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High-affinity HPMA copolymers with tailored *N*-acetyllactosamine presentation discriminate between galectins

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ABSTRACT

N-Acetyllactosamine (LacNAc; Galβ4GlcNAc) is a typical disaccharide ligand of galectins. The most abundant members of these human lectins, galectin-1 (Gal-1) and galectin-3 (Gal-3), participate in a number of pathologies including cancerogenesis and metastatic formation. In this study we synthesized a series of fifteen *N*-(2-hydroxypropyl) methacrylamide (HPMA) based glycopolymers with varying LacNAc amount and presentation, and evaluated the impact of their architecture on the binding affinity to Gal-1 and Gal-3. The controlled radical RAFT (reversible addition-fragmentation chain transfer) copolymerization technique afforded linear polymer precursors with comparable molecular weights (M_n ≈ 22,000 g mol^{-1}) and a narrow dispersity ($Đ$ ≈ 1.1). The precursors were conjugated with the functionalized LacNAc disaccharide (4-22 mol. % content in glycopolymer), prepared by enzymatic synthesis under the catalysis by β-galactosidase from *Bacillus circulans*. The structure-affinity relationship study based on ELISA-type assay revealed that the type of LacNAc presentation, individual or clustered on bi- or trivalent linkers, brings a clear discrimination (almost 300-fold) between Gal-1 over Gal-3, reaching avidity to Gal-1 in

nanomolar range. While Gal-1 strongly preferred a dense presentation of individually distributed LacNAc epitopes, Gal-3 preferred clustered LacNAc presentation. Such a strong galectin preference based just on the structure of a multivalent glycopolymer type is exceptional. The prepared non-toxic, non-immunogenic and biocompatible glycopolymers are prospective for therapeutic applications requiring selectivity for one particular galectin.

KEYWORDS

HPMA copolymer; galectin; glycopolymer; *N*-acetyllactosamine; selectivity

INTRODUCTION

Current research devoted to cancer treatment has been facing challenges to overcome the limitations of conventional anti-cancer therapeutics including the lack of selectivity, toxicity, and the resistance of tumor cells. Therefore, the development of biocompatible nanocarrier systems selectively targeting tumor tissues triggers an increasing interest aiming to improve the efficacy and safety of anti-cancer therapy.[1,2] Moreover, the therapeutic potential of these systems may further be improved by attaching specific targeting moieties[2–4] such as antibodies,[5] oligopeptides,[6,7] or carbohydrates.[8]

Polymer carriers based on *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers have been receiving considerable attention for their ability to influence the pharmacokinetics of various bioactive compounds, prolong their circulation time *in vivo* and deliver them to their biological targets.[2,4,9] Their potential as carbohydrate carriers targeting lectins was shown previously.[10] Among other favorable properties, HPMA copolymers are biocompatible, non-immunogenic, non-toxic and water-soluble biomaterials. The powerful technique of the controlled radical RAFT (reversible addition-fragmentation chain transfer) polymerization enables the synthesis of well-defined polymers carrying suitable functions for attaching bioactive molecules.[2,4,9,11] These polymers have adjustable molar mass, narrow molar mass distribution and high end-group fidelity – important characteristics for the polymer conjugates designed for medical purposes.[11]

Galectins are glycan-binding soluble proteins overexpressed in many types of cancer both inside the cancer cells and extracellularly.[12] They are involved in tumor cell adhesion, metastasis,[13] tumor angiogenesis,[14] proliferation and growth of cancer cells[15] as well as in

their escape from the host immune system.[16] Alterations in the galectin expression in human cells have been linked to the development of cancer. Therefore, galectins are considered a promising target for the anti-cancer therapy and their presence in the patient blood can indicate the prognosis of the ongoing disease,[12,17–19] thus meeting basic requirements for a cancer biomarker.[12,18–20] There are twelve human galectins known.[21] However, most studies focus on the two most common and best known representatives – prototype-type galectin-1 (Gal-1)[22] and chimera-type galectin-3 (Gal-3).[23]

The carbohydrate binding domains of both Gal-1 and Gal-3 contain a complex diversified binding groove for carbohydrate ligands based on the β-galactoside moiety. Gal-3 naturally recognizes poly-LacNAc, i.e. $[\beta 3Gal\beta 4GlcNAc]_n$ structure, in contrast to Gal-1, which only binds the terminal disaccharide motif of LacNAc type. Due to the similar binding preferences of these two most abundant galectins, it is very difficult to prepare selective inhibitors acting on one of them only. In the case of chimera-type Gal-3, the multivalency effect evoked by interaction with multivalent disaccharide ligands probably occurs primarily via the statistical rebinding mechanism, and, therefore, it results in rather low avidities.[24] Avidity is a result of cumulative affinities of individual glycans on the joint carrier. In contrast, in lectins with several binding sites such as Gal-1, the multivalency effect[12,19,25] may often reach up to 10^6-fold as a result of the chelation mechanism.[12] However, the molecular fundaments of galectin function are not fully understood yet and novel information and questions still arise, suggesting a highly complex nature of these processes.[26]

Selective galectin targeting, based on a known molecular mechanism, helps to better intercept and decode the underlying biological processes in their complexity. Moreover,

robust selectivity, in combination with high binding affinity, enables to exercise efficient targeted galectin inhibition in the presence of less specific cell surface glycans and abundant serum glycoproteins, which is a prerequisite for a successful translation into future clinical practice. The variety of published multivalent systems for galectin inhibition, which are based on readily available natural ligands such as lactose (Galβ4Glc) or LacNAc (Galβ4GlcNAc), exhibit no considerable selectivity between galectins.[12] Selectivity can only be accomplished by appropriate choice of the glycan epitope. For example, TF antigen (Galβ3GalNAc),[12] LacdiNAc (GalNAcβ4GlcNAc) disaccharide[8,27–29] or the complex tetrasaccharide of GalNAcβ4GlcNAcβ3Galβ4GlcNAc[12,18,19,25] are reported to be selective for Gal-3 in contrast to Gal-1. A high selectivity has also been demonstrated in small-molecule inhibitors of galectins, especially those based on the aryl-substituted galactose scaffold.[30,31] These compounds, however, pose a considerable synthetic challenge, which is also reflected on the overall synthetic yields. Very recently, impact of glycan density on graft polymers on the selectivity between galectins has been shown by Zhou et al.[32] To our knowledge, no macromolecular ligands selective for Gal-1 have been reported up to date.

LacNAc is a common disaccharide ligand for both Gal-1 and Gal-3.[12] We present here a novel synthetic approach using LacNAc disaccharide that affords HPMA-based glycopolymers acting as selective high-affinity ligands of galectins. Their avidity to Gal-1 and Gal-3 was tuned not only by the amount of LacNAc epitope on the polymer carrier (further termed as “epitope density”) but also by the structure of the linker that presents LacNAc either individually or in bi- or trivalent clusters. As a result, the best

glycopolymers reached avidities to Gal-1 in nanomolar range. It is an outstanding example of a multivalent biocompatible carrier that can reach selectivity between these two galectins of over two orders of magnitude, particularly, using a disaccharide ligand as simple as LacNAc. The straightforward synthesis and properties of the present glycopolymers predestine them for biomedical applications, possibly leading from the bench to the bedside.

EXPERIMENTAL SECTION

Materials

The preparation of the β-galactosidase from *Bacillus circulans* was from Daiwa Kasei, Ltd. (Shiga, Japan). 2,2′-Azobisisobutyronitrile (AIBN), 2-cyanopropan-2-yl dithiobenzoate (CTA), 2-thiazoline-2-thiol, 4-(dimethylamino)pyridine (DMAP), β-alanine, CuBr, dimethyl sulfoxide (DMSO), methacryloyl chloride, methanol, methyl 3,5-dihydroxybenzoate (**8**), methyl-3,4,5-trihydroxybenzoate (**9**), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC.HCl), *N*-Boc-ethylenediamine, *N*,*N*-diisopropylethylamine (DIPEA), *N*,*N*-dimethylacetamide (DMA), *N*,*N*-dimethylformamide (DMF), and *t*-butanol were purchased from Sigma-Aldrich (Czech Republic). 1-Aminopropan-2-ol was from TCI Europe (Belgium) and propargyl bromide from Acros Organics (Czech Republic). Benzotriazole-1-yl-oxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and trifluoroacetic acid (TFA) were from Iris Biotech (Germany). The solvents for NMR were from the following producers: D_2O (99.96 atom % D) from VWR Chemicals (Leuven, Belgium), and CD_3OD (99.80 atom % D) from VWR Chemicals (Leuven, Belgium). LacNAc (**7**; Galβ4GlcNAc; β-D-

galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose) was from Carbosynth (UK). All other solvents and chemicals were of analytical grade.

Characterization Methods

Mass Spectrometry (MS)

Mass spectra of compounds **4-6** and **14-17** were acquired with an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, USA) using an electrospray ion source and injection through a 2 μL loop (**4-6**) or 5 μL loop (**14-17**). Methanol/water (4:1 v/v) was used as a mobile phase at a flow rate of 30 μL min^{-1} (**4-6**) or 100 μL min^{-1} (**14-17**). Internal calibration of the mass in the negative mode was performed with a lock mass of deprotonated palmitic acid. For the negative ion mode, spray voltage, capillary voltage, tube lens voltage, and capillary temperature were 5.0 kV, -25 V, -125 V, and 275 °C, respectively. For the positive ion mode, spray voltage, capillary voltage, tube lens voltage, and capillary temperature were 5.0 kV, 9 V, 150 V, and 275 °C, respectively. The Orbitrap mass spectra were recorded at the resolution of 100,000.

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra of carbohydrates and glycosylated branched linkers were measured by Bruker Avance III 600 MHz spectrometer (^{1}H: 600.23 MHz; ^{13}C: 150.93 MHz for compounds **4**, **5**, **16**, and **17**) or by Bruker AVANCE III 700 MHz spectrometer (^{1}H: 700.13 MHz; ^{13}C: 176.05 MHz for compound **6**) in D_2O (for compounds **4**, **5**, **6**, and **17**) and CD_3OD (for compound **16**) at 303 K (compounds **4**, **6**, **16**, and **17**) or 298 K

(compound **5**). ^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC, gHMBC, and 1D-TOCSY spectra were obtained using standard manufacturer's software.

^{1}H NMR spectra of polymer precursors and glycopolymers were acquired with Bruker Avance III 600 (or Bruker Avance DPX 300) spectrometer operating at 600.2 MHz (or 300.1 MHz). The NMR spectra of all samples were measured in 5 mm NMR tubes using $CDCl_3$, CD_3OD, DMSO-d_6 or D_2O. Typical conditions for measurements of the spectra were as follows: $\pi/2$ pulse width 18 μs (15.6 μs), relaxation delay 15 s (10 s), spectral width 12 kHz (6.6 kHz), acquisition time 2.73 s (4.95 s), 16-200 scans. The Bruker Avance DPX 300 MHz spectrometer was used for recording spectra of compounds **10** and **11** ($CDCl_3$) and compound **12** (CD_3OD). Bruker Avance III 600 MHz spectrometer was applied for measurements of compounds **13**-**15** and **20**-**24** (DMSO-d_6) as well as compounds **18**-**19** and **25**-**39** (D_2O).

High Performance Liquid Chromatography (HPLC)

The composition of transglycosylation reaction mixtures as well as the purity of prepared carbohydrates was analyzed by hydrophilic interaction liquid chromatography (HILIC) using Shimadzu Prominence LC analytical system. It was composed of the binary HPLC pump Shimadzu LC-20AD, cooling autosampler Shimadzu SIL-20ACHT, the column oven Shimadzu CTO-10AS column oven, the system controller Shimadzu CBM-20A, and the diode array detector Shimadzu SPD-M20A (Shimadzu, Japan). Samples (1 μL) dissolved in acetonitrile/water (3:1, v/v) were run through the HILIC column TSKgel Amide80 5 μm (250 × 4.6 mm, Tosoh Bioscience, Germany) at a flow rate of 1 mL min^{-1} at 27 °C. For elution, binary gradient was employed: 22% B for 0-7 min, 22-35% B for 7-

20 min; 35% B for 20-25 min; and 35-22% B for 25-26 min where A is 100% acetonitrile and B is water. The detection was performed at 200 nm. The purity of the monomers HPMA (**20**) and *N*-methacryloyl-β-alanine thiazolidine-2-thione (MA-AP-TT; **21**) for polymer synthesis and the course of reactions to obtain compounds **16-19** and **25-39** were monitored on a Shimadzu HPLC system equipped with a C18 reversed-phase Chromolith Performance RP-18e column (4.6 × 100 mm, Merck Millipore) and a diode array detector (Shimadzu SPD-M20A), in a mobile phase of water/acetonitrile/0.1% TFA with a gradient of 5-95% v/v acetonitrile at a flow rate of 5 mL min^{-1}.

Size Exclusion Chromatography (SEC)

The number-average molecular weight (M_n), weight-average molecular weight (M_w) and dispersity ($Đ$) of polymer precursors **22-24** and glycopolymers **25-39** were determined by a Shimadzu HPLC system equipped with size exclusion chromatography (SEC) columns (TSKgel Super SW3000, 4.6 × 300 mm, 4 μm, for polymers **22-27**, **30-32** and **35-37**; Superose 6, 10 x 300 mm for glycopolymers **28**, **29**, **33**, **34**, **38** and **39**). Optilab-rEX differential refractometer index (RI) detector, multi-angle light scattering (MALS) detector (DAWN HELEOS II, Wyatt Technology Co., USA) and SPD-M20A photodiode array detector (Shimadzu, Japan) were used. methanol/0.3 M sodium acetate buffer, pH 6.5 (4/1, v/v) at a flow rate of 0.3 mL min^{-1} was used as a mobile phase for the TSKgel column, and 0.3 M sodium acetate buffer, pH 6.5 at a flow rate of 0.5 mL min^{-1} was used for the Superose column. The M_w and $Đ$ were calculated using ASTRA VI software. The Optilab®-rEX detector enabled direct determination of refractive increment (*dn/dc*) of the polymers, and the solvent refractive index provided 100 % recovery of the injected sample from the column.

Dynamic Light Scattering (DLS)

The hydrodynamic diameter (D_h) of copolymers and glycopolymers were measured by dissolving the sample in water (5 mg mL^{-1}) and filtering through a 0.45 μm PVDF filter. Nano-ZS instrument (ZEN3600, Malvern, UK) was used and the intensity of the scattered light was detected at angle $\theta = 173°$ using laser of a wavelength of 632.8 nm. The values were determined as a mean of at least three independent measurements.

UV–VIS Spectrophotometry

The content of thiazolidine-2-thione (TT) groups in the copolymer precursors were determined by UV–VIS spectrophotometry in methanol using Specord 205 ST, Analytic Jena AG, Germany. The molar absorption coefficient used was ε(TT) = 10,300 L mol^{-1} cm^{-1} (λ_{max} = 305 nm) estimated for 3,3′-disulfanediyl*bis*[1-(2-thioxothiazolidin-3-yl)propan-1-one].

Enzyme Activity Assay

The activity of the β-galactosidase of *Bacillus circulans* in the commercial preparation Biolacta BN5 (Daiwa Kasei, Ltd) was measured in an end-point assay with *p*-nitrophenyl β-D-galactopyranoside (1; *p*NP-Gal; Glycon Biochemicals, Germany) as a substrate at a starting concentration of 2 mM. The reaction mixture was incubated in 50 mM sodium acetate buffer pH 5 for 10 min at 35 °C and 850 rpm. Then, the reaction (50 μL) was stopped by adding 0.1 M Na_2CO_3 (1 mL) and the amount of released *p*-nitrophenol was determined spectrophotometrically at 420 nm. One unit of enzymatic activity corresponds to the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the above conditions. The commercial preparation contained a side activity of β-*N*-acetylhexosaminidase, which was

determined analogously using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as a substrate (*p*NP-GlcNAc; Sigma-Aldrich, USA). In reactions containing co-solvents, the respective amount of buffer was substituted by the respective co-solvent (acetone or acetonitrile) in 0-40% v/v, and the enzyme activity was determined as described above. All data were measured in triplicate.

Synthetic Procedures

2-Acetamido-2-deoxy-β-D-glucopyranosyl azide (2) and (*t*-butoxycarbonylamido)ethylthioureidyl 2-acetamido-2-deoxy-β-D-glucopyranoside (3)

Compound **2**[33] and compound **3**[34] were prepared from 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc; Acros Organics) using an optimized procedure described previously. The ^{1}H and ^{13}C NMR data corresponded to the structure.

Optimization of Enzymatic Synthesis of Disaccharides 4 and 5 at Analytical Scale

*p*NP-Gal donor (**1**; 30-50 mM) was combined with 2-acetamido-2-deoxy-β-D-glucopyranosyl azide acceptor (**2**; 150–250 mM) or with (*t*-butoxycarbonylamido)ethylthioureidyl 2-acetamido-2-deoxy-β-D-glucopyranoside acceptor (**3**; 150–250 mM). The mixtures were dissolved in 50 mM sodium acetate buffer (pH 5.0-7.5) with the addition of acetonitrile (0-40 % v/v). The β-galactosidase from *B. circulans* (0.03–0.16 mg mL^{-1}, 0.05–0.3 U mL^{-1}) was added and the reaction mixtures were shaken at 35 °C and 850 rpm for up to 36 h. Aliquots (10 µL) were regularly analyzed by TLC (propan-2-ol/H_2O/NH_4OH, 7/2/1, v/v/v) with detection under UV light and by carbonization, and by HPLC.

β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl azide (**4**)

Compound *p*NP-Gal (**1**; 50 mg, 0.17 mmol), acceptor **2** (185 mg, 0.75 mmol) and the β-galactosidase from *B. circulans* (0.4 U, 0.75 mg, 15 μL) were incubated in a mixture of acetonitrile (20 % v/v) in 50 mM sodium acetate buffer pH 5.0 (total reaction volume 5 mL) at 35 °C and 850 rpm under monitoring by HPLC and TLC. After 24 h, another portion of enzyme (0.04 U, 0.38 mg, 8 μL) was added and the reaction ran for additional 4 h. Then the reaction mixture was boiled for 2 min to inactivate the enzyme, cooled down to room temperature (r.t.), and centrifuged (13,500 rpm; 10 min). For product isolation, it was loaded onto a Biogel P-2 column (26 × 1000 mm, Bio-Rad, USA) with water mobile phase at 7 mL h^{-1}. Pure acceptor **2** was partially recovered (115 mg). The fractions containing the product were combined and lyophilized; the title compound **4** was obtained as a white solid (29 mg, 0.07 mmol, 56 % isolated yield). HRMS (ESI^-): found *m*/*z* 407.14148 (expected 407.14197 for $[M-H]^-$, $C_{14}H_{22}O_{10}N_4$-), Fig. S5 in the ESI. For the respective NMR data, see the ESI: Table S1 and Figures S1a and S1b.

(*t*-Butoxycarbonylamido)ethylthioureidyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (5) and (2-aminoethyl)thioureidyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (6)

Compound *p*NP-Gal (**1**; 50 mg, 0.17 mmol), acceptor **3** (318 mg, 0.75 mmol) and the β-galactosidase from *B. circulans* (0.3 U, 0.56 mg, 11 μL) were incubated in 50 mM sodium acetate buffer pH 5.0 (total reaction volume 5 mL) at 35 °C and 850 rpm under monitoring by HPLC and TLC. After 5 h, the reaction was boiled for 2 min to inactivate the enzyme, cooled down to r.t., and centrifuged (13,500 rpm; 10 min). For product isolation, it was loaded onto a Biogel P-2 column with water mobile phase at 7 mL h^{-1}. Pure acceptor **3** was partially recovered

(195 mg). The fractions containing the product were combined and lyophilized; the title compound **5** was obtained as a white solid (31 mg, 0.05 mmol, 31 % isolated yield). HRMS (ESI^-): found *m*/*z* 583.22814 (expected 583.22907 for $[M-H]^-$, $C_{22}H_{39}O_{12}N_4S^-$), Fig. S6 in the ESI. For the respective NMR data, see the ESI: Table 3 and Figures S3a and S3b.

For conjugation to the polymer carrier, the *t*-butoxycarbonyl (*t*-Boc) protecting group was deprotected by dissolving disaccharide **5** (10 mM) in 1M HCl and incubating under stirring at 4 °C for 48 h. Then, neutralization by Amberlite anex resin (OH^- cycle) was performed and the deprotected compound **6** (16 mg, 0.03 mmol, 64 % yield) was lyophilized. HRMS (ESI^-): found *m*/*z* 483.17664 (expected 483.17615 for $[M-H]^-$, $C_{17}H_{31}O_{10}N_4S^-$), Fig. S7 in the ESI. The deprotected amine **6** (see the ESI: Table 4, Figs. S4a and S4b) should be readily used for conjugation as it tends to decompose under long-term storage.

Synthesis of Branched Linkers 14 and 15

The branched linkers containing either two triple bonds (**14**) or three triple bonds (**15**) were prepared in three steps. In the first step, compounds **10** (470 mg, 65 %) and **11** (181 mg, 56 %) containing alkyne were prepared from methyl 3,5-dihydroxybenzoate (**8**) or methyl-3,4,5-trihydroxybenzoate (**9**), respectively, using propargyl bromide and K_2CO_3 in acetone, as previously described.[35] Then, intermediates **10** and **11** reacted with KOH in ethanol/water according to Deng et al.,[36] yielding intermediates **12** (184 mg, 83 %) and **13** (113 mg, 65 %), respectively. The title branched linkers containing *t*-Boc group protecting primary amino group, namely compounds **14** (198 mg, 67 %) and **15** (82 mg, 64%) were obtained by amide formation of compounds **12** and **13**, respectively, using *N*-

Boc-ethylenediamine *via* a modified procedure previously described.[37] The detailed synthetic procedures, NMR and MS data are shown in the ESI, Section 3.1.

Synthesis of Glycosylated Branched Linkers 18 and 19

Glycosylated branched linkers **18** and **19** were synthesized in two steps. First, branched linkers **14** or **15** reacted with disaccharide **4** by Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) in DMF overnight, affording compounds **16** and **17**, respectively. Then, the *t*-Boc protecting group was removed under acidic conditions in CH_2Cl_2 for 2 h, yielding primary amines (**18** and **19**) to be coupled to polymer precursors.

The detailed synthetic procedure for compound **18** is as follows: Compounds **14** (15 mg, 40 μmol) and **4** (37.8 mg, 93 μmol) were dissolved in 1 mL of dry DMF and the mixture was bubbled with argon for 30 min. Then, CuBr (3.5 mg, 24 μmol; powder form) was added, and the reaction mixture was kept under argon for 1.5 h. Then, after changing the septum, the reaction was carried out under argon atmosphere and stirring at r.t. for 20 h. DMF was removed under vacuum by repeated co-distillation with methanol and water and purification was performed using gel permeation chromatography on Biogel P-2 column with water as a mobile phase (7 mL h^{-1}), yielding compound **16** (40 mg, 83%). For NMR data, see Table S5 and Figs. S11a and S11b in the ESI, Section 3.2. For MS data, see Fig. S11c in the ESI, Section 3.2. For *t*-Boc deprotection, compound **16** (40 mg, 33.6 μmol) was mixed with CH_2Cl_2 (4 mL), then TFA (342 μL, 4.5 mmol) was added dropwise in three steps (1.5 mmol in each step) with 30 min intervals under cooling and stirring. The reaction was monitored by HPLC and TLC (propan-2-ol/H_2O/NH_4OH, 7/2/1, detection by carbonization using 5% v/v H_2SO_4 in ethanol). After shaking at r.t. for

2 h, CH_2Cl_2 was evaporated under vacuum. TFA was removed by repeated co-distillation with methanol and water under vacuum until neutral pH was reached. Evaporation under vacuum yielded the title compound **18** (34 mg, 85%). ^{1}H NMR (600.2 MHz, D_2O): $\delta \approx$ 8.28 ppm (s, 2H, C*H* from triazol of both meta positions), $\delta \approx$ 7.04 ppm (s, 2H, C*H* from *ortho* positions of aromatic portion), $\delta \approx$ 6.84 ppm (s, 1H, C*H* *para* position of aromatic portion), $\delta \approx$ 5.85 ppm (d, 2H, C*H* from C-1 of GlcNAc units), $\delta \approx$ 5.26 ppm (s, 4H, CH_2 next to triazol of *meta* positions), $\delta \approx$ 4.52 ppm (d, 2H, C*H* from C-1 of Gal units), $\delta \approx$ 4.30 ppm (t, 2H, C*H* of C-2 of GlcNAc units), $\delta \approx$ 3.93 ppm (m, 12H, C*H* of C-3 and C-4 of GlcNAc units, CH_2 of C-6 of GlcNAc units, C*H* of C-4 of Gal units and 1H, CH_2 of C-6 of Gal units), $\delta \approx$ 3.75 ppm (m, 6H, C*H* of C-5 of GlcNAc units, C*H* of C-5 of Gal units and 1H, CH_2 of C-6 of Gal units), $\delta \approx$ 3.68 ppm (m, 4H, C*H* of C-2 of Gal units and 2H, CH_2–NH), $\delta \approx$ 3.57 ppm (t, 2H, C*H* of C-3 of Gal units), $\delta \approx$ 3.25 ppm (t, 2H, CH_2–NH_2), $\delta \approx$ 1.67 ppm (s, 6H, CH_3CONH~ of GlcNAc units). See also Fig. S12 in the ESI, Section 3.2.

The synthetic procedure for compound **19** was analogous to **18**. The reaction of compounds **15** (11 mg, 25 μmol) and **4** (33 mg, 81 μmol), using CuBr catalysis (3.5 mg, 24 μmol; powder form) in dry DMF (1.2 mL) afforded compound **17** (29 mg, 66%). For NMR data, see Table S6 and Figs. S13a and S13b in the ESI, Section 3.2. For MS data, see Fig. S13c in the ESI, Section 3.2. For *t*-Boc deprotection, compound **17** (29 mg, 17.5 μmol) reacted with TFA (170 μL, 2.2 mmol) in CH_2Cl_2 (4 mL), yielding the title compound **19** (28 mg, 98%). ^{1}H NMR (600.2 MHz, D_2O): $\delta \approx$ 8.26 ppm (s, 2H, C*H* from triazol of both *meta* positions), $\delta \approx$ 8.14 ppm (s, 1H, C*H* from triazol of *para* position), $\delta \approx$ 7.20 ppm (s, 2H, C*H* from *ortho* positions of aromatic portion), $\delta \approx$ 5.86 ppm (d, 2H,

C*H* from C-1 of GlcNAc units from *meta* positions), $\delta \approx 5.80$ ppm (d, 1H, C*H* from C-1 of GlcNAc moiety from *para* position), $\delta \approx 5.25$ ppm (br, 4H, CH_2 next to triazol of *meta* positions), $\delta \approx 5.16$ ppm (br, 2H, CH_2 next to triazol of *para* position), $\delta \approx 4.53$ ppm (d, 3H, C*H* from -1 of Gal units), $\delta \approx 4.30$ ppm (m, 3H, C*H* from C-2 of GlcNAc units), $\delta \approx$ 3.93 ppm (m, 18H, C*H* from C-3 and C-4 of GlcNAc units, CH_2 from C-6 of GlcNAc units, C*H* of C-4 of Gal units and 1H, CH_2 of C-6 of Gal units), $\delta \approx 3.76$ ppm (m, 9H, C*H* of C-5 of GlcNAc units, C*H* of C-5 of Gal units and 1H, CH_2 of C-6 of Gal units), $\delta \approx$ 3.68 ppm (m, 5H, C*H* of C-2 of Gal units and 2H, CH_2–NH), $\delta \approx 3.57$ ppm (t, 3H, C*H* of C-3 of Gal units), $\delta \approx 3.25$ ppm (t, 2H, CH_2–NH_2), $\delta \approx 1.75$ ppm (s, 3H, CH_3CONH~ of GlcNAc unit of *para* position), $\delta \approx 1.65$ ppm (s, 6H, CH_3CONH~ of GlcNAc units of *meta* positions). See also Fig. S14 in the ESI, Section 3.2.

Synthesis of Copolymer Precursors 22-24

The monomers HPMA (**20**) and MA-AP-TT (**21**) were synthesized as described previously.[38,39,40] The poly(HPMA-*co*-MA-AP-TT) polymer precursors **22-24** were prepared by RAFT polymerization using CTA and the initiator AIBN, followed by dithiobenzoate end group removal as shown before.[41] The reaction conditions were adopted from our previous work[40] and the molar ratio of monomer/CTA/AIBN was 350/2/1. The molar ratio of monomers HPMA/MA-AP-TT varied as follows: 90/10 (copolymer **22**; 9 mol. % of TT groups), 80/20 (copolymer **23**; 17 mol. % of TT groups) or 75/25 (copolymer **24**; 23 mol. % of TT groups). The detailed synthetic procedures for copolymers **22-24** are described in the ESI, Section 4.1.

Synthesis of Glycopolymers with Individual LacNAc Presentation (25-29)

The glycopolymers **25-29** with individual presentation of LacNAc were prepared by aminolysis of TT groups of polymer precursors **22-24** with the corresponding amount of disaccharide **6**. Glycopolymers **25** and **26** were prepared from polymer precursor **22**, glycopolymers **27** and **28** from precursor **23** and glycopolymer **29** from precursor **24**. A representative synthetic procedure for glycopolymer **28** is as follows. Disaccharide **6** (12 mg, 22 μmol) was dissolved in a mixture of dry methanol and dry DMA (1/3; 800 μL) and added dropwise to the solution of copolymer precursor **23** (20 mg; 27 μmol of TT groups) in dry methanol (200 μL). After stirring for 30 min, DIPEA was added (4.9 μL, 28 μmol) and the reaction was carried out under stirring for 20 h at 24 °C. Then, 1-aminopropan-2-ol (2 μl, 27 μmol) was added, and the reaction was left stirring for 30 min to remove the unreacted TT groups. Crude glycopolymer **28** was diluted with methanol (1.2 mL), and purified on Sephadex LH-20 column in methanol mobile phase under UV detection. The combined polymer fractions were evaporated under vacuum, dissolved in distilled water and freeze-dried (26 mg, 88 %). NMR data for glycopolymer **28** are shown in the ESI, Section 4.2., Fig. S15. For the calculation of LacNAc content by NMR analysis, the integral intensity of signal at δ = 3.92 ppm (1H, C*H*–OH) or δ = 3.00-3.28 ppm (2H, CH_2–NH) of the HPMA unit, and of signal at $\delta \approx$ 4.48 ppm (1H, C*H* of C-1 of Gal) of LacNAc were used. Molecular weights and dispersities of glycopolymers **25-29** are shown in Table 1.

Synthesis of Glycopolymers with Clustered LacNAc Presentation: Bivalent (30-34) and Trivalent (35-39) Branching

The glycopolymers **30-34** carrying bivalent branching and the glycopolymers **35-39** carrying trivalent branching were prepared by aminolysis of the TT groups of the polymer precursor **23** with the primary amino group of compounds **18** and **19**, respectively. A representative synthetic procedure for glycopolymer **36** with trivalent branching is as follows. Copolymer **23** (24 mg; 25 μmol of TT groups) was dissolved in dry DMA (200 μL) and compound **19** (6.0 mg, 3.6 μmol) dissolved in dry DMA/methanol (3/1; 1 mL) was added. After stirring for 1 h, DIPEA (0.9 μL, 5 μmol) was added, and the reaction was carried out at 24 °C for 20 h. To remove the unreacted TT groups, of 1-aminopropan-2-ol (1.8 μL, 24 μmol) was added and the reaction mixture was stirred for 30 min. Crude glycopolymer **36** was purified on Sephadex LH-20 column with methanol mobile phase under UV detection. The collected polymer fraction was evaporated under vacuum, dissolved in water and freeze-dried (25 mg, 86 %). NMR data for glycopolymers **31** and **38** are shown in the ESI, Section 4.2., Figs. S16 and S17, respectively. For the calculation of disaccharide content by NMR analysis, the integral intensity of signal at δ = 3.00-3.28 ppm (2H, CH_2–NH) of the HPMA unit were used. The LacNAc content in glycopolymers **30-34** was calculated using signals at $\delta \approx$ 8.31 ppm (2H, C*H* of both triazol moieties), $\delta \approx$ 5.86 ppm (2H, C*H* of C-1 of GlcNAc), $\delta \approx$ 4.51 ppm (2H, C*H* of C-1 of Gal), $\delta \approx$ 4.30 ppm (2H, C*H* of C-2 of GlcNAc). The LacNAc content in glycopolymers **35-39** was calculated using signals at $\delta \approx$ 8.30 ppm (2H, C*H* of triazol of both *meta* positions), $\delta \approx$ 8.14 ppm (1H, C*H* of triazol of *para* position), $\delta \approx$ 7.20 ppm (2H, C*H* of *ortho* position of aromatic portion), $\delta \approx$ 5.90 ppm (2H, C*H* of C-1 of GlcNAc in *meta* positions), $\delta \approx$ 5.81 ppm (1H, C*H* of C-1 of GlcNAc in *para* position), $\delta \approx$ 4.32 ppm (3H, C*H* of C-2 of

GlcNAc). Molecular weights and dispersities of glycopolymers **30-39** are shown in Table 1.

Protein Production

Recombinant Human His_6-tagged Galectin-1 and Galectin-3

Recombinant His-tagged constructs of Gal-1 and Gal-3 were produced and purified as described previously.[8] Gal-1 construct contained Cys2Ser mutation for increasing its stability.[42,43] Briefly, both galectins were recombinantly expressed in the cells of *E. coli* Rosetta 2 (DE3) pLysS. The cultivation of pre-cultures (60 mL in 0.5 L baffled flasks) was done overnight in Lysogeny broth (LB) medium (0.5 w/v yeast extract, 1.0 v/w tryptone, 0.5% w/v NaCl pH 7.4) at 220 rpm and 37 °C. The medium contained ampicillin (100 mg L^{-1}) and chloramphenicol (34 mg L^{-1}). After 17 h, main cultures (600 mL in 3L baffled flasks) in Terrific broth (TB) medium (2.4 w/v yeast extract, 1.2% w/v tryptone, 0.4% v/v glycerin, 17 mM KH_2PO_4, 72 mM K_2HPO_4 pH 7.0) containing antibiotics were inoculated with pre-cultures and incubated at 37 °C and 150 rpm until they reached optical density (OD_{600}) of 0.6–0.8. Then, the expression of galectins was induced by isopropyl 1-thio-β-D-galactopyranoside (0.5 mM). After 24 h, the cells were centrifuged (5,000 rpm, 20 min, 4 °C) and frozen at -20 °C. For purification of galectins, *E. coli* cells were suspended in ice-cold equilibration buffer (20 mM Na_2HPO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4) and sonicated on ice (six 30 s cycles, 52% amplitude). After removing cell debris (centrifugation at 13,400 rpm, 15 min, 10 °C), the supernatant was filtered through 0.8 μm syringe filter and loaded on HisTrap™ HP 5 mL column (GE Healthcare) according to manufacturer`s instructions in equilibration buffer. Galectins

were eluted by elution buffer (20 mM Na_2HPO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4). The combined fractions containing galectin were dialyzed in SnakeSkin™ Dialysis Tubing (10 kDa MWCO, ThermoFisher Scientific) overnight against 50 mM NaH_2PO_4/150 mM NaCl/2 mM ethylenediaminetetraacetic acid pH 7.5 (EPBS buffer), and for additional 4 h against 50 mM NaH_2PO_4/150 mM NaCl pH 7.5 (PBS buffer). A usual yield was ca 7-10 g of cells per 1 L medium and 5 mg of pure Gal-3 and 15 mg of pure Gal-1 per 1 g cells. After sterile filtration, galectins (100-200 μM solutions) were stable for 1 - 2 months; due to lower stability, galectin-1 solution contained glycerol (20 % v/v). The galectin samples contained ca 9000-10,000 E.U. (i.e. 900-1000 ng) lipopolysaccharide (LPS) per mg protein as determined by chromogenic Endpoint Chromogenic Limulus Amebocyte Lyzate (LAL) Kit (Charles River Endosafe, Charleston, USA). These LPS concentrations were confirmed to have no influence on the outcome of ELISA-type measurements. Alternatively, LPS-free purification of galectins was performed by modifying the standard procedure: an additional step of washing the loaded sample with 0.5% v/v Triton X-100 in equilibration buffer (10 column volumes), followed by extensive washing with equilibration buffer (20 column volumes) was included, reducing LPS content ca 20-times. This optional step had no impact on galectin viability or biological activity.

Competitive ELISA Assay with Galectin-1 and Galectin-3

The ability of glycopolymers **25-39** to inhibit binding of soluble human Gal-1 or Gal-3 to immobilized standard ligand asialofetuin (ASF, Sigma Aldrich, Steinheim, Germany) was determined in a competitive ELISA assay. For reference, binding affinities of both

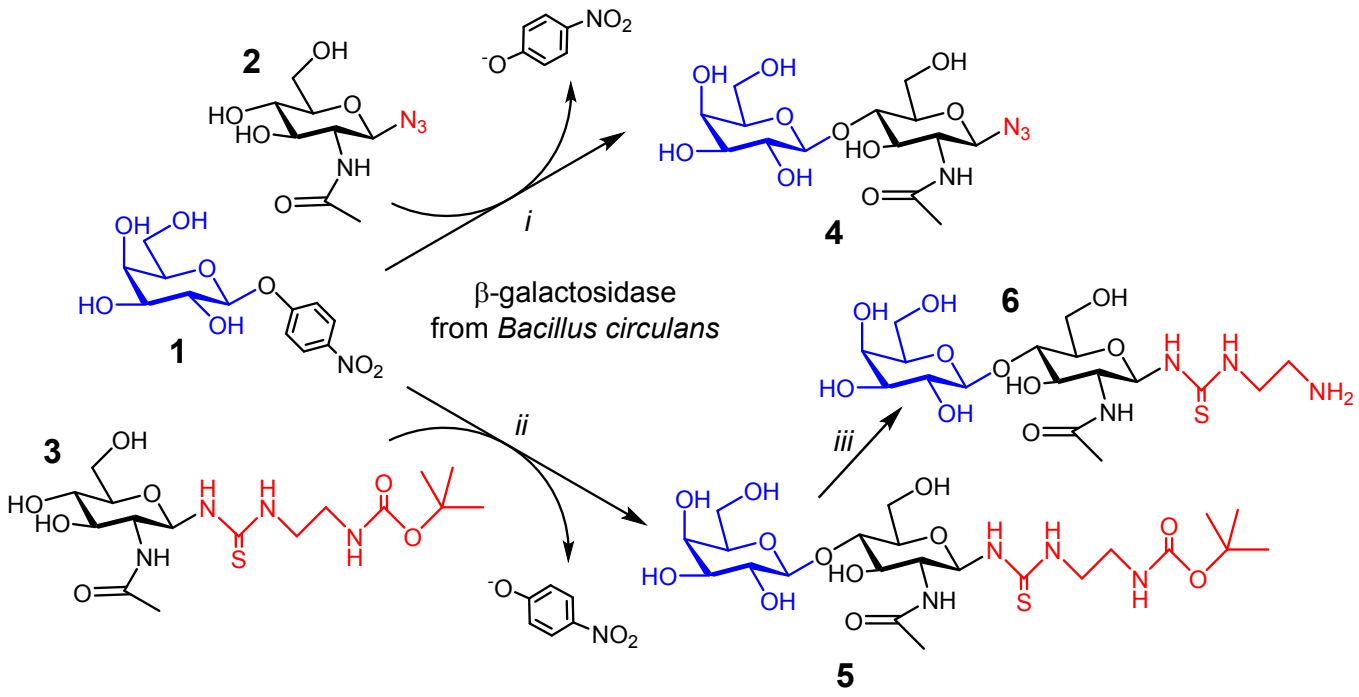
galectins to immobilized ASF ($K_{d,app}$) were determined in a direct ELISA assay,[19,44] and were in correlation with previously published results[44] acquired by the same method: $K_{d,app}$ = (2.6 ± 0.6) μM for Gal-1 vs. (3.7 ± 0.5) μM for Gal-3. The F16 Maxisorp NUNC-Immuno Modules (Thermo Scientific, Roskilde, Denmark) were coated with ASF (0.1 μM, 50 μL/well) in PBS buffer (50 mM NaH_2PO_4/150 mM NaCl pH 7.5) overnight. Then, blocking with BSA (2% w/v) in PBS followed (1 h, r.t.). Afterwards, respective glycopolymers in various concentrations and Gal-1 or Gal-3 (total volume 50 μL; 4.5 μM final galectin concentration) in EPBS buffer were added, and incubated for 2 h. After each step, the wells were extensively rinsed with Tween 20 (0.05% v/v) in PBS. Bound galectins were detected using anti-His_6-IgG1 mouse antibody conjugated to horseradish peroxidase (Roche Diagnostics, Mannheim, Germany) in PBS (1/500 for Gal-1 and 1/1000 for Gal-3, 1 h, r.t., 50 μL/well). The IgG-conjugated peroxidase was quantified by TMB One substrate (Kem-En-Tec, Taastrup, Denmark). The reaction was stopped by adding 3M HCl (50 μL) for colorimetric detection at 450 nm. LacNAc (**7**) was used as a positive control.

RESULTS AND DISCUSSION

Chemoenzymatic Synthesis of Functionalized Disaccharides 4 and 5

In order to compare two different presentations of LacNAc (**7**; Galβ4GlcNAc; for structure see Scheme 2) epitope on the HPMA copolymer backbone, we prepared LacNAc disaccharide with two different functionalities by enzymatic synthesis. Glycosidases are robust glycosylation tools tolerating a variety of modifications in the substrate molecule,[45,46] although they are unable to catalyze multifold glycosylation of a

multivalent acceptor.[47] The amino function in disaccharide **6** was used, after *t*-Boc deprotection, for direct coupling to HPMA polymer precursors **22**-**24** by aminolysis whereas the azido function in disaccharide **4** enabled nested presentation on a bi- or trivalent aromatic linkers (**14** and **15**, respectively) using CuAAC. Both functionalized disaccharides **4** and **6** were prepared from respective monosaccharide precursors **3** and **2** by one-step synthesis in aqueous buffer using a commercial preparation Biolacta® with β-galactosidase from *Bacillus circulans*. This enzyme is well known as a robust and potent galactosylation tool used especially for industrial preparation of long-chain galactooligosaccharides.[48,49] In this case, our task was different since we aimed at a regioselective and high-yielding mono-galactosylation of two different functionalized acceptors. Both acceptors **2** and **3** were prepared as described previously.[33,34] The *t*-Boc protected compound **3** proved to be an attractive acceptor for β-galactosylation, yielding the desired disaccharide **5** in a yield of 31 %. After *t*-Boc deprotection, the resulting compound **6** was readily coupled to respective polymer precursors.



Scheme 1. Enzymatic synthesis of functionalized LacNAc epitope. *i*, 20% CH_3CN in 50 mM sodium acetate buffer pH 5.0, 28 h, 35 °C; *ii*, 50 mM sodium acetate buffer pH 5.0, 5 h, 35 °C; *iii*, 1M HCl, 4 °C, 48 h.

The synthesis of compound **4** was much more challenging. Besides β-galactosidase, the Biolacta preparation also contains contaminating β-*N*-acetylhexosaminidase. The latter activity is known to cleave acceptor **2**,[33] which considerably lowered product yields. Moreover, depending on its set-up, the transgalactosylation was not fully regioselective since the Galβ6GlcNAc azide regioisomer was sometimes formed (see the ESI, Table S2 and Figs. S2a and S2b). Therefore, we applied extensive reaction engineering in order to increase transgalactosylation yields and render the reaction fully regioselective. The dependence of β-galactosidase and β-*N*-acetylhexosaminidase activities on reaction pH (see the ESI, Fig. S8A) indicated pH ≤ 5 or ≥ 7 as the most selective for β-galactosidase activity. Analysis of transgalactosylation reactions at various pH revealed that the reaction regioselectivity strongly depends on pH; whereas at slightly basic pH a mixture

of β4 (compound **4**) and β6 regioisomers were formed in a ratio of ca 3/1, the reaction at pH 5 proved to be fully regioselective yielding exclusively compound **4**. Since at pH 5 the residual β-*N*-acetylhexosaminidase activity still lowered the synthetic yield, we screened various co-solvents for its further suppression (ESI, Fig. S8B, C). Whereas increasing concentrations of acetone (0-40% v/v) suppressed both activities rather equally, acetonitrile discriminated β-*N*-acetylhexosaminidase faster than β-galactosidase. Thus, in 20 % v/v acetonitrile, residual β-*N*-acetylhexosaminidase activity was only 6% compared to 60% maintained β-galactosidase activity. Additionally, acetonitrile has a higher boiling point than acetone, and it had already been found useful in similar reactions. Therefore, we opted for a final setup of 20% v/v acetonitrile/acetate buffer pH 5.0. It afforded the desired product **4** in 56% yield, to be coupled to branched linkers **14** and **15**.

Synthesis of Glycosylated Branched Linkers 18 and 19

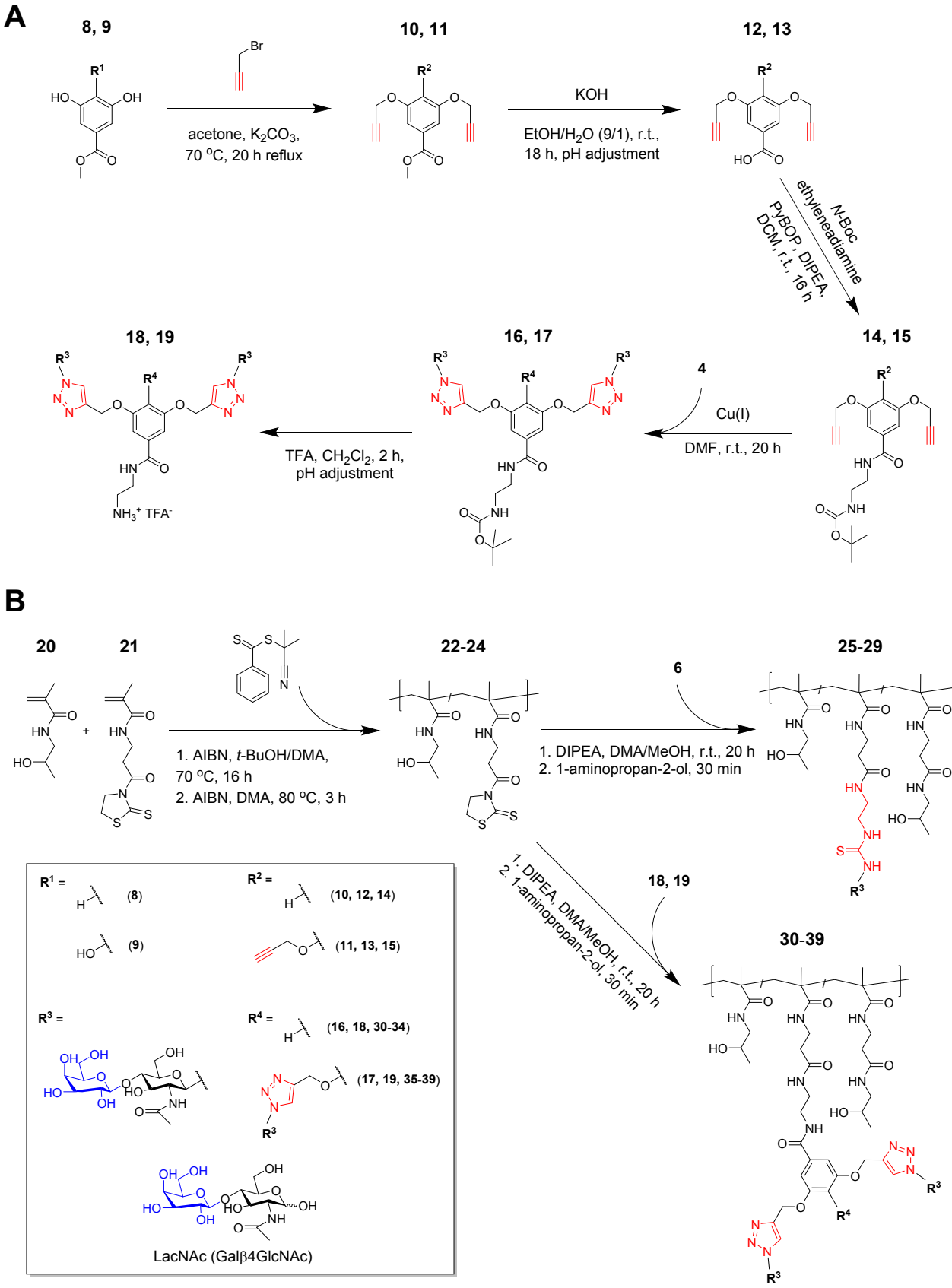
Bi- and trivalent branched linkers **14** and **15**, respectively, (Scheme 2A) were prepared by an original procedure in three steps. First, intermediates **10** and **11** were afforded by propargylation of compounds **8** and **9**, respectively. Complete propargylation was confirmed by the appearance of respective alkyne signals in ^{1}H NMR spectrum. Subsequent methyl ester hydrolysis yielded intermediates **12** and **13**, and we observed the disappearance of methyl ester signal in ^{1}H NMR spectrum. The final step, amide formation yielding **14** and **15**, was accompanied by appearance of signals of amide, urethane and *t*-Boc groups in ^{1}H NMR spectrum. Azido-functionalized LacNAc (**4**) was conjugated to the available propargyl moieties on **14** or **15** by CuAAC (one set-up of "click chemistry"),[50] accompanied by the appearance of the triazole moiety signal in ^{1}H

NMR spectrum. The subsequent *t*-Boc deprotection afforded the title compounds **18** and **19** with two or three LacNAc moieties, respectively, and an amino group for the attachment to polymer precursors. The *t*-Boc deprotection proved to be the most challenging part of the synthesis. Here, we had to optimize the method to prevent the cleavage of the glycosidic bond and achieve the complete deprotection. Finally, we found that a reasonable approach to tackle this issue is to perform the reaction by a careful addition of a total of 132 equivalents of TFA in three steps (44 eq. added dropwise in each step) under cooling, and terminate the reaction after a maximum of 2 h under monitoring by HPLC.

Synthesis and characterization of polymer precursors 22-24 and glycopolymers 25-39

Polymer precursors **22-24** containing reactive TT groups with comparable molecular weights ($M_n \approx$ 19,000 - 24,600 g mol^{-1}) and narrow dispersity ($Đ \approx 1.1$) were prepared by controlled RAFT polymerization technique. The copolymers differed in the content of the TT reactive groups, *i.e.*, 9 mol. % in **22**, 17 mol. % in **23** and 23 mol. % in **24.** The varying content of TT groups enabled the covalent attachment of the amine-functionalized LacNAc (**6**) or amine-terminated branched linkers bearing two or three LacNAc glycans (**18** and **19**, respectively) in a wide content range (see Table 1).

The aminolysis of TT groups by disaccharide **6** afforded glycopolymers **25-29** containing ca 5 to 19 mol. % LacNAc statistically distributed along the polymer chain (Scheme 2B).


A
8, 9
10, 11
12, 13
acetone, K2CO3, 70 °C, 20 h reflux
KOH
EtOH/H2O (9/1), r.t., 18 h, pH adjustment
N-Boc ethyleneadiamine
PyBOP, DIPEA, DCM, r.t., 16 h
14, 15
16, 17
18, 19
4
Cu(I)
DMF, r.t., 20 h
TFA, CH2Cl2, 2 h, pH adjustment
B
20
21
22-24
25-29
1. AIBN, t-BuOH/DMA, 70 °C, 16 h
2. AIBN, DMA, 80 °C, 3 h
6
1. DIPEA, DMA/MeOH, r.t., 20 h
2. 1-aminopropan-2-ol, 30 min
18, 19
30-39
1. DIPEA, DMA/MeOH, r.t., 20 h
2. 1-aminopropan-2-ol, 30 min
R1 = (8) (9)
R2 = (10, 12, 14) (11, 13, 15)
R3 =
R4 = (16, 18, 30-34) (17, 19, 35-39)
LacNAc (Galβ4GlcNAc)

Scheme 2 (**A**), Synthesis of glycosylated branched linkers **18** and **19**; (**B**), synthesis of glycopolymers **25-29** with individual presentation of LacNAc, and glycopolymers **30-39** with LacNAc presented on bi- and trivalent branched linkers. Structure of LacNAc disaccharide is shown in the box.

The molar mass and size of the polymers increased with the increasing amount of LacNAc content, showing the slight expansion of the polymer coil. Only in glycopolymers **28** and **29** with a high LacNAc content, the dispersity slightly increased. We assumed that this increase could be ascribed to the statistical distribution of disaccharide along the polymer backbone since any slight difference in the disaccharide content between various polymer chains will be more notable at higher LacNAc contents. Moreover, we also observed limited solubility of **28** and **29** in methanol, which might have been caused by the high amount of LacNAc. Therefore, the molecular weight of **28** and **29** was determined in aqueous mobile phase on the Superose column.

The glycopolymers carrying LacNAc epitope presented on dibranched (**30-34**) or tribranched (**35-39**) linkers were prepared by conjugation of primary amino group of the glycosylated compounds **18** or **19**, respectively, with the reactive TT groups of polymer precursor **23** (Scheme 2B). The content of LacNAc ranged from ca 4 to 22 mol. %, and the introduction of branched linkers containing LacNAc in bi- or trivalent presentation led to a slight increase in the polymer molar mass. Similar to glycopolymers with disaccharide **6**, a slight increase in the hydrodynamic size of glycopolymers **30-39** was observed with increasing amount of di- or tribranched LacNAc epitopes. In glycopolymers **33**, **34**, **38,** and **39** with a high content of LacNAc we also observed a

limited solubility in methanol and slightly enhanced molecular weight dispersity, probably due to the statistical reasons described above.

We prepared a total of fifteen glycopolymers presenting LacNAc in five different molar contents and three different presentations, i.e. statistical distribution of individual units or of bivalent and trivalent branched units, to be tested for affinity to galectins. The physico-chemical characteristics of all polymers precursors and glycopolymers, namely molar mass, hydrodynamic diameter and the content of LacNAc, are summarized in Table 1.

Table 1 Characteristics of polymer precursors used for the conjugation of LacNAc epitope and glycopolymers

Cmpd	Prepared from	Content of TT groups [mol. %][a]	Content of LacNAc [mol. %][b]	M_n [g mol^{-1}][c]	M_w [g mol^{-1}] [c]	$Đ$ [c]	$D_h \pm$ SD[d] [nm]
22	**20, 21**	9	-	21,900	23,100	1.1	5.2 ± 0.7
23	**20, 21**	17	-	19,000	21,300	1.1	5.3 ± 0.7
24	**20, 21**	23	-	24,600	28,000	1.1	5.9 ± 0.4
25	**6, 22**	-	5.0	23,600	25,400	1.1	6.4 ± 0.3
26	**6, 22**	-	6.9	21,500	22,500	1.1	6.3 ± 0.2
27	**6, 23**	-	13.2	25,600	26,900	1.1	7.7 ± 0.3
28	**6, 23**	-	16.7	26,600	34,400	1.3	7.8 ± 0.5
29	**6, 24**	-	19.3	25,200	33,600	1.3	8.2 ± 1.1
30	**18, 23**	-	3.8	25,800	28,400	1.1	6.2 ± 0.7
31	**18, 23**	-	8.2	23,500	24,900	1.1	6.2 ± 1.0
32	**18, 23**	-	12.1	28,000	28,900	1.1	6.5 ± 0.4
33	**18, 23**	-	18.7	28,200	38,200	1.3	7.3 ± 0.1
34	**18, 23**	-	20.9	30,200	37,900	1.3	8.2 ± 0.5
35	**19, 23**	-	4.6	25,100	26,300	1.1	5.8 ± 0.3
36	**19, 23**	-	7.3	23,100	24,900	1.1	5.9 ± 0.9
37	**19, 23**	-	12.8	21,700	24,800	1.1	5.9 ± 1.0
38	**19, 23**	-	15.1	28,400	32,200	1.2	8.2 ± 0.4
39	**19, 23**	-	22.0	32,400	38,400	1.2	7.3 ± 0.8

[a] The content of TT groups was determined by UV-Vis spectrophotometry in methanol, using the molar absorption coefficient of ε(TT) = 10,300 L mol^{-1} cm^{-1} (λ_{max} = 305 nm);

[b] The content of glycan was determined by NMR (600.2 MHz) using D_2O as solvent;

[c] The number-average molecular weight (M_n), weight-average molecular weight (M_w) and dispersity ($Đ$) of polymer precursors and glycopolymers were determined using SEC with RI and MALS detection. For precursors **22-24** and glycopolymers **25-27**, **30-32** and **35-37**, TSKgel Super SW3000 column was used with methanol/ 0.3 M sodium acetate

buffer, pH 6.5 (4/1, v/v) as a mobile phase. For glycopolymers **28**, **29**, **33**, **34**, **38** and **39**, Superose 6 was used with 0.3 M sodium acetate buffer, pH 6.5 as a mobile phase;

[d] Hydrodynamic diameter (D_h) were evaluated in water (5 mg mL^{-1}) using a Nano-ZS instrument, Malvern. The intensity of scattered light was detected at angle $\theta = 173$ °.

Inhibitory Potency of Glycopolymers 25-39 with Galectins

Glycopolymers **25-39** (M_n = 21,500-32,400 g mol^{-1}) were assayed for their ability to inhibit binding of human Gal-1 and Gal-3 to immobilized asialofetuin (ASF) glycoprotein in a competitive ELISA-type assay. The inhibitory potencies were compared to those of the respective monovalent disaccharide, namely LacNAc (**7**), and to the *t*-Boc protected branched linkers carrying two or three LacNAc molecules (**16** and **17**, respectively). Two main structural aspects were studied in the present glycopolymer library from the viewpoint of their avidity and/ or selectivity to galectins. First, we aimed to determine the impact of LacNAc density, and, second, we aimed to reveal the influence of its varying structural presentation, from a simple individual presentation to the clustered presentation on bivalent and trivalent linkers. The acquired results are shown in Table 2 and in Figure 1.

Table 2 Competitive inhibition of galectin binding to ASF by to glycopolymers determined by ELISA[a]

Cmpd	Sugar motif	*n* (glycan content [mol.%])	Gal-1		Gal-3		Selectivity for Gal-1
			IC_{50}/glycan [μM] (*rp*/*n*)	**IC_{50}** [μM] (*rp*)	IC_{50}/glycan [μM] (*rp*/*n*)	**IC_{50}** [μM] (*rp*)	
-	Lactose	1	n.a.	**357 ± 48**	n.a.	**143 ± 28**	0.4
7	LacNAc	1	n.a.	**78 ± 23**	n.a.	**44 ± 8**	0.6
16	$(LacNAc)_2$	2	37 ± 10 (*2.1*)	**19 ± 5** (*4*)	24 ± 5 (*1.8*)	**12 ± 2** (*4*)	0.6
17	$(LacNAc)_3$	3	28 ± 9 (*2.8*)	**9 ± 3** (*8*)	13 ± 5 (*3.4*)	**4.4 ± 1.6** (*10*)	0.5
25	LacNAc-*NH*	7.1 (5.0 %)	41 ± 14 (*1.9*)	**5.8 ± 1.9** (*13*)	27 ± 7 (*1.6*)	**4.4 ± 1.0** (*10*)	0.8

26	LacNAc-*NH*	8.4 (6.9 %)	41 ± 14 (*1.9*)	**4.9 ± 1.6** (*16*)	14 ± 5 (*3.1*)	**1.7 ± 0.6** (*26*)	0.3
27	LacNAc-*NH*	16.4 (13.2 %)	17 ± 8 (*5*)	**1.0 ± 0.5** (*76*)	71 ± 17 *(0.6)*	**4.3 ± 1.0** (*10*)	4.3
28	LacNAc-*NH*	19.9 (16.7 %)	0.47 ± 0.13 (*166*)	**0.024 ± 0.006** (*3318*)	135 ± 46 (*0.3*)	**6.8 ± 2.3** (*6*)	283
29	LacNAc-*NH*	20.6 (19.3 %)	1.8 ± 1.0 (*43*)	**0.086 ± 0.05** (*910*)	221 ± 27 (*0.2*)	**11 ± 1** (*4*)	128
30	$(LacNAc)_2$-triazole	6.0 (3.8 %)	48 ± 13 (*1.6*)	**8.1 ± 2.2** (*10*)	9 ± 2 (*5*)	**1.4 ± 0.3** (*30*)	0.2
31	$(LacNAc)_2$-triazole	10.3 (8.2 %)	48 ± 21 (*1.6*)	**4.7 ± 2.0** (*17*)	92 ± 18 (*0.5*)	**9 ± 2** (*5*)	2
32	$(LacNAc)_2$-triazole	16.2 (12.1 %)	9.2 ± 1.6 (*9*)	**0.57 ± 0.10** (*138*)	76 ± 26 (*0.6*)	**4.7 ± 1.6** (*9*)	8
33	$(LacNAc)_2$-triazole	21.6 (18.7 %)	5.7 ± 1.1 (*14*)	**0.27 ± 0.05** (*293*)	49 ± 11 (*0.9*)	**2.3 ± 0.5** (*19*)	9
34	$(LacNAc)_2$-triazole	24.6 (20.9 %)	10.1 ± 1.9 (*8*)	**0.41 ± 0.08** (*191*)	113 ± 31 (*0.4*)	**4.6 ± 1.3** (*10*)	11
35	$(LacNAc)_3$-triazole	6.9 (4.6 %)	65 ± 17 (*1.2*)	**9.4 ± 2.5** (*8*)	6 ± 1 (*7*)	**0.9 ± 0.2** (*48*)	0.1
36	$(LacNAc)_3$-triazole	9.3 (7.3 %)	29 ± 12 (*2.7*)	**3.1 ± 1.4** (*25*)	77 ± 29 (*0.6*)	**8.3 ± 3.1** (*5*)	3
37	$(LacNAc)_3$-triazole	13.3 (12.8 %)	6.1 ± 1.4 (*13*)	**0.46 ± 0.10** (*170*)	74 ± 8 (*0.6*)	**5.6 ± 0.6** (*8*)	12
38	$(LacNAc)_3$-triazole	19.4 (15.1 %)	3.5 ± 0.9 (*22*)	**0.18 ± 0.04** (*432*)	45 ± 6 (*1*)	**2.3 ± 0.3** (*19*)	13
39	$(LacNAc)_3$-triazole	27.8 (22.0 %)	2.3 ± 0.4 (*34*)	**0.082 ± 0.02** (*951*)	48 ± 12 (*0.9*)	**1.7 ± 0.4** (*26*)	21

[a] *n*, average number of glycans per polymer chain (glycan content, mol. %); *n* = 1, monovalent standard;

rp, relative potency, *i.e.* IC_{50} (LacNAc)/IC_{50} (glycopolymer); *rp*/*n* relative potency per glycan, *i.e.* IC_{50} (LacNAc)/IC_{50}/glycan (glycopolymer)

n.a., not applicable;

Selectivity for Gal-1 was defined as a ratio $IC_{50\ (Gal\text{-}3)}$ / $IC_{50\ (Gal\text{-}1)}$. It expresses how many times the avidity of a particular glycopolymer is higher to Gal-1 compared to Gal-3.

The results show a significant impact of both studied parameters on the glycopolymer affinity to galectins, and, moreover, huge differences in the structural preferences between both studied galectins. Gal-3 is a monomeric lectin (30 kDa) able to oligomerize in solution upon contact with multivalent ligands. According to the literature,[12] it is relatively difficult to evoke its multivalency effect especially with disaccharide ligands. In this study, Gal-3 clearly preferred low LacNAc content in the glycopolymers, reaching the highest relative potency (and also the highest avidity) with glycopolymers containing as few as 7 mol. % of individually presented LacNAc (glycopolymer **26**) or 5 mol. % of LacNAc clustered on bi- and trivalent linkers (glycopolymers **30**, and **35**, respectively). These compounds exhibited an up to 7-times increase in relative potency per glycan. Probably due to steric hindrance, the increasing amount of LacNAc on the polymer

carrier brought a slight (see **34**, **39**) or a more pronounced (see **29**) avidity decrease. If we speculate that Gal-3 exhibits a more pronounced multivalency effect in its pentamer form[51] of ca 150 kDa, the size of the polymer carrier (20-30 kDa) might not be sufficiently large to bind several binding sites of the pentamer and evoke the multivalency effect.

In sum, we suggest that with a higher glycan load, Gal-3 prefers LacNAc presentation on bi- and especially trivalent branching on the polymer chain: in those cases, the clustered presentation enabled to reach higher relative potencies (both per glycan and total) and higher avidities than in the individual LacNAc presentation (cf. **29**, **34**, and **39**). In accordance with our previous results,[8] Gal-3 does not exhibit a notable multivalency effect with simple disaccharides such as LacNAc and therefore, in the whole set the best affinities are in single-digit μM range (Table 2, Fig. 1). The best compound in the series is the glycopolymer **35** with 5 mol. % LacNAc on trivalent branched linker (on average 6.9 LacNAc moieties per polymer chain) with IC_{50} of 0.9 μM. This glycopolymer showed a 48-fold stronger inhibition effect compared to the monovalent LacNAc standard (7-fold stronger on a per-glycan basis), which is a good result compared to other multivalent ligands of Gal-3 decorated with unmodified disaccharides.[8,12,19,52,53] The present study insinuates that epitope presentation on branched linkers may be more suitable than a simple individual presentation for Gal-3. This information may be further developed in future studies.

The situation is different for Gal-1. In contrast to Gal-3, Gal-1 is an electrostatically bound dimer of two identical CRD domains (14 kDa) and in this study it strongly preferred the LacNAc presentation in a high density on the polymer chain. As a result, we

can observe a significant avidity increase (both per glycan and total) in glycopolymers from low to high LacNAc content within all studied structural presentations (individual: **25** to **29**, bivalent branching: **30** to **34**, trivalent branching: **35** to **39**), reaching IC_{50} values in nanomolar range when LacNAc content exceeded 12 mol. % (Table 2, Fig. 1) and tenfold to over hundredfold increased relative potencies per glycan (cf. **28**, **29**, **39**). Thus, Gal-1 appears to exhibit a strong multivalent effect with the present glycopolymer ligands. Interestingly, contrary to the traditional pattern of natural glycans such as in ASF glycoprotein (LacNAc presented on three triantennary glycans), Gal-1 seems to prefer individual LacNAc presentation over the branched linkers in the studied set. Thus, the best ligand of Gal-1 is glycopolymer **28** with 17 mol. % of individually presented LacNAc (on average 19.9 LacNAc moieties per polymer chain), featuring IC_{50} of 24 nM and an excellent relative potency of 3318. This corresponds to 166-times increased relative potency per glycan. Thus, glycopolymer **28** ranks among the strongest multivalent ligands of Gal-1 ever prepared.

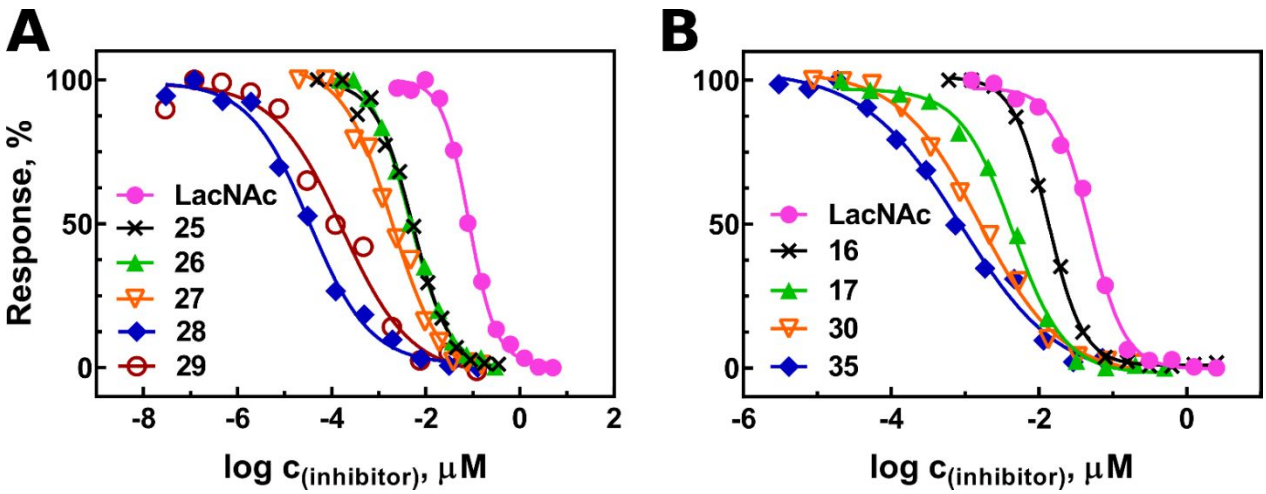


Figure. 1 Dose-response curves of competitive inhibition of galectin binding to ASF by selected glycopolymers. (**A**), Inhibition of Gal-1 binding by monovalent LacNAc, and by

glycopolymers **25-29** decorated with individually presented LacNAc (5.0-19.3 mol. %). (**B**), Inhibition of Gal-3 binding by monovalent LacNAc, by *t*-Boc protected dibranched linker **16**, tribranched linker **17**, and by glycopolymers **30** and **35** (two best inhibitors in the series). The sigmoidal curves yield the values of IC_{50} for the respective compounds.

Importantly, the found structural preferences of both studied galectins, Gal-1 and Gal-3 are so distinctive that with an epitope as simple as LacNAc, we were able to reach a notable discrimination between Gal-1 over Gal-3. In the ELISA-type assay with the immobilized asialofetuin competitor, glycopolymer **29** (19 mol. % of individually presented LacNAc) exhibited a 128-fold stronger avidity to Gal-1 than to Gal-3 (as demonstrated in the ratio of respective IC_{50} values), and glycopolymer **28** (17 mol. % of individually presented LacNAc) showed the highest selectivity of the set, with a 283-fold higher avidity (ratio of IC_{50} values) for Gal-1 compared to Gal-3. Such a selectivity found in the present glycopolymers is unusual as in the majority of previous studies, selectivity between galectins was reached through a laborious choice of glycan epitopes, often very specific and not easy to synthesize,[27,54,55] which may naturally pose a bottleneck for any broader application. In the present work, we have been able to steer the selectivity of a multivalent ligand by manipulation in glycan density and a pattern of glycan presentation. Another similar case has been shown in glycocalixarenes[56] where the selectivity to galectins was steered by the calixarene structure (cone or alternate) and by the ring size,[57] and also, very recently, in lactose-decorated graft polymers.[32] The water-soluble HPMA-based glycopolymers represent a straightforward and elegant option in galectin inhibition, also due to their *in vivo* applicability resulting from their non-toxic, non-immunogenic nature and prolonged half-life in the blood stream.

CONCLUSION

This work demonstrates an example of steering the avidity and selectivity of a multivalent glyco-nanomaterial between galectins. The type of presentation of a common disaccharide ligand, LacNAc, along the HPMA copolymer backbone, either individual or clustered on bi- or trivalent linkers, brought a difference between glycopolymer avidity to Gal-1 in contrast to Gal-3 of up to 300-fold. While Gal-1 preferred a dense presentation of individually distributed LacNAc epitopes, Gal-3 favored clustered LacNAc presentation.

The controlled RAFT polymerization afforded synthesis of HPMA copolymer precursors with a low dispersity and tunable composition. The LacNAc disaccharide derivatives bearing the appropriate functions for coupling to HPMA copolymer was conveniently prepared in a one-step enzymatic synthesis in a high yield.

In sum, we present here multivalent water-soluble LacNAc-carrying glycopolymers that are suitable for *in vivo* applications due to the biocompatible HPMA scaffold. For their nanomolar avidity to Gal-1, these glycopolymers may thus be prospective for therapeutic applications requiring efficient inhibition of Gal-1.

Supporting Information

1. **Functionalized disaccharides – structural characterization**
   1.1. NMR data and spectra
   1.2. MS spectra
2. **Enzymatic synthesis of functionalized disaccharide 4**
3. **Glycosylated branched linkers 18 and 19**
   3.1. Synthesis of branched linkers **14** and **15, NMR and MS spectra**
   3.2. Synthesis of glycosylated branched linkers **18** and **19, NMR and MS spectra**

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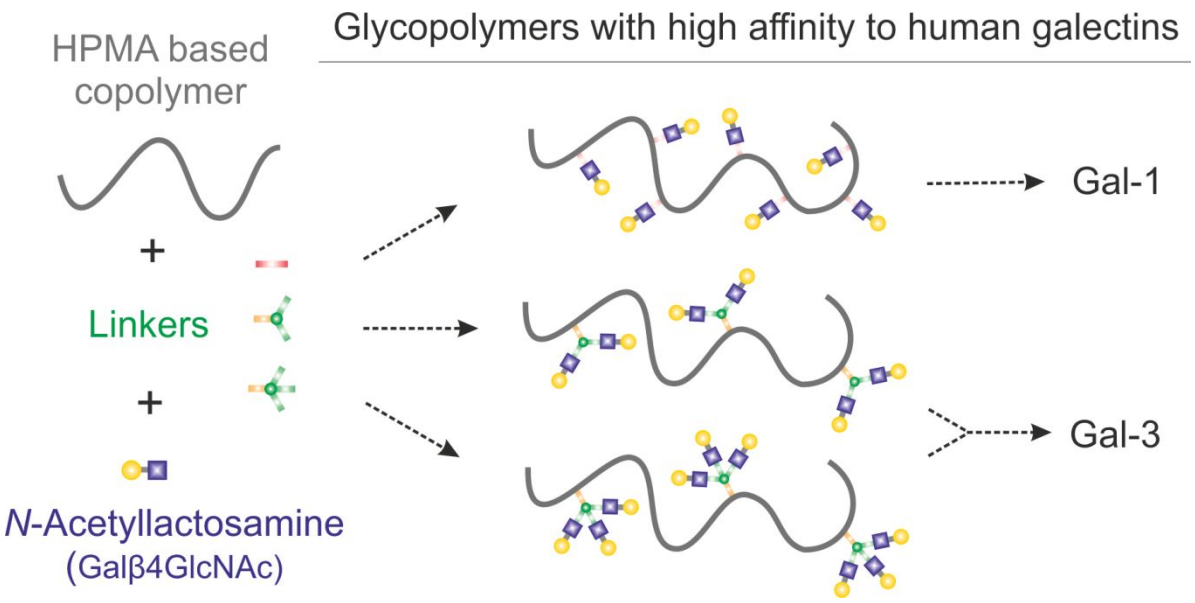
Glycopolymers with high affinity to human galectins
HPMA based copolymer
+
Linkers
+
N-Acetyllactosamine
(Galβ4GlcNAc)
Gal-1
Gal-3