCN, Bu_4NHSO_4, CH_3CN, RT, 4 h; *iii*) $NH_2CH_2CH_2NH_2$, Boc_2O, $C_4H_8O_2$, 6 h, 4°C; *iv*) $BrC_2H_4NH_2$, NaN_3, H_2O, 18 h, 80°C; *v*) CH_3CN, RT, 16 h; ***vi***) CH_3ONa/CH_3OH, RT, 30 min

signal for quantification at 254 nm and a hydrophobic character for easy chromatographic purification. In addition, deprotection can be easily achieved *in situ* before coupling to surfaces resulting in the final amino-functionalized product with over 90% yield. Therefore, both products appeared to be ideal for further use as acceptor substrates of poly-LacNAc synthesizing enzymes (Schemes 2 and 3).

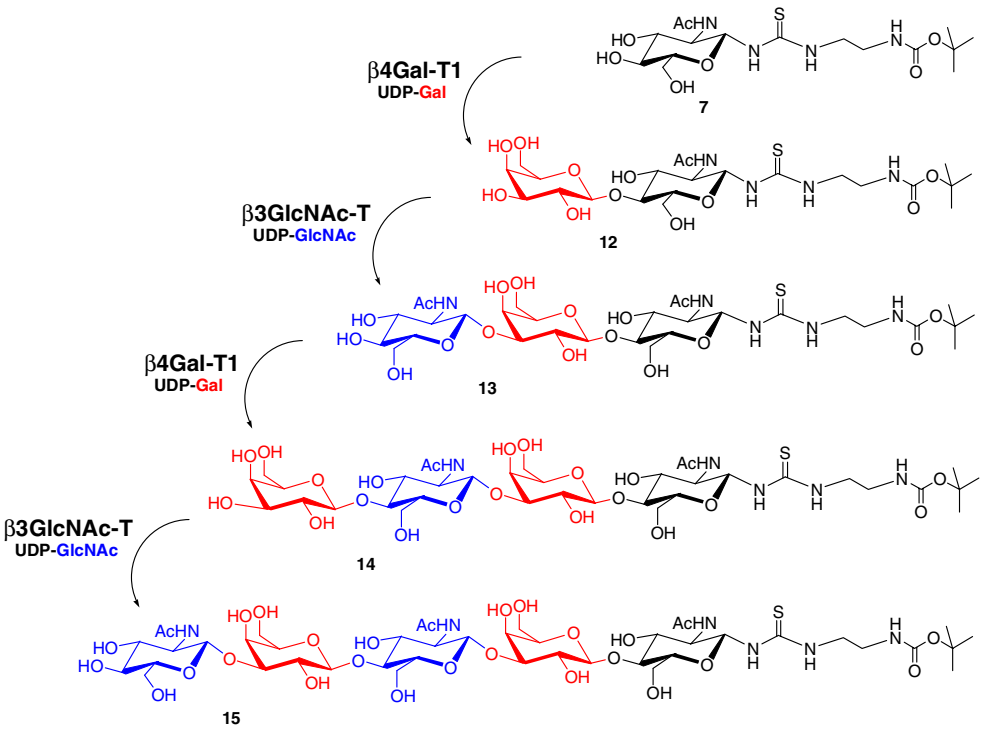
Testing of GlcNAc-linker-NH_2-*t*Boc and GlcNAc-linker-azide as acceptor substrates of different β4GalT enzyme constructs

In our previous studies we investigated three different constructs of the human β4GalT-1 and mutants for their kinetic characteristics towards different GlcNAc acceptors and UDP-Gal(NAc) donor substrates [31]. We could demonstrate that the fusion construct of human β4GalT-1, His_6-propeptide-catβ4GalT-1, has a favorable catalytic efficiency with hydrophobic acceptor substrates. Therefore, the β-*N*-acetylglucosamides **7** and **11** were tested for their conversion with these three human β4GalT-1 constructs to form LacNAc with *in situ* regeneration of UDP-Gal [27] (Scheme 2). The His_6-propeptide-catβ4GalT-1 appeared as the best biocatalyst with quantitative conversion of **7** (5 mM) within 2 h (Fig. 1). In contrast, the conversion of **7** with the other β4GalT-1 constructs, the prepropeptide-natβ4GalT-1 and the lumβ4GalT-1, reached not more than 15%. The conversion of compound **11** was lower with all three enzymes and not sufficient for preparative synthesis.

Compound **7** was also tested as acceptor substrate of bacterial β4GalTs. Compared to mammalian sources, bacterial enzymes have the advantage of an efficient production in



Scheme 2 Testing of compounds **7** and **11** as acceptor substrates with *in situ* regeneration of UDP-Gal. The assay comprised *i*) β4GalT-1 construct, *ii*) sucrose synthase, and *iii*) UDP-glucose 4′-epimerase



Scheme 3 Synthesis of poly-LacNAc structures of defined length with human His_6Propeptide-catβ4GalT-1 and β3GlcNAcT from *H. pylori*. The compounds **12** to **15** were subsequently used for kinetic characterization of the glycosyltransferases, for further enzymatic synthesis and lectin-binding assays, respectively

bacterial systems with usually higher activities. The kinetic studies included HP0826 (β4GalT from *Helicobacter pylori*), MalE-HP0826 (MalE-β4GalT from *Helicobacter pylori*), and LgtB (β4GalT from *Neisseria meningitidis*), as well as the recombinant human enzymes His_6-propeptide-catβ4GalT-1 and HB4GT (human MalE-β4GalT-1) (Table 1). Within the tested concentration range the affinity of HP0826 and LgtB for **7** was too low to determine significant values. However, the fusion protein MalE-HP0826 showed the lowest activity and the highest K_m value for the acceptor substrate **7**. In comparison, both recombinant human enzyme constructs turned out as the best enzymes for LacNAc synthesis. Furthermore, our data reveal that His_6-propeptide-catβ4GalT-1 is the favorable enzyme construct due to the higher specific activity confirming our previous findings [31].

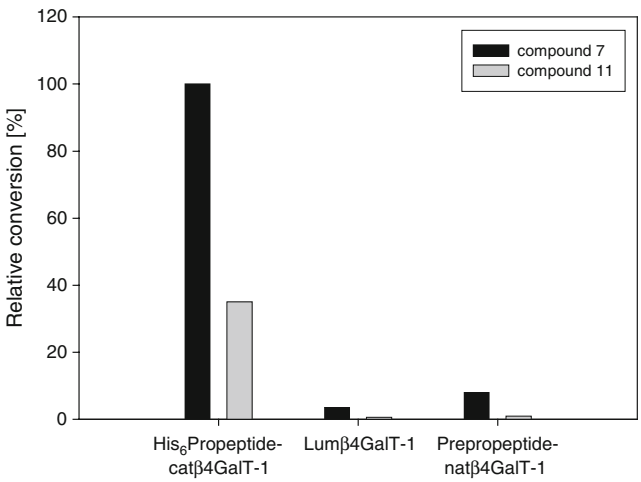


Fig. 1 Conversion of compound **7** and **11** as acceptor substrates of three different constructs of the human β4GalT-1 after 2 h incubation. The assays included *in situ* regeneration of UDP-Gal as depicted in Scheme 2

Preparative synthesis of LacNAc and defined poly-LacNAc structures

His_6-propeptide-catβ4GalT-1 was selected for the preparative synthesis of LacNAc-linker-NH_2-*t*Boc (**12**) starting with compound **7** (Scheme 3). The preparative synthesis of **12** is affected by the substrate inhibition of compound **7**. Therefore, a 5 mM solution of **7** was used, which could be completely converted in an overnight incubation under the applied conditions. The synthesis gave a relative high productivity of 1.5 g product per U enzyme with a space-

Table 1 Kinetic data of bacterial and human β4GalTs for compound **7**

Enzyme	V_{max} [U mg^{-1}]	K_m [mM]	K_{iS} [mM]	V_{max}/K_m
HP21	–[a]	–[a]	–[a]	–[a]
HP27	0.014	8.3	–	1.7×10^{-3}
lgtB	–[a]	–[a]	–[a]	–[a]
HB4GT	0.059	1.12	2.0	0.05
His_6Propeptide-catβ4GalT-1	1.30	1.20	1.40	1.10

HP21: β4GalT from *Helicobacter pylori*; HP27: MalE-β4GalT from *Helicobacter pylori*; lgtB: β4GalT from *Neisseria meningitidis*; HB4GT: human MalE-β4GalT-1; human His_6-propeptide-catβ4GalT-1

[a] Measured data too low to determine significant values

time-yield of 2 g product L^{-1}·day^{-1}. The synthesis of **12** was also achieved in a smaller scale with *in situ* regeneration of UDP-Gal (Scheme 2) [27] without changing the productivity. The space-time-yield was decreased to 1 g L^{-1} day^{-1}.

Starting from compound **12** the synthesis of defined poly-LacNAc structures was performed by subsequent addition of the β3GlcNAcT from *Helicobacter pylori* (JHP1032) and His_6-propeptide-catβ4GalT-1 (Scheme 3). The reactions were monitored by HPLC for the conversion of the donor substrates UDP-Gal and UDP-GlcNAc, respectively, and the complete conversion of the corresponding acceptor substrate. The products **12**, **13**, **14**, and **15** were synthesized in 10 mg-scale (Scheme 3) and analysis by mass spectrometry and NMR confirmed the structures of the (poly)-LacNAc oligosaccharide products **12**–**15** as depicted. They were subsequently used for the kinetic characterization of the bacterial β3GlcNAcT, different βGalTs, for further enzymatic modifications as well as for binding studies of lectins.

Kinetic characterization of β3GlcNAcT from *Helicobacter pylori* and His_6-propeptide-catβ4GalT-1 with defined LacNAc oligosaccharide structures

The kinetic data of the JHP1032-β3GlcNAcT for the LacNAc (**12**) and di-LacNAc (**14**) acceptor substrates revealed a decrease in affinity for the higher oligosaccharide with only a slightly lower reaction rate (Table 2). The three-fold lower catalytic efficiency was also found for the donor substrate UDP-GlcNAc. Most interestingly here is that the affinity of β3GlcNAcT for the UDP-GlcNAc in combination with **14** is lowered by a factor of about 20, whereas the activity is seven-fold increased. This may reflect the regulation of the poly-LacNAc synthesis by the bacterial enzyme. A decreased catalytic efficiency for LacNAc, di-LacNAc and tri-LacNAc structures were also described for the human iGnT enzyme involved in the synthesis of poly-LacNAc [38]. However, in contrast to the bacterial enzyme iGnT has the same affinity for the acceptor substrates LacNAc, di-LacNAc and tri-LacNAc structures with a significant lower V_{max} reduced by a factor of 2 for di-LacNAc and 2.5 for tri-LacNAc.

The kinetic characterization of the human β4GalT-1 constructs His_6-propeptide-catβ4GalT-1 and HB4GT with the acceptor substrates **13** and **15** revealed no influence of the oligosaccharide length on the catalytic efficiencies (Table 2). Also Ujita *et al.* described that the human β4GalT-1 only marginally decreased its efficiency when the acceptor contains an increasing number of LacNAc-repeats [38]. In contrast, for the bacterial LgtB-β4GalT from *Neisseria meningitidis* the catalytic efficiencies decreased significantly for the acceptor substrate **15** and for the donor substrate UDP-Gal. In comparison, His_6-propeptide-catβ4-GalT-1 is the enzyme with the highest activity for the acceptor substrates **13** and **15**. Also important is that substrate inhibition decreases constantly for the acceptors **7** and **13** with no inhibition for the pentasaccharide **15** (Tables 1 and 2). Obviously, the inhibitory effect of the hydrophobic aglycon in **7** is compensated by the elongation of the sugar chain in compound **13** and **15**. In comparison, HB4GT still showed significant substrate inhibition for **13** and **15** (Table 2). These results confirm our previous results for the novel construct His_6-propeptide-catβ4GalT-1 [31] and its favorable use of for the efficient synthesis of poly-LacNAc structures.

Table 2 Kinetic characterisation of β3GlcNAcT and β4GalTs with different poly-LacNAc structures as acceptor substrates and their corresponding donor substrates

Acceptor or donor substrate	V_{max} [U mg^{-1}]	K_m [mM]	K_{iS} [mM]	V_{max}/K_m
β3GlcNAcT				
Compound **12**	0.058	2.2	–	0.026
UDP-GlcNAc	0.091	1.9	–	0.048
Compound **14**	0.038	4.5	–	0.008
UDP-GlcNAc	0.626	38.9	–	0.016
His_6Propeptide-catβ4GalT-1				
Compound **13**	0.800	1.10	3.40	0.727
UDP-Gal	1.200	0.30	–	4.000
Compound **15**	1.100	1.70	–	0.624
UDP-Gal	1.040	0.28	–	3.714
HB4GT				
Compound **13**	0.140	0.9	1.1	0.156
UDP-Gal	0.752	4.5	–	0.167
Compound **15**	0.160	1.0	2.0	0.160
UDP-Gal	0.973	3.9	–	0.249
lgtB				
Compound **13**	0.308	1.5	–	0.205
UDP-Gal	0.440	1.8	–	0.244
Compound **15**	0.319	3.9	–	0.082
UDP-Gal	0.420	6.8	–	0.063

In conclusion, the kinetic data confirm the His_6-propeptide-catβ4GalT-1 and the JHP1032-β3GlcNAcT as the best enzyme combination for the synthesis of the poly-LacNAc structures. The critical step in the synthesis is the addition of the first galactose because the hydrophobic linker contributes to the inhibitory effect of the substrate **7** in higher concentrations. As far as we know, this is the first report on comprehensive kinetic studies for human and bacterial enzymes using defined poly-LacNAc acceptor structures.

Preparative synthesis of poly-LacNAc structures by a one-pot-synthesis

Since the focus of our approach is to imitate the distribution of natural sugar chains of different size on the surface of a cell, the synthesis of a mixture of poly-LacNAc with

different lengths may be more favorable than defined sugar structures of defined lengths. Therefore, we combined His_6-propeptide-catβ4GalT-1 with the β3GlcNAcT and both donor substrates in a one-pot synthesis starting from compound **12** as acceptor substrate. The oligosaccharide mixtures were analyzed by HPLC (Fig. 2a). Incubation of the product mixture with a glycosidase helped to identify the oligosaccharide structures (Fig. 2b). Assignment of the products with their retention times was performed according to the synthesized compounds **7**, **12**, **13**, **14**, and **15** (Scheme 3) and incubation with β-*N*-acetylglucosaminidase (Fig. 2c). The latter reveals the presence of LacNAc, di-, tri-, tetra-, and penta-LacNAc structures. In the synthesized poly-LacNAc mixture differently sized sugar structures from tri- to heptasaccharide appear as main components (Fig. 2a). The one-pot synthesis is reproducible with defined enzyme activities and a defined incubation time as well as by subsequent glycosidase treatment. As far as we know, this is the first report of a successful one-pot-synthesis of defined mixture of poly-LacNAc structures. Together with defined poly-LacNAc oligosaccharides these glycans are important for further enzymatic modifications and finally for immobilization onto surfaces and characterization by lectin binding experiments.

Sialylation of poly-LacNAc structures

Since higher affinities of the galectin CGL2 towards α2-3-sialylated LacNAc structures were already described (see data by Markus Künzler, ETH Zürich, in the consortium of functional glycomics: http://www.functionalglycomics.org/glycomics/publicdata.jsp), we characterized two bacterial α2,3-sialyltransferases (SiaT) from *Campylobacter jejuni* (CST-I) and *Neisseria meningitidis* (NST) with LacNAc **12** and di-LacNAc **14** as acceptor substrates (Scheme 4). Both enzymes convert a 5 mM solution of the acceptor substrate completely, but CST-I showed better kinetic data and a higher catalytic efficiency. However, the kinetic efficiency decreased with the longer acceptor sugar chain (data not shown). Both α2-3-sialylated products (**17**) and (**18**) were synthesized, purified and analyzed by MS successfully (Scheme 4). Conclusively, the poly-LacNAc structures are suitable for the introduction of further modifications that are of physiological relevance. Work is in progress, to introduce further modifications like fucosylation and sulfation, and to test these and the α2-3-sialylated structures in lectin binding experiments.

Immobilization of poly-LacNAc oligosaccharide structures and lectin binding experiments

The covalent binding of sugars to surfaces in microarrays has been described several times including different chemical coupling methods and subsequent analysis by carbohydrate binding proteins [39–43]. In our studies we used for the first time *t*Boc-protected amino-linker GlcNAc of the different poly-LacNAc structures which can be deprotected under mild acid conditions giving the free amino group for immobilization onto amino-reactive microtiter plates.

First experiments in the microtiter plate were done with deprotected compound **7**, GlcNAcβ-linker-NH_2. Biotin-labeled lectin II from *Griffonia simplicifolia* followed by a streptavidin–peroxidase conjugate was used for testing the accessibility of the immobilized sugar by a lectin as well as the binding capacity of the microtiter plate (Fig. 3a). The half maximal capacity was 2.1 mM sugar. Inhibition of lectin binding by different concentrations of soluble GlcNAc confirmed the sugar-induced binding of the lectin onto the sugar-modified microtiter plate. In a further step the binding of the lectin to the immobilized sugar was inhibited by soluble sugar. The experiments with added soluble GlcNAc proved that we could detect the sugar–protein binding in a specific way (Fig. 3b).

The deprotection of product **12** gave LacNAc-linker-NH_2 (**19**), which was coupled at different concentrations onto the amino-reactive microtiter plate. The detection by a commercial biotin labeled lectin from *Psophocarpus tetragonolobus* proved the presence of immobilized LacNAc (Fig. 4a); however, a saturation curve was not obtained. As an alternative for the detection of immobilized LacNAc the specific labeling of the terminal galactose with recombinant α3GalT and UDP-Gal-biotin (Scheme 4b) [44, 45] as donor substrate was investigated. Activity measurements using UDP-Gal and compound (**12**) revealed good acceptance ($V_{max\ app}$=0.38 U/mg; $K_{m\ app}$=1.9 mM) despite the linker modification of reducing GlcNAc. The broad acceptance of sugar modifications of the acceptor substrate is also described by Stults *et al*. [46]. Testing recombinant α3GalT with UDP-Gal-biotin gave a specific activity of 11 mU/mg which was sufficient for the labeling of LacNAc structures terminated by galactose *via* an enzymatic linked biotin–streptavidin assay. The enzymatic labeling with the Galili-enzyme was more sensitive giving a saturation curve with a linear response between 0.5 and 2.0 mM immobilized LacNAc (Fig. 4b). In comparison the linear response for the LacNAc detection by the lectin started at 2 mM immobilized sugar (Fig. 4a). We concluded that labeling of terminal sugars by an enzymatic reaction using a labeled donor substrate is the more sensitive method. As far as we know, this is a novel method to analyze immobilized glycan structures on surfaces. We currently study different β4GalT constructs for the enzymatic labeling of GlcNAc terminated immobilized glycan structures using UDP-Gal-biotin as modified donor substrates. This would then provide a highly specific method for the labeling of terminated poly-LacNAc structures terminated by GlcNAc and Gal, respectively.

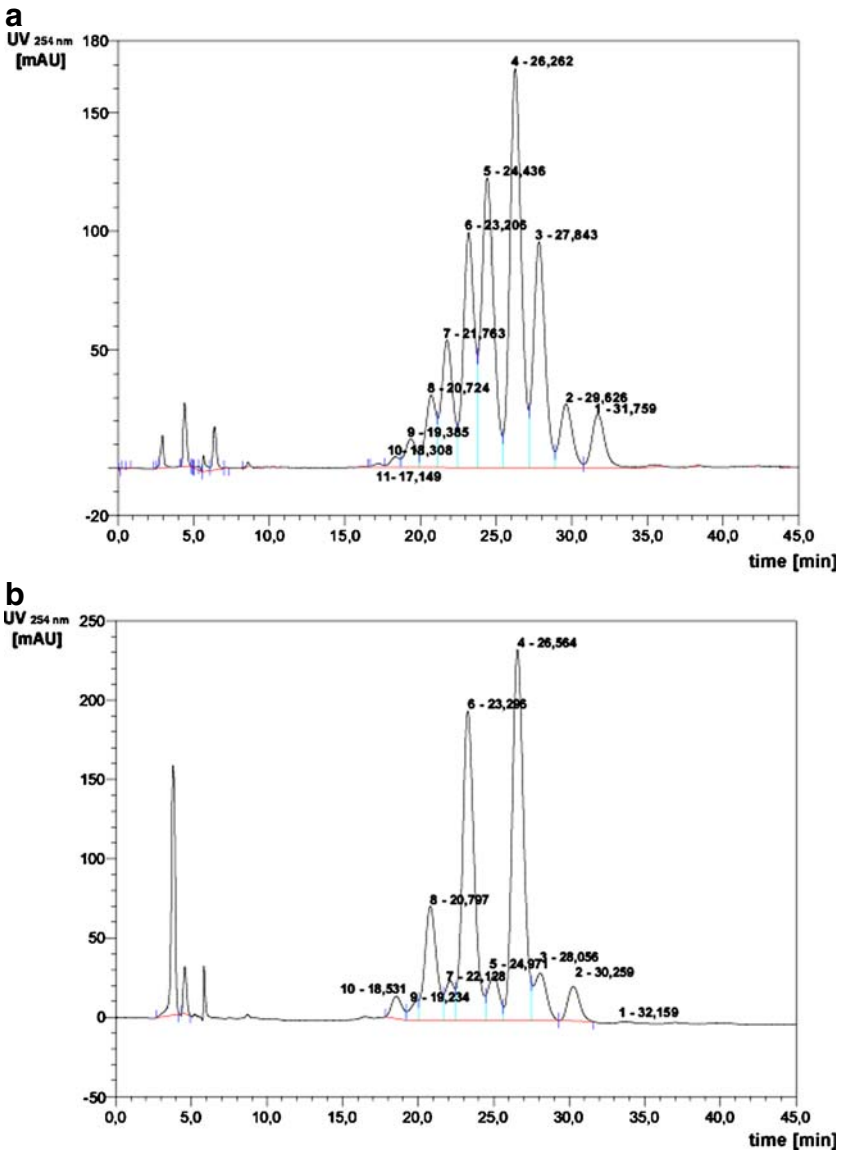
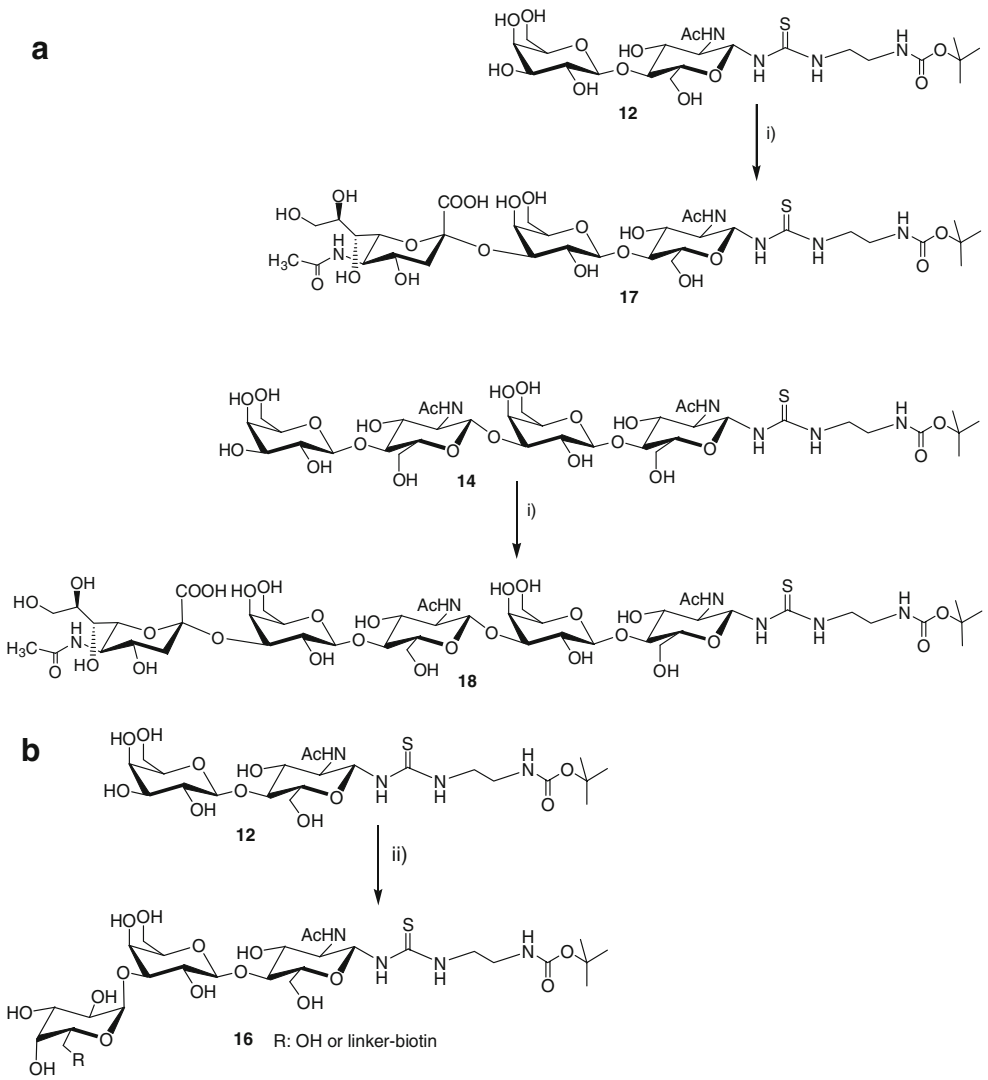


Fig. 2 Enzymatic one-pot synthesis of a defined mixture of poly-LacNAc with 24 h incubation. **a** HPLC analysis of the poly-LacNAc-linker-NH_2-*t*Boc mixture. **b** HPLC analysis of poly-LacNAc-linker-NH_2-*t*Boc mixture after treatment with β-*N*-acetylglucosaminidase. **c** Assignment of products with retention times

c

Peak-No./Retention time [min]	Compound	Number of sugars
1/31.8	**7**	1
2/29,6	LacNAc **12**	2
3/27.8	**13**	3
4/26.2	di-LacNAc **14**	4
5/24.4	**15**	5
6/23.2	tri-LacNAc	6
7/21.7		7
8/20.7	tetra-LacNAc	8
9/19.4		9
10/18.3	penta-LacNAc	10
11/17.1		11



Scheme 4 Enzymatic modification of LacNAc **12** and di-LacNAc **14** with: **a** α2,3-sialyltransferase, i) using CMP-Neu5Ac as donor substrate; **b** α1,3-galactosyltransferase, ii) using UDP-Gal or UDP-Gal-biotin as donor substrates

In the following experiments defined poly-LacNAc oligosaccharides were immobilized onto microtiter plates for binding studies with our model galectin His_6CGL2. The highest binding signals for CGL2 were obtained with a tri-LacNAc-structure (hexa-oligosaccharide) and a hepta-oligosaccharide (Fig. 5). Since tri-LacNAc has a significantly higher binding signal than the GlcNAc-terminated hepta-oligosaccharide we may conclude that CGL2 prefers poly-LacNAc structures with terminal galactose. A similar relation is observed for the other defined oligosaccharide structures; the binding signals of LacNAc (disaccharide) and di-LacNAc (tetrasaccharides) are higher than those for GlcNAc and the GlcNAc-terminated tri- and pentasaccharide (Fig. 5).

In order to test the binding of CGL2 to different mixtures of poly-LacNAc structures the enzymatic synthesis was started from compound **12** and terminated after different incubation times. The length of the oligosaccharides in the poly-LacNAc mixtures were subsequently assigned by their retention times in HPLC analysis as depicted in Fig. 2. In this way poly-LacNAc mixtures with oligosaccharides of different lengths were obtained as depicted in Fig. 6a. After 2 h synthesis a poly-LacNAc mixture was obtained with oligosaccharides ranging from tri- to hexasaccharides including relative fractions of 53% trisaccharide **13** and 31% di-LacNAc **14**. Termination of the poly-LacNAc synthesis after 7 h gave a range of tri- to octasaccharides with relative fractions of 20%, 35%, and 26% for the tri-, tetra-(di-LacNAc **14**), and pentasaccharide, respectively. The broadest distribution of oligosaccharides ranging from tri- to nonasaccharides was obtained after 24 h synthesis. The fractions of oligosaccharides comprised di-LacNAc **14** (12%), pentasaccharide (21%), tri-LacNAc (22%), hepta-saccharide (18%), tetra-LacNAc (12%), and nonasaccharide (7%).

Each poly-LacNAc mixture was immobilized after deprotection of the *t*Boc-terminating linker and tested for binding of CGL2. Figure 6b illustrates that binding efficiency of CGL2 improved constantly with poly-LacNAc mixtures having higher oligosaccharides as their main

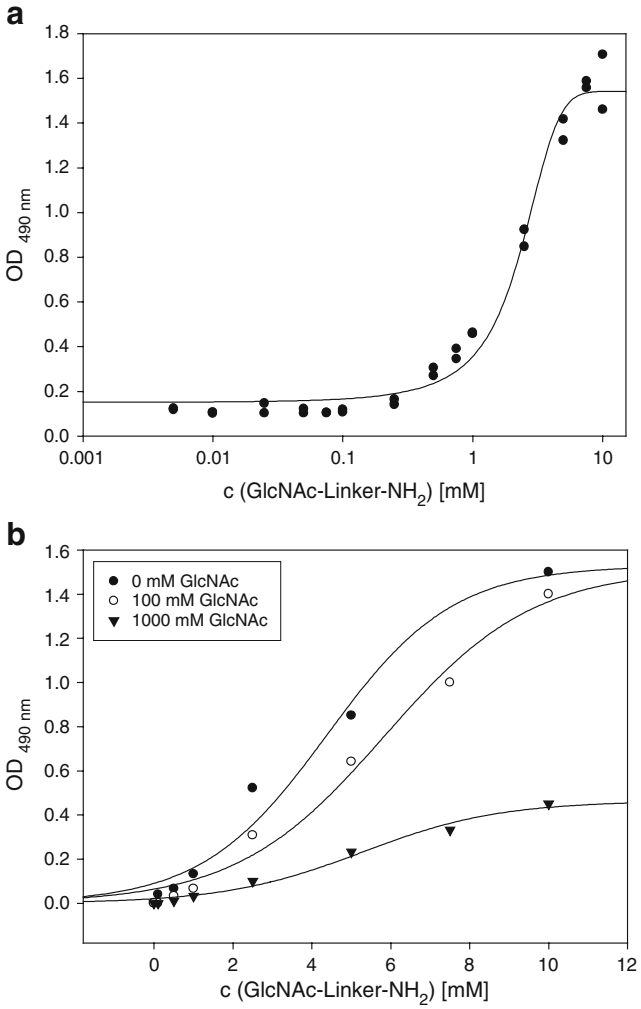


Fig. 3 **a** Binding assay of lectin II from *Griffonia simplicifolia* with deprotected compound **7** as ligand immobilized onto amino-reactive microtiter plates. **b** Inhibition of lectin binding by addition of soluble GlcNAc as competitive sugar ligand

fractions. The best binding for CGL2 was reached with an oligosaccharide mixture showing the broadest distribution of poly-LacNAc structures containing di-, tri-, and tetra-LacNAc structures. This mixture of poly-LacNAc-structures with up to a penta-LacNAc unit **22** still showed the highest binding signal of CGL2 in comparison to poly-LacNAc structures with a length of up a hexasaccharide (Fig. 7).

The structure and ligand binding of the fungal galectin CGL2 was recently characterized [34, 47]. Screening by a glycan microarray gave a broad range of different glycan binding partners for CGL2. The microarray comprised 203 different glycan structures containing also a defined LacNAc, di- and tri-LacNAc structure coupled to different aglycone linkers. In this study differences between the binding efficiency depended not only on the repeating LacNAc units, but also on the chemical linker at the reducing end. A step-wise significant increase of binding was described for the GlcNAc-Sp0, LacNAc-Sp0 and di-LacNAc-Sp0. The binding of CGL2 to the trisaccharide-Sp0 was similar to that for LacNAc. No further improvement of binding could be detected with the tri-LacNAc-Sp0. Changing the linker molecule from Sp0 to Sp8 increased the binding by a factor of 1.5. These studies support our results that higher LacNAc structures bind better to CGL2; however, defined mixtures of poly-LacNAc structures were not investigated in the CFG microarray analysis.

Poly-LacNAc mixture should be also useful for efficient binding of human galectins. Hirabayashi *et al.* described optimal binding structures including di-, tri-, and penta-LacNAc for binding studies of seven galectins [48].

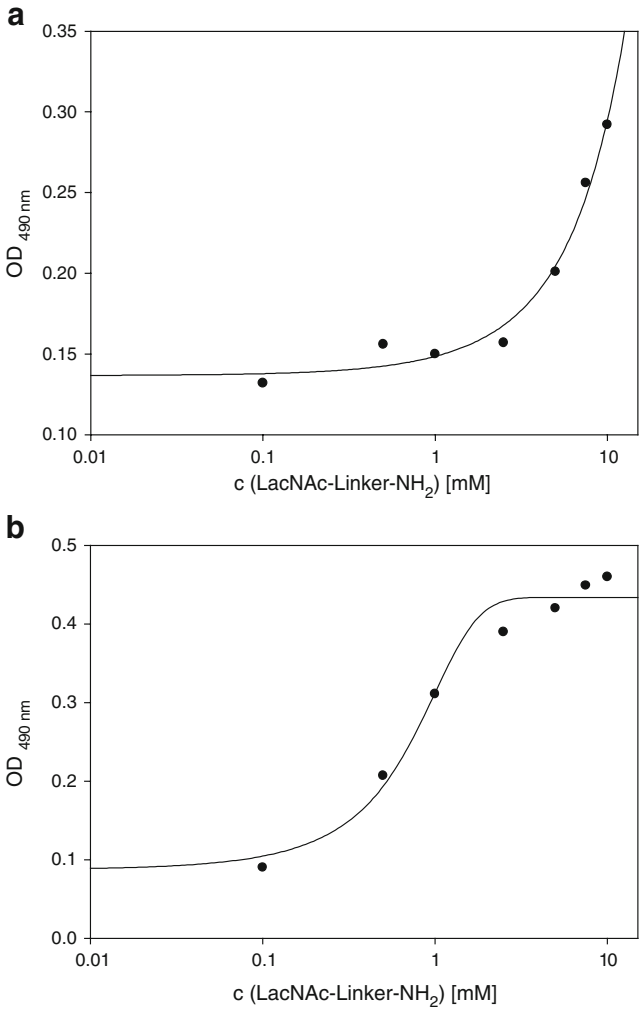


Fig. 4 **a** Binding assay of lectin from *Psophocarpus tetragonolobus* with deprotected compound **12** (LacNAc-linker-NH_2, **19**) as ligand covalently immobilized onto amino-reactive microtiter plates. **b** Enzymatic labeling of terminal Gal of covalently immobilized compound **19** by α3GalT-mediated transfer of UDP-Gal-biotin (Scheme 4b) and subsequent detection by streptavidin–peroxidase conjugate

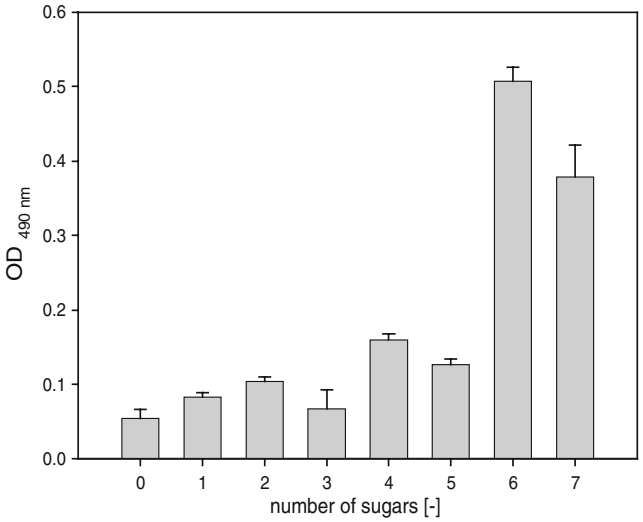


Fig. 5 Binding of His_6CGL2 galectin to defined (poly)-LacNAc structures. Compound **7** (1 sugar), compounds **12** to **15** (2 to 5 sugars) as well as a tri-LacNAc and a heptasaccharide structure were deprotected and bound onto an amino-reactive microtiter plate

Concerning the effect of a LacNAc repetition sequence on the binding constant the galectins showed big differences. For galectin-9 the highest enhancement in affinity was seen depending on the length of poly-LacNAc. In contrast, the affinity of galectin-1 to higher poly-LacNAc structures was only poorly enhanced. However, mixtures of poly-LacNAc structures were not investigated in these studies.

Therefore, we conclude that a defined mixture of poly-LacNAc structures is the best choice for biofunctionalisation of biomaterial surfaces. The synthesis is easy and efficient, the deprotection can be done with over 90% yield and the affinity towards the galectin CGL2 is higher in contrast to other sugar structures.

CGL2-mediated binding of laminin and fibronectin onto immobilized mixed poly-LacNAc structures

In order to demonstrate that an artificial ECM can be built up *via* sugar–galectin interactions in a microtiter plate, the mixture of poly-LacNAc structures was utilized for binding the galectin CGL2 which facilitates subsequent binding of ECM glycoproteins. Laminin (from mouse EHS tumor) is known to contain 25–30% by weight of carbohydrate with bi- and tri-antennary N-glycans carrying poly-LacNAc chains [49]. Laminin was also characterized as a high-affinity

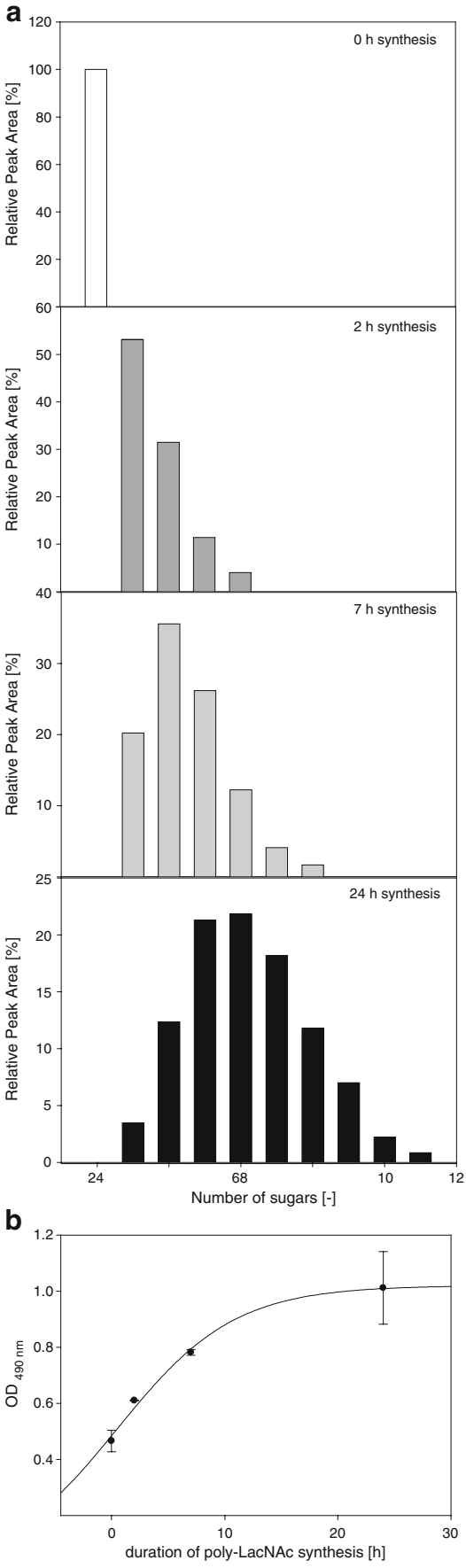


Fig. 6 **a** Different mixtures of poly-LacNAc structures starting from compound **12** were synthesized by variation of the incubation time. The relative distribution of the different poly-LacNAc structures is depicted showing increasing oligosaccharide length with longer incubation time. **b** Binding of His_6CGL2 galectin to the different poly-LacNAc structures covalently immobilized onto amino-reactive microtiter plates. Binding of His_6CGL2 was detected by an anti-His_6-peroxidase conjugate

substrate for galectin 1 and 3 [50, 51] and should therefore also be bound by our model lectin CGL2. In a first experiment, a poly-LacNAc mixture (from a 24 h synthesis) was covalently bound onto amino-reactive microtiter plates. After binding of CGL2 onto immobilized poly-LacNAc the ECM glycoprotein laminin was applied and analyzed for binding by an anti-laminin antibody conjugated to peroxidase. Figure 8a reveals that the tetrameric CGL2 mediates the binding of laminin by cross-linking the ECM glycoprotein to the surface-bound poly-LacNAc. The binding of laminin to CGL2 can be interfered by addition of LacNAc as competitive sugar ligand. However, our data also show that laminin interacts also directly with the poly-LacNAc surface without CGL2 as mediator. This result may be explained by the described alpha-dystroglycan lectin binding domain in laminin [52, 53].

In a second experiment the human serum fibronectin was investigated for binding to immobilized poly-LacNAc and CGL2. The glycosylation pattern of fibronectin was recently described by Tajiri *et al.* [54]. The specific binding of fibronectin could be detected and decreased by addition of soluble competitive LacNAc sugar (Fig. 8b). However, the background signal was relative high due to the antibody binding. Fibronectin was also directly bound to the poly-LacNAc structures, which may be due to the cryptic lectin site in the cell-binding domain described by Hörmann *et al.* [55]. In summary, we could demonstrate that poly-LacNAc functionalized surfaces bind ECM glycoproteins *via* galectin-crosslinking. Work is in progress to utilize poly-LacNAc structures together with galectins for building up artificial ECM layers on biomaterial surfaces and to explore the application potential of these biohybrid surfaces for the adhesion of different cell types.

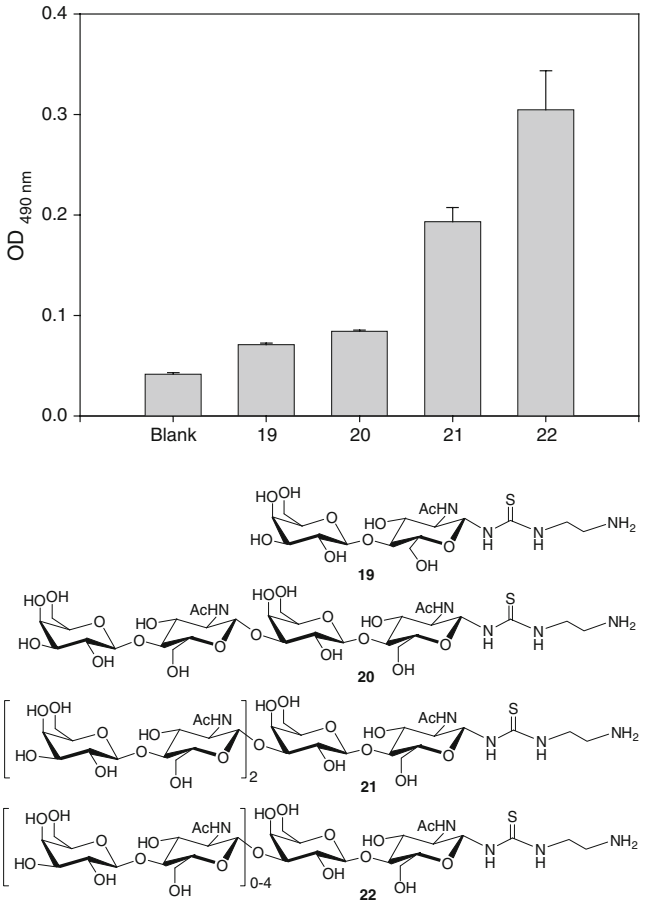


Fig. 7 Binding of the galectin His_6CGL2 to 1 mM LacNAc-linker-NH_2 (**19**), tetrasaccharide-linker-NH_2 (**20**), hexasaccharide-linker-NH_2 (**21**), and poly-LacNAc-linker-NH_2 (**22**), respectively, covalently immobilized onto amino-reactive microtiter plates

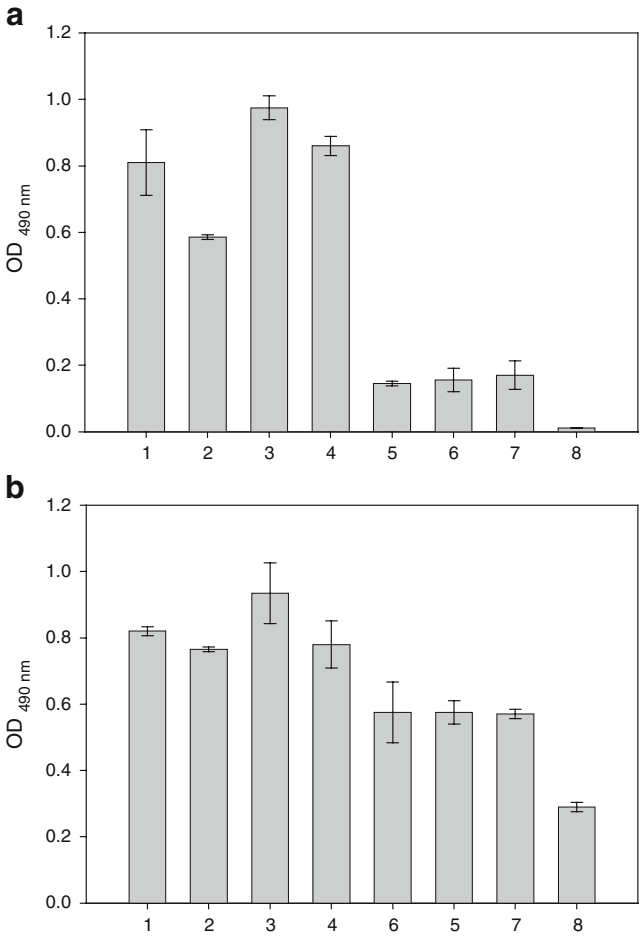


Fig. 8 Binding of ECM glycoproteins onto poly-LacNAc functionalized microtiter plates. **a** Laminin: *1*: Poly-LacNAc+His_6CGL2+laminin, *2*: Poly-LacNAc+His_6CGL2+laminin+10 mM LacNAc, *3*: poly-LacNAc+laminin, *4*: Poly-LacNAc+laminin+10 mM LacNAc, *5*: poly-LacNAc+His_6CGL2, *6*: poly-LacNAc, *7*: blocked microtiter plate+His_6CGL2+laminin, *8*: blocked aminoreactive microtiter plate. **b** Fibronectin: *1*: Poly-LacNAc+His_6CGL2+fibronectin, *2*: poly-LacNAc+His_6CGL2+fibronectin+10 mM LacNAc, *3*: poly-LacNAc+fibronectin, *4*: poly-LacNAc+fibronectin+10 mM LacNAc, *5*: poly-LacNAc+His_6CGL2, *6*: poly-LacNAc, *7*: blocked microtiter plate+His_6CGL2+fibronectin, *8*: blocked aminoreactive microtiter plate

Conclusions

Our studies present a novel approach for the determination of the best poly-LacNAc binding structure for galectins. The defined mixtures of poly-LacNAc structures may reflect similar distributions of oligosaccharides present on cell surfaces. Therefore, we are convinced that mixtures of poly-LacNAc structures are efficient signals for targeting galectins to biofunctionalized surfaces which can be also exploited for galectin-mediated immobilization of ECM glycoproteins and subsequent cell adhesion onto biomaterial surfaces.

The chemical modified compound **7** can be easily produced and has many advantages for the biocatalytic synthesis and immobilization of poly-LacNAc structures on various bio-functional materials. The recombinant His_6-propeptide-catβ4GalT-1 showed optimal conversion rates for **7** and efficient synthesis of poly-LacNAc in combination with β3GlcNAcT from *H. pylori*. Here, our study underlines the importance of specified enzyme constructs for the conversion of chemically modified and extended acceptor substrates. The resulting products can be readily quantified and purified by its hydrophobic modification. After a one-step deprotection the poly-LacNAc structures are used for subsequent covalent binding onto amino-reactive surfaces. With amino-reactive microtiter plates we developed a small-scale-test system for the determination of optimal binding poly-LacNAc structures for the lectin CGL as a model for galectins. We could also demonstrate that CGL2 mediates binding of laminin and fibronectin, ECM glycoproteins, to surface bound poly-LacNAc structures. Work is in progress to perform such studies with human galectins and to immobilize poly-LacNAc structures onto hydrogels. The poly-LacNAc structured hydrogel surfaces will be analyzed for galectin binding and used for targeted cell adhesion.

Experimental section

*Synthesis of compound (**2**)* Starting from *N*-acetylglucosamine (**1**) the synthesis of compound **2** was done according to Horton [56]. The final yield was 50% (20 g, 55 mmol).

*Synthesis of compound (**3**)* For the synthesis of compound **3** 10.64 g KSCN (109.6 mmol), 18.6 g Bu_4NHSO_4 (54.8 mmol), and 20 g molecular sieves (4 Å) were dissolved and stirred in 300 ml dried acetonitrile for half an hour at room temperature. After addition of 20 g of compound **2** (54.67 mmol) the reaction mixture was heated under reflux at 85°C. The reaction was checked by TLC (silica gel 60 F_{254} aluminium sheets; mobile phase petrol ether/ethyl acetate 2:1; detection by UV light at 254 nm and charring with 5% H_2SO_4 in ethanol). After 4 h the insoluble part was filtered off from the reaction mixture, filter cake was washed with additional portion of dichloromethane. The combined filtrates were evaporated *in vacuo*. The product was purified by a flash column chromatography (200 g silica gel 60 (40–63 μm, 230–400 mesh), mobile phase petrol ether/ethyl acetate 2:1) yielding **3** as 6.5 g of light yellow solid (30%, 17 mmol).

*Synthesis of compound (**5**)* In 800 ml dioxane, 665.6 mmol (40 g) of **4** was dissolved. A solution of 219.19 mmol (48 g) Boc-anhydride in the remaining 200 ml dioxane was added drop wise over 6 h to the stirred solution of **4** at 4°C. After over night incubation the assay was filtered and evaporated until no further dioxane could be removed. For the final purification the roar product was re-suspended in 100 ml water and extracted three-times with 100 ml dichloromethane respectively. The combined organic phases were dried for 15 min by the addition of some Na_2SO_4, filtered and evaporated as described above to obtain compound **5**. The product was a colorless, highly viscous solution which turned white when frozen. After weighting the sample (34.86 g, 217.71 mmol) was stored at −20°C for the further use in the synthesis of **6**.

*Synthesis of compound (**6**)* For the synthesis of compound **6** 1 mol of compound **3** was mixed with 1.1 mol of compound **5** in dry acetonitrile. The reaction was stirred over night at room temperature and checked by TLC the next day (silica gel 60 F_{254} aluminium sheets; mobile phase chloroform/acetone 4:1; and detection by UV light at 254 nm and charring with 5% H_2SO_4 in ethanol). After complete conversion of **3** the reaction mixture was evaporated. The purified product could be obtained after a flash column chromatography (silica gel 60 [40–63 μm, 230–400 mesh] mobile phase chloroform: acetone=4:1). The final yield of the product was 87% (2.4 g, 4.3 mmol). The reaction was repeated twice resulting in similar yields.

*Synthesis of GlcNAc-linker-NH_2-tBoc (**7**)* Compound **6** was stirred with CH_3ONa/CH_3OH for 0.5 h. The reaction was stopped by adding Dowex 50 WX-200 and checked by TLC (silica gel 60 F_{254} aluminium sheets; mobile phase chloroform/acetone 4:1 or 2-propanol/water/25% NH_3 7:2:1; detection by UV light at 254 nm and charring with 5% H_2SO_4 in ethanol). The ion exchanger was filtered off and washed with methanol. The filtrate was dried by evaporation. The dried product was dissolved in some water and lyophilized over night (white product). The yield was exactly 90% (2.5 g, 5.9 mmol). ^{1}H NMR (399.87 MHz, DMSO-d_6, 30°C): δ 1.377 (9H, s, $(CH_3)_3C$), 1.829 (3H, s, Ac), 3.043 (2H, m, H-2), 3.14* (1H, m H-5^A), 3.15 (1H, m, H-4^A), 3.32 (1H, m, H-3^A), 3.44* (2H, m, H-1), 3.475 (1H, m, H-6^{A}u), 3.560 (1H, m, H-2^A), 3.621 (1H, m, H-6^{A}d), 4.949 (1H, br s, 3^A-OH), 4.96* (1H, m, H-1^A), 4.984 (1H, br s, 4^A-OH),

4.467 (1H, t, J=5.6 Hz, 6^A-OH), 6.802 (1H, br s, 2-NH), 7.497 (1H, d, J=7.3 Hz, 1^A-NH), 7.893 (1H, br s, 1-NH), 7.951 (1H, br s, 2^A-NH).

^{13}C NMR (100.55 MHz, DMSO-d_6, 30°C, HMQC and HMBC readouts): δ 23.1 (2^A-Ac), 28.5 (($\mathbf{C}H_3)_3C$), 39.5 (C-2), 43.9 (C-1), 54.6 (C-2^A), 60.7 (C-6^A), 70.3 (C-4^A), 74.3 (C-3^A), 77.8 ($(CH_3)_3\mathbf{C}$), 78.1 (C-5^A), 83.2 (C-1^A), 155.8 (N–CO–O), 170.6 (C=O).

*Synthesis of compound (***9***)* The synthesis of the alternative coupling reagent 2-azidoethanamine was carried out according to the protocol of Benalil *et al.* [57]. The yield was 38% (0.32 g, 3.7 mmol).

*Synthesis of compound (***10***) and (***11***)* For the synthesis of **10** 1.86 mmol of **9** and 2.31 mmol of **3** were dissolved in anhydrous acetonitrile respectively. The reaction was performed over night at room temperature with constant stirring. The success of the synthesis was judged by TLC analysis (silica gel 60 F_{254} from Merck with mobile phase chloroform/acetone 3:1, and detection charring with 5% H_2SO_4 in ethanol). The reaction mixture was evaporated and the product was isolated by flash chromatography (silica gel 60 with mobile phase chloroform/acetone 10:1). The fractions containing the product were pooled and again evaporated to dryness. The deacetylation reaction was done analogues to (**7**). The final yield was about 70% (0.2 g, 0.57 mmol). The product was analyzed by ^{1}H and ^{13}C NMR.

^{1}H NMR (399.87 MHz, D_2O, 30°C): δ 1.786 (3H, s, Ac), 3.264 (1H, dd, J=9.8, 8.7 Hz, H-4^A), 3.29* (2H, m, H-2), 3.329 (1H, ddd, J=9.8, 5.0, 2.1 Hz, H-5^A), 3.422 (1H, dd, J=10.2, 8.7 Hz, H-3^A), 3.540 (1H, dd, J=12.4, 5.0 Hz, H-6^Au), 3.61* (2H, m, H-1), 3.67* (1H, m, H-2^A), 3.680 (1H, dd, J=12.4, 2.1 Hz, H-6^Ad), 5.286 (1H, m, H-1^A).

^{13}C NMR (100.55 MHz, D_2O, 30°C): δ 22.26 (Ac), 44.21 (C-1), 50.10 (C-2), 54.70 (C-2^A), 60.85 (C-6^A), 69.87 (C-4^A), 74.33 (C-3^A), 77.54 (C-5^A), 83.02 (C-1^A), 175.31 (C=O).

*Enzyme production and purification*The recombinant enzymes used in this study were produced and purified as described previously: Sucrose Synthase 1 (SuSy1) [58], UDP-Glc 4′-epimerase [59], the fusion protein of human β1-4-galactosyltransferase 1 His_6-Propeptide-catβ4GalT-1 [31], the *Helicobacter pylori* β1-3-*N*-acetylglucosaminyltransferase (β3GlcNAcT, JHP1032, gene number from the strain J99 genome sequence) [32], the bacterial β1-4-galactosyltransferases *H. pylori* HP0826, MalE-HP0826 (gene number from the strain 26695 genome sequence), *Neisseria meningitides* LgtB (gene number from the genome strain NMB 1928) [32, 60], the soluble MalE fusion protein of human β1-4-galactosyltransferase 1 (HB4GT) [61], and the *Neisseria meningitidis* α2-3-sialyltransferase NST [62]. The construct expressing CST-I (protein sequence database #AAF13495) from *Campylobacter jejuni* OH4384 contained the full length sequence of a α2-3-sialyltransferase fused to MalE in the vector pCW [63]. The plasmid encoding recombinant murine α1-3GalT (pET12a-α3GalT) was kindly provided by Prof. Berger (University of Zürich) [64]. The expression plasmid was transformed into *E. coli* BL21(DE3). Production, purification and analysis were performed as described elsewhere [31].

Galectin production and purification The plasmid YEplac195-PvuIICGL2inverse encoding the fungal galectin CGL2 from *Coprinus cinereus* was a generous gift from Prof. Aebi (ETH Zürich) [34]. CGL2 was cloned as a His_6-tagged protein using the two primers 3′-P-CCGCTCGA GCGGATGCTCTACCACCTTTT-5′ and 5′-P-CGC GGATCCGCGCTAAGCAGGGGGAAG-3′ and the plasmid YEplac195-PvuIICGL2inverse as template for PCR amplification. The PCR product and pET14b were digested by *Xho*I/*Bam*HI and ligated giving the expression plasmid pET14b-His_6CGL2. CGL2 was produced in *E. coli* BL21 (DE3) transformed with pET14b-His_6CGL2. Purification by IMAC (Ni^{2+}–NTA) and analysis were performed as described elsewhere [31]. The purified protein was washed and stored in PBS (50 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) at 4°C for further use.

First testing of the compound **7** *and* **11** *as acceptor substrates of the human β1,4GalT-1* Compound **7** and **11** were tested as acceptor substrates of human β1,4GalT-1. The assays were prepared analogously to Zervosen *et al.* [27]. The conversion of 5 mM acceptor substrate was analyzed by HPLC (LiChrospher® 100 RP18, LiChroCart, Merck, 0.5 ml/min, RT, isocratic with 15% acetonitrile).

Kinetic characterization of glycosyltransferases A continuous photometric assay was applied for kinetic characterization of glycosyltransferases. The enzymatic activity was measured at a constant concentration of 5 mM acceptor substrate with 0–10 mM donor substrate to determine the donor substrate kinetic. The acceptor substrate kinetic was measured with 0–5 mM acceptor substrate with a constant concentration of 5 mM donor substrate. Donor and acceptor substrates at the indicated concentrations were mixed with 100 mM HEPES–NaOH buffer, pH 7.2, containing 25 mM KCl, 1 mM DTT, 1 mM PEP, 2 mM K_2HPO_4, 4 mM $MgCl_2$, 2 mM $MnCl_2$, 0.25 mM NADH, 5 U lactate dehydrogenase, and 5 U pyruvate kinase [31]. Kinetic constants were obtained by non-linear regression of the data points using Sigma Plot10 (SPSS GmbH Software, München, Germany). Alternatively, CE or HPLC product analysis was also used kinetic measurements in a discontinuous assay as indicated.

Enzymatic synthesis, analysis and characterization of defined poly-LacNAc structures The enzymatic synthesis of defined poly-LacNAc structures was performed as described below in detail. The reaction assays were incubated at 30°C and donor substrate was added until the acceptor substrate was consumed. Product formation was analyzed by HPLC (LiChrospher® 100 RP18, LiChroCart, Merck, 0.5 ml/min, RT, gradient elution with 0% or 11% to 50% *v*/*v* acetonitrile or isocratic with 15% *v*/*v* acetonitrile) or CE (CE running buffer 25 mM $Na_2B_4O_7$ pH 9.4 or 20 mM $Na_2B_4O_7$, 50 mM SDS pH 9.4 at 25 kV [65]) (see also Supporting Information). The *t*Boc-linker-sugars were purified by Sep-Pak® Vac C18 1cc columns (Waters GmbH, Eschborn, Germany). The columns were equilibrated with 3 ml methanol and water. After applying the sample (maximum volume 1 ml) a washing step with 3 ml water was followed by the elution with 3 ml 50% (*v*/*v*) acetonitrile. The fractions were analyzed by HPLC or CE. The resulting defined sugar structures were analyzed by MS and/or NMR. Mass spectra were measured on a matrix-assisted laser desorption/ionisation reflectron time-of-flight (MALDI-TOF) mass spectrometer BIFLEX (Bruker-Franzen, Bremen, Germany) equipped with a nitrogen laser (337 nm) and griddles delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Spectra were calibrated externally using the mono-isotopic $[M+H]^+$ ions of matrix peak 379.1 *m*/*z* and Angiotensin I 1296.7 *m*/*z*. A saturated solution of α-cyano-4-hydroxy-cinnamic acid or 2,5-dihydroxy benzoic acid in 50% MeCN/0.3% acetic acid was used as a MALDI matrix. A 1 μl of matrix solution was mixed with a 1 μl of sample diluted in water and a 1 μl of premix was loaded on the target, the droplet was allowed to dry at ambient temperature. The MALDI-TOF spectra were collected in reflectron mode.

NMR spectra were recorded on a Varian ^{UNITY}*Inova*-400 MHz spectrometer (399.87 MHz for ^{1}H, 100.55 MHz for ^{13}C) in D_2O (99.98%D, ARMAR Chemicals, Döttingen, CH), DMSO-d_6 (99.9%D, Aldrich, Steinheim, DE), CD_3OD (99.8%D, Chemtrade, Leipzig, DE) at 30°C. Residual signals of solvents were used as internal standard (D_2O: δ_H 4.508 ppm, DMSO-d_6: δ_H 3.330 ppm, δ_C 39.60 ppm, CD_3OD: δ_H 2.500 ppm, δ_C 49.30 ppm). Carbon chemical shifts in D_2O were referenced to acetone (δ_C 30.50 ppm). NMR experiments: COSY, TOCSY, HMQC, HSQC, HMBC, gHMBCAD, and HMQCTOCSY were performed using the manufacturer's software. For selective 1D-TOCSY was used sequence published by Uhrín *et al.* [66]. ^{1}H NMR and ^{13}C NMR spectra were zero filled to fourfold data points and multiplied by window function before Fourier transformation. Two-parameter double-exponential Lorentz–Gauss function was applied for ^{1}H to improve resolution and line broadening (1 Hz) was applied for ^{13}C get better signal-to-noise ratio.

Proton spin systems of individual sugar units were assigned by COSY, TOCSY, 1D-TOCSY. This assignment was transferred to carbons by HMQC or HSQC and further improved by HMQCTOCSY. The linkage position was deduced from heteronuclear correlations in the HMBC or from the downfield glycosylation shifts of the involved carbons (C-3 or C-4), type of glycosidic linkage (β) was determined from the $J_{H\text{-}1,H\text{-}2}$.

Chemical shifts are given in δ-scale [ppm], and coupling constants in Hertz. Digital resolution allowed us to give chemical shifts of protons on three, carbons on two and coupling constants on one decimal place. Carbon chemical shifts reported to one decimal place were read out from HMQC or HSQC (protonated carbons) and HMBC (quaternary carbons). Some proton chemical shifts accurate two decimal places (marked by asterisk) were extracted from HMQC or HSQC. Signals of respective carbohydrate moieties in the NMR spectra are denoted by capitals A–E in the order from the thioureido-linkage (see Scheme 3).

*Enzymatic synthesis of LacNAc-linker-NH_2-tBoc (***12***)* Compound **7** (5 mM) was mixed with 5 mM UDP-Gal in buffer A (50 mM HEPES–NaOH buffer, pH 7.2., with 25 mM KCl, 1 mM DTT, 2 mM $MnCl_2$, 2 U alkaline phosphatase), and 20 mU His_6-Propeptide-catβ4GalT-1. The reaction mixture was incubated over night at 30°C. Compound **12** (10 mg, 17.1 μmol)) was synthesized with quantitative conversion of **7**. Assays with *in situ* regeneration of the donor substrate were carried out according to Zervosen *et al.* [27].

The molecular mass of **12** was determined by MALDI-TOF: found: *m*/*z* 585.3 for $[M+H]^+$ and *m*/*z* 607.3 for $[M+Na]^+$; calculated for [M]: *m*/*z* 584.2.

^{1}H NMR (399.87 MHz, CD_3OD, 30°C): δ 1.460 (9H, s, $(CH_3)_3C$), 1.995 (3H, s, 2^A-Ac), 3.243 (2H, dd, ΣJ=12.5 Hz, H-2), 3.510 (1H, dd, J=9.7, 3.3 Hz, H-3^B), 3.52* (1H, m, H-5^A), 3.578 (1H, dd, J=9.7, 7.5 Hz, H-2^B), 3.617 (1H, m, H-5^B), 3.632 (2H, m, H-1), 3.645 (1H, dd, ΣJ=18.2 Hz, H-4^A), 3.693 (1H, dd, ΣJ=18.5, H-3^A), 3.720 (1H, dd, J=11.4, 4.6 Hz, H-6^Bu), 3.788 (1H, dd, J=11.4, 7.4 Hz, H-6^Bd), 3.842 (1H, dd, J=3.3, 0.7 Hz, H-4^B), 3.876 (1H, dd, J=12.1, 3.9 Hz, H-6^Au), 3.89* (1H, m, H-2^A), 3.929 (1H, dd, J=12.1, 2.4 Hz, H-6^Ad), 4.410 (1H, d, J=7.5 Hz, H-1^B), 5.363 (1H, br m, H-1^A).

^{13}C NMR (100.55 MHz, CD_3OD, 30°C, gHSQC and HMBC readouts): δ 22.9 (2^A-Ac), 28.9 (($\mathbf{C}H_3)_3C$), 40.9 (C-2), 45.6 (C-1), 55.8 (C-2^A), 62.0 (C-6^A), 62.7 (C-6^B), 70.5 (C-4^B), 72.7 (C-2^B), 74.5 (C-3^A), 75.0 (C-3^B), 77.3 (C-5^B), 77.9 (C-5^A), 80.5 (($CH_3)_3\mathbf{C}$), 80.9 (C-4^A), 105.3 (C-1^B), 158.8 (N–CO–O), 174.9 (2^A-CO).

*Enzymatic synthesis of GlcNAc(β1-3)Gal(β1-4)GlcNAcβ1-linker-NH_2-tBoc (***13***)* LacNAc-linker-*t*Boc (**12**, 7.5 mM) was mixed with 8 mM UDP-GlcNAc in buffer B (50 mM

HEPES–NaOH buffer, pH 7.2, with 25 mM KCl, 1 mM DTT, 2 mM $MgCl_2$, 2 U alkaline phosphatase), and 20 mU β3GlcNAcT (JHP1032). The reaction mixture was incubated over night at 30°C. Compound **13** (10 mg, 12.7 μmol) was synthesized with quantitative conversion of **12**.

MALDI-TOF of **13**; found: 788.6 for $[M+H]^+$ and *m/z* 810.6 for$[M+Na]^+$; calculated for [M] *m/z* 787.3.

^{1}H NMR (399.87 MHz, D_2O, 30°C): δ 1.184 (9H, s, (C**H$_3$**)$_3$C), 1.769 (3H, s, Ac), 1.794 (3H, s, Ac), 3.018 (2H, m, H-2), 3.205 (1H, m, H-5^C),3.226 (1H, m, H-4^C), 3.324 (1H, dd, J=10.3, 9.0 Hz, H-3^C), 3.354 (1H, dd, J=9.9, 7.8 Hz, H-2^B), 3.417 (2H, m, H-1), 3.432 (1H, m, H-5^A), 3.48* (1H, m, H-5^B), 3.484 (1H, dd, J=9.9, 3.3 Hz, H-3^B), 3.497 (1H, m, H-4^A), 3.50* (2H, m, H-6^B), 3.509 (1H, dd, J=10.3, 8.4 Hz, H-2^C), 3.514 (1H, dd, J=12.3, 4.9 Hz, H-6^{C}u), 3.560 (1H, m, H-3^A), 3.598 (1H, dd, J=12.6, 4.4 Hz, H-6^{A}u), 3.651 (1H, dd, J=12.3, 1.9 Hz, H-6^{C}d), 3.672 (1H, m, H-2^A), 3.711 (1H, dd, J=12.6, 1.9 Hz, H-6^{A}d), 3.906 (1H, m, H-4^B), 4.231 (1H, d, J=7.8 Hz, H-1^B), 4.443 (1H, d, J=8.4 Hz, H-1^C), 5.335, 5.232 (1H, m, H-1^A).

^{13}C NMR (100.55 MHz, D_2O, 30°C): δ 22.28 (Ac), 22.42 (Ac), 27.93 ((**C**H$_3$)$_3$C), 39.50 (C-2), 44.43 (C-1), 54.22 (C-2^A), 55.90 (C-2^C), 60.15 (C-6^A), 60.74 (C-6^C), 61.18 (C-6^B), 68.57 (C-4^B), 69.95 (C-4^C), 70.25 (C-2^B), 72.84 (C-3^A), 73.81 (C-3^C), 75.12 (C-5^B),75.90 (C-5^C), 76.28 (C-5^A), 78.32 (C-4^A), 81.32 ((CH$_3$)$_3$**C**), 82.14 (C-3^B), 84.28, 82.86 (C-1^A), 103.04 (C-1^C), 103.13 (C-1^B), 158.41 (N-CO-O), 175.16 (C=O), 175.20 (C=O), 183.08 (C=S).

*Enzymatic synthesis of di-LacNAc-linker-NH$_2$-tBoc (***14***)* The enzymatic synthesis of compound **14** was carried out as described for compound **12** taking 6.7 mM of **13** and 7 mM UDP-Gal. Compound (**14**) was obtained with quantitative yield (10 mg, 10.53 μmol).

MALDI-TOF of **14**; found: *m/z* 950.7 for $M-H^+$ and *m/z* 972.7 for $M-Na^+$; calculated for $M-H^+$ *m/z* 949.4.

^{1}H NMR (399.87 MHz, D_2O, 30°C): δ 1.192 (9H, s, (C**H$_3$**)$_3$C), 1.777 (3H, s, Ac), 1.799 (3H, s, Ac), 3.024 (2H, m, C-2), 3.304 (1H, dd, J=9.9, 7.8 Hz, H-2^D), 3.356 (1H, m, H-5^C), 3.363 (1H, dd, J=10.0, 7.8 Hz, H-2^B), 3.422 (2H, m, C-1), 3.431 (1H, dd, J=9.9, 3.4 Hz, H-3^D), 3.454 (1H, m, H-5^A), 3.48* (1H, m, H-5^B), 3.49* (2H, m, H-3^C, H-5^D), 3.491 (1H, dd, J=10.0, 3.2 Hz, H-3^B), 3.50* (1H, m, H-4^C), 3.509 (1H, m, H-4^A), 3.52* (4H, m, H-6^B, H-6^D), 3.568 (1H, m, H-3^A), 3.577 (1H, dd, J=10.3, 8.3 Hz, H-2^C), 3.611 (1H, m, H-6^{A}u), 3.687 (1H, m, H-2^A), 3.688 (1H, m, H-4^D), 3.617 (1H, dd, J=12.3, 4.5 Hz, H-6^{C}u), 3.723 (1H, m, H-6^{A}d), 3.727 (1H, dd, J=12.3, 2.2 Hz, H-6^{C}d), 3.917 (1H, m, H-4^B), 4.240 (2H, d, J=7.8 Hz, H-1^B, H-1^D), 4.472 (1H, d, J=8.3 Hz, H-1^C), 5.240, 5.347 (1H, m, H-1^A).

^{13}C NMR (100.55 MHz, D_2O, 30°C): δ 22.28 (Ac), 22.43 (Ac), 27.93 ((**C**H$_3$)$_3$C), 39.51 (C-2), 44.43 (C-1), 54.22 (C-2^A), 55.43 (C-2^C), 60.14 (C-6^A, C-6^C), 61.18 (C-6^B or C-6^D), 61.24 (C-6^B or C-6^D), 68.56 (C-4^B), 68.79 (C-4^D), 70.21 (C-2^B), 71.20 (C-2^D), 72.41 (C-5^D), 72.76 (C-3^D), 72.84 (C-3^A), 74.79 (C-5^C), 75.11 (C-5^B),75.58 (C-3^C), 76.28 (C-5^A), 78.31 (C-4^A), 78.49 (C-4^C), 81.28 ((CH$_3$)$_3$**C**), 82.22 (C-3^B), 82.86 (C-1^A), 102.94 (C-1^C), 103.11 (C-1^D), 103.13 (C-1^B), 158.41 (N-CO-O), 175.10 (C=O), 175.20 (C=O), 183.11 (C=S).

Enzymatic synthesis of GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAcβ1-linker-NH$_2$-tBoc **15** The enzymatic synthesis of compound **15** was carried as described for **13** taking 4.5 mM of di-LacNAc-linker-NH$_2$-*t*Boc (**14**) and 5 mM UDP-GlcNAc. Compound **15** was obtained in quantitative yield (10 mg, 8.67 μmol).

MALDI-TOF of **15**; found: *m/z* 1175.7 for $M-Na^+$, calculated: *m/z* 1175.5 for $M-Na^+$;

^{1}H NMR (399.87 MHz, D_2O, 30°C): δ 1.192 (9H, s, (C**H$_3$**)$_3$C), 1.777 (3H, s, Ac), 1.796 (3H, s, Ac), 1.798 (3H, s, Ac), 3.029 (2H, m, H-2), 3.20* (1H, m, H-5^E), 3.22* (1H, m, H-4^E), 3.33* (1H, m, H-3^E), 3.347 (1H, dd, J=9.9, 7.8 Hz, H-2^D), 3.35* (1H, m, H-5^C), 3.360 (1H, dd, J=9.9, 7.8 Hz, H-2^B), 3.431 (2H, m, H-1), 3.44* (1H, m, H-5^A), 3.48* (3H, m, H-5^B, H-4^C, H-5^D), 3.487 (2H, dd, J=9.9, 3.2 Hz, H-3^B, H-3^D), 3.49* (1H, m, H-3^C), 3.50* (1H, m, H-4^A), 3.51* (5H, m, H-6^B, H-6^D, H-2^E), 3.53* (1H, m, H-6^{E}u), 3.56* (2H, m, H-3^A, H-2^C), 3.60* (2H, m, H-6^{A}u, H-6^{C}u), 3.66* (1H, m, H-6^{E}d), 3.68* (1H, m, H-2^A), 3.71* (2H, m, H-6^{A}d, H-6^{C}d), 3.913 (2H, m, H-4^B, H-4^D), 4.229 (1H, d, J=7.8 Hz, H-1^D), 4.237 (1H, d, J=7.8 Hz, H-1^B), 4.447 (1H, d, J=8.5 Hz, H-1^E), 4.468 (1H, d, J=8.5 Hz, H-1^C), 5.235, 5.341 (1H, m, H-1^A).

^{13}C NMR (100.55 MHz, D_2O, 30°C): δ 21.88, 22.03 (3×Ac), 27.53 ((**C**H$_3$)$_3$C), 39.08 (C-2), 44.04 (C-1), 53.84 (C-2^A), 55.01 (C-2^C), 55.52 (C-2^E), 59.76 (C-6^A, C-6^C), 60.35 (C-6^E), 60.79 (C-6^B, C-6^D), 68.17 (C-4^B, C-4^D), 69.56 (C-4^E), 69.85 (C-2^B, C-2^D), 72.02 (C-3^C), 72.47 (C-3^A), 73.43 (C-3^E), 74.40 (C-5^C), 74.73 (C-5^B, C-5^D), 75.51 (C-5^E), 75.89 (C-5^A), 77.91 (C-4^A), 78.12 (C-4^C), 80.91 ((CH$_3$)$_3$**C**), 81.83 (C-3^B, C-3^D), 82.44 (C-1^A), 102.55 (C-1^C), 102.66 (C-1^E), 102.74 (C-1^B, C-1^D), 158.04 (N–CO–O), 174.73, 174.78, 174.83 (3×C=O).

Enzymatic synthesis of mixed poly-LacNAc structures A mixture of poly-LacNAc structures was synthesized in a one-pot reaction by mixing 2 mM of acceptor substrate **12**, 10 mM UDP-Gal, and 10 mM UDP-GlcNAc in 50 mM HEPES–NaOH buffer, pH 7.6, containing 2 mM $MnCl_2$, 2 mM $MgCl_2$, 2 U alkaline phosphatase, 10 mU His$_6$-Propeptide-catβ4GalT-1, and 10 mU β3GlcNAcT (JHP1032). The reaction mixture was incubated over night at 30°C and analyzed by HPLC. To determine the degree of poly-LacNAc polymerisation deglycosylation assays were

performed as follows: 30 µl the purified poly-LacNAc mixture (2 mM) were mixed with 500 mU β-*N*-acetylglucosaminidase from Jack bean (Sigma). After overnight incubation at 30°C the mixture was analyzed by HPLC (LiChrospher® 100 RP18, LiChroCart, Merck, gradient with 11–50% *v/v* acetonitrile, 40 min, 0.5 ml/min at room temperature). The resulting poly-LacNAc structures deglycosylated by one sugar were analyzed by HPLC–ESI-MS (LiChrospher® 100 RP18, LiChroCart, Merck, 15% acetonitrile, 45 min, 0.5 ml/min, RT with coupled Thermo Finnigan ESI-MS, negative mode, 400°C, 50–200 V cone voltage) according to their molecular masses and corresponding retention times.

*Enzymatic synthesis of Gal(α1-3)Gal(β1-4)GlcNAc(β1-linker-NH_2-tBoc (***16***)* LacNAc-linker-*t*Boc (**12**) was further modified with the recombinant α3GalT as described previously [64]. The trisaccharide **16** was obtained in quantitative yield (15 mg, 20.1 µmol). After isolation with Sep-Pak® Vac C18 **16** was characterized by HPLC–ESI-MS as described above.

ESI-MS of 16; found: *m/z* 745.2 for $M-H^-$; calculated *m/z* 745 for $M-H^-$.

*Enzymatic synthesis of Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-linker-NH_2-tBoc (***17***) and Neu5Ac(α2-3)Gal(β1-4)GlcNAc (β1-3)Gal(β1-4)GlcNAc(β1-linker-NH_2-tBoc (***18***)* The synthesis of α2–3-sialylated structures performed in 50 mM ammonium acetate buffer, pH 7.5, by mixing 5 mM of **12** or **14**, respectively, with 5.5 mM CMP-Neu5Ac, 10 mM $MgCl_2$, and 50 mU CST-I and incubation over night at 30° C. After isolation with Sep-Pak® Vac C18 the products **17** (3 mg, 3.42 µmol, 95% yield) and **18** (4 mg, 3.22 µmol, 95% yield) were characterized by MALDI-TOF (Elite-STR MALDI-TOF instrument, Perkin-Elmer Biosystems, Fragmingham, MA). Approximately 2 µg of each oligosaccharide was mixed with a matrix containing a saturated solution of dihydroxybenzoic acid. Negative mass spectra were acquired using the reflector mode.

MALDI-TOF of **17**, found: *m/z* 874 for $M-H^-$; calculated: *m/z* 875 for $M-H^-$.

MALDI-TOF of **18**, found: *m/z* 1239 for $M-H^-$; calculated: *m/z* 1240 for $M-H^-$.

Kinetic studies were done with a discontinuous assay as previously described [62].

Deprotection and purification of the tBoc-protected oligosaccharides To remove the *t*Boc protection group 1 ml of 10 mM sugar solution was mixed with 1 ml of 2 M HCl and incubated over night at 4°C. The deprotection step was controlled by TLC (silica gel 60, mobile phase acetonitrile/0.1 M ammonia chloride 75:25). The de-protected sugar was isolated by elution with distilled H_2O from MTO-Dowex® M43 anion exchange resins (SUPELCO, Bellefont, PA, USA), subsequently lyophilized. Analysis of tBoc-protected saccharides, *e.g.* compound **7**, **12**, and **13**, and their corresponding deprotected saccharides, by HPLC/ESI-MS and CE revealed 80–99% yield (depending on CE or HPLC analysis) for the deprotection step and no detectable hydrolysis of the saccharides (see supporting information).

Characterization of immobilized poly-LacNAc structures on microtiter plates The amino-functionalized sugar structures were dissolved in 100 mM carbonate buffer, pH 9.6, and coupled to Nunc Immobilizer™ amino-reactive microtiter plates (Nunc GmbH, Wiesbaden, Germany) over night (100 µl/well). The next day the plates were blocked with 10 mM ethanolamine in the same buffer for 1 h at room temperature (300 µl/well). The microtiter plates with immobilized glycan structures were washed with PBS and used for further lectin binding experiments.

Lectins were selected for the detection of specific glycan structures: lectin II from *Griffonia simplicifolia* for terminal GlcNAc, lectin from *Erythrina crystagalli* for LacNAc, and galectin His_6CGL2 for poly-LacNAc [34]. The different commercial biotin-labeled lectin solutions (20 µg/ml in PBS with 1 mM $CaCl_2$, $MnCl_2$, and $MgCl_2$,respectively, Vector Laboratories Inc., Burlingame, USA) and the galectin His_6CGL2 (100 µg/ml in PBS) were added to the immobilized glycans and incubated for 2 h at room temperature (100 µl/well). For inhibition experiments soluble sugar was added to the incubation mixture in concentration between 1–1000 mM). After a washing step lectin binding was detected by incubation with streptavidin–peroxidase (1:1,000 dilution of 1 mg/ml, Roche Diagnostics GmbH, Mannheim, Germany). Bound His_6CGL2 was detected by an anti-His_6-peroxidase antibody conjugate (1 mg/ml, 100 µl/well of a 1:1,000 dilution in PBS, Roche Diagnostics GmbH, Mannheim, Germany).

The colorimetric detection was done with OPD substrate from DakoCytomation (Glostrup, Denmark) and stopped with 3 M HCl. The microtiter plates were measured at $OD_{490\ nm}$. The binding data were analyzed with Sigma Plot10 (SPSS GmbH Software, München, Germany).

Labeling of immobilized LacNAc by UDP-Gal-biotin The targeting of terminal galactose with an enzymatic transfer of UDP-Gal-biotin [44] by the recombinant α3GalT was done starting from a LacNAc functionalized amino reactive microtiter plate. After blocking with ethanolamine the plate was incubated with 0.8 mM UDP-Gal-biotin, 5.5 mU α3GalT, 1 mM $MgCl_2$ over night at 37°C. The analysis of the reaction was done by an ELISA with recombinant streptavidin–peroxidase and OPD substrate analogues to the tests described above.

Artificial ECM in a functionalized microtiter plate Amino-reactive microtiter plates were functionalized with 0.2 mM amino-terminating poly-LacNAc structures dissolved in 100 mM carbonate buffer over night (100 μl/well). The next day the plates were blocked with 10 mM ethanolamine in 100 mM carbonate buffer pH 9.6 for 1 h at room temperature (300 μl/well). The microtiter plates with immobilized glycan structures were washed with PBS and used for further galectin and ECM-protein binding: analogue the method described above. The model galectin His_6CGL2 (5 μg/ml in PBS) was added to the immobilized glycans and incubated for 1 h at room temperature (100 μl/well). After another washing step laminin (from mouse EHS tumor cell line, Sigma) or fibronectin (from plasma, Sigma) were added at a final concentration of 1 μg/ml protein to the assays (100 μl/well). Some wells were incubated with soluble glycans for inhibition studies. Detection of the built-up ECM could be done by a specific antibody against the ECM-protein (produced in rabbit) and a second antibody specific for rabbit IgG coupled with a peroxidase (both antibodies from Sigma). The colorimetric detection was done with OPD substrate from DakoCytomation (Glostrup, Denmark) and stopped with 3 M HCl. The assay was analyzed by measuring the $OD_{490\ nm}$. The binding data were calculated with Sigma Plot10 (SPSS GmbH Software, München, Germany).

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