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Biomacromolecules, **Just Accepted Manuscript** • DOI: 10.1021/acs.biomac.0c00676 • Publication Date (Web): 16 Jun 2020

Temperature switchable glycopolymers and their conformation-dependent binding to receptor targets

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Keywords

LCST, temperature stimulus, PNIPAM, carbohydrate polymer, inhibitors, *E. coli*, lectin

Abstract

The temperature-dependent binding of copolymers from poly(*N*-isopropylacrylamide) (PNIPAM) and mannose ligands to *E. coli* and concanavalin A (ConA) is determined. Through polymer analogous reactions using poly(*N*-acryloxysuccinimide) and amine-linked mannose residues with different linkers, glycopolymers are prepared under variation of the mannose density. Quantitative adhesion-inhibition assays show the inhibitory potential of the glycopolymers as a function of the mannose/NIPAM ratio and linker type above and below their lower critical solution temperature (LCST). Intriguingly, opposite temperature effects on the binding to *E. coli* and ConA are observed. While the *E. coli* inhibition is stronger above the LCST, the ConA inhibition is overall weaker at elevated temperature. When going beyond the LCST, the polymers undergo a coil-to-globule transition, forming microphases with surface-enriched hydrophilic sugar moieties explaining increased *E. coli* inhibition by steric shielding. However, the formation of such microphases above the LCST renders a fraction of carbohydrate ligands inaccessibleand polymers remaining in the solution phase then have coil sizes below the minimum binding site spacing of the ConA receptor explaining reduced ConA inhibition. Overall these results suggest that the coil-to-globule transition of glycopolymers may induce lower or higher inhibitory potentials due to the adverse effects of steric shielding and carbohydrate ligand accessibly.

1. Introduction

Interactions between carbohydrates and proteins at the surface of cells or pathogens control numerous biological processes including infections, fertilization, recognition or signaling.[1] Lectins, as a class of carbohydrate binding proteins, decorate the pathogen surface and form complexes with glycans at the cell's glycocalyx, which is a critical step in the development of infectious diseases. The macromolecular glycans involved in these processes interact with the pathogen receptors through multiple weak interactions generating sufficient adhesion across large

interfacial areas[2, 3] to drive pathogen invasion and disease progression. The ability to suppress such lectin-glycan mediated adhesion processes by multivalent carbohydrate presenting inhibitors has been proposed as a strategy to fight infections.[4-6] Different glycoconjugates have been developed for this purpose, e.g. nanogels,[7] gold nanoparticles,[8, 9] and many more, see recent reviews.[10-12] To improve the viability of such inhibitors in practice, being able to increase or decrease their affinity toward pathogens by remote stimulus is desired. For example, to first capture pathogens at a high affinity state of the glycoconjugate and to release the pathogen afterward for diagnosis by switching to a low affinity state. Remote stimuli could also reduce side effects, e.g. by locally restricting bacterial inhibition/capture to the inflamed tissues. For controlled drug release many of such remotely switchable and site specific scaffolds were successfully established.[13] Recent studies also transferred this concept to glyco-conjugated thermo-responsive polymers in order to control the interactions to lectins, bacteria or viruses via temperature stimulus.[14-23] The involved polymers have a lower critical solution temperature (LCST) in the physiological temperature range. They form extended coils below the LCST, and attain a collapsed globule conformation above the LCST. It was reasonably assumed that the temperature-controlled coil-to-globule transition affects the presentation of carbohydrate ligands as well as the size of the scaffold and thus controls the binding affinity of the glycoconjugate. However, the literature shows diverging results on how temperature changes affect the affinity. One the one hand, affinities toward single lectins or bacteria were shown to decrease above the LCST,[14-16] whereas other studies showed increasing affinities.[19-23] For example, using linear copolymers composed of *N*-isopropylacrylamide (NIPAM) and mannose derivatives Pasparakis *et al*. showed that binding to *E. coli* and their clustering is preferred below the LCST.[14] Using a similar pair of monomers, and additional crosslinker to form microgels, we showed that their binding and clustering of *E. coli* is

preferred above the LCST.[19] Other work showed no clear effect of the temperature transition on the binding efficiency.[24-26]

This work is aimed at investigating the changes in the temperature dependent affinity of thermosensitive glycopolymers to understand the diverging findings and interpretations in the literature. It could be argued that carbohydrate units become inaccessible at elevated temperature when linear LCST-polymers attain a globule conformation and aggregate to explain reduced affinity. On the other hand, the surface presentation of hydrophilic carbohydrate units at such globules might increase above the LCST when the polymer becomes hydrophobic. In addition the size ratio between the glycopolymer ligands and their targets are shifted by the coil-to-globule transition, which may affect their inhibitory potential due to steric shielding effects.[27] Such steric shielding effects describe the ability of a large inhibitor particle to block the binding between ligand and receptor decorated surfaces due to the steric screening of binding sites.[28] Furthermore, the multivalent binding to receptor sites could be affected because of the change of the polymer coil size when crossing the LCST, e.g. when the coil size falls below the minimum binding site of the receptor. Therefore, here we systematically vary the compositions of linear PNIPAM/mannose copolymers as well as the linker between mannose units and the polymer backbone. We study their binding via inhibition assays with concanavalin A (ConA) as a well-known mannose specific lectin with a minimum binding site distance of 7.2 nm [29] and *E. coli*, a bacteria binding to mannose via monovalent FimH receptors.[30] By choosing these targets with broadly different properties and systematically varying the glycopolymer mannose density and linker type we aim to elucidate phase transition effects in glycopolymer binding.

2. Experimental

Materials

α-D-mannopyranoside (99%, Acros Organics), β-D-galactose pentaacetate (95%, Fluorochem) acetonitrile (≥99.9%, Panreac AppliChem) p-toluenesulfonic acid (98%, Alfa Aesar), sodium methanolate (98%, Alfa Aesar), isopropylamine (99+%, Alfa Aesar), acetic anhydride (≥98%, VWR Chemicals), *n*-hexane (99%, VWR Chemicals), ethyl acetate (freshly distilled), sodium bicarbonate (100%, Fisher Chemicals), Amberlite-IR120® (Fisher Chemicals), magnesium sulfate (62-70%, Fisher Chemicals), tetrahydrofuran (99.99%, Fisher Chemicals), chloroform (99.97%, Fisher Chemicals), dimethylsulfoxide (99.99%, Fisher Chemicals) allyl alcohol (≥99%, Merck KGaA), trimethylamine (>99.0%, Merck KGaA), acryloyl chloride (96%, Merck KGaA), boron trifluoride diethyl etherate (≥98%, TCI), hydrogen (Air Liquide), *N,N*-dimethylformamide (≥99.8%, Biosolve-Chemicals). All other chemicals were obtained from Sigma-Aldrich (Germany).

Synthesis of N-acryloxysuccinimide (NAS)

The synthesis of NAS was adapted from published protocols.[16] In 200 mL of chloroform *N*-Hydroxysuccinimide (14.4 g, 125 mmol) and triethylamine (22 mL, 155 mmol) were dissolved at 0 °C. Then acryloyl chloride (10 mL, 125 mmol) was added dropwise under stirring at 0 °C for 30 min. The organic phase was washed three times with saturated sodium bicarbonate solution and dried over sodium sulfate. The volume was then reduced to 1/3 and NAS was precipitated by addition of n-hexane. The supernatant n-hexane was removed under reduced pressure. The remaining yellow oil was dissolved in chloroform. This cycle was repeated until the remaining oil was not soluble in chloroform giving a yield of 18.09 g (88%).^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.74 - 6.67 (dd, $^{3}J_{HH}$ = 17.4, 0.8 Hz, 1H, *H1*), δ 6.38 - 6.27 (dd, $^{3}J_{HH}$= 16.6, 10.3 Hz, 1H, *H2*), δ 6.20 - 6.14 (dd, $^{3}J_{HH}$ = 10.7, 0.8 Hz, 1H, *H3*), δ 2.89 - 2.81 (s, 4H, *H4-7*) (Supporting Information S1).

Synthesis of Poly(N-acryloxysuccinimide) (PNAS)

NAS (15.3 g, 90 mmol) in 140 mL *N*,*N*-dimethylformamide (DMF) was heated to 80 °C and flushed with nitrogen for 15 min. The polymerization was initiated by addition of 4,4'-Azobis(4-cyanovaleric acid) (255.6 mg, 0.9 mmol) in 10 mL DMF. After 20 hrs the polymer was precipitated in cold tetrahydrofuran, filtered and dried under vacuum. A brown solid was obtained (yield 12.21 g, 79%, $\bar{M}_n$ = 15340 Da).^{1}H-NMR (600 MHz, $CDCl_3$) δ = 12.38 - 12.14 (s, 1H, *H9*) 3.28 - 3.00 (s, 1H, *H3*), 2.85 - 2.74 (s, 4H, *H4-7*), 2.25 - 1.90 (s, 2H, *H1+2*) 1.40 - 1.33 (s, 3H, *H8*) (Supporting Information S2).

Synthesis of the glycopolymers

Poly(*N*-acryloxysuccinimide) (1.00 g, 65 µmol) was dissolved in 10 mL dimethyl sulfoxide (DMSO) and heated to 40 °C. Depending on the Man/Gal functionalization degree, different amounts of amine functionalized sugar (Supporting Information S4) were added. After 2 hrs of reaction, isopropylamine (1.5 mL, 17.7 mmol) was added reacting for an additional 2 hrs. The reaction solution was cooled down and diluted with 35 mL water and dialyzed for 48 hrs followed by freeze drying. The Supporting Information shows the ^{1}H NMR analysis (Supporting Information S4) and the size exclusion chromatography (Supporting Information S5) of the polymers.

Phenol sulfuric acid (PSA) method

To determine the glycopolymer Man/Gal functionalization degree, the PSA method was used.[31] At first a calibration curve was measured using a methyl α-$_D$-mannopyranoside (MeMan) dilution series in water (160 µM, 80 µM, 40 µM and 20 µM). To 125 µL of each MeMan solution 125 µL of a 5wt% phenol solution in water was added and vigorously shaken. Afterward, 625 µL of concentrated sulfuric acid was added, vigorously shaken and reacted at 30 °C for 30 min. Next,

the absorbance at a wavelength of 490 nm was measured. For analyzing the functionalization degree, 125 µL of the polymer solution at known concentration was used (Supporting Information S6).

Turbidimetry

Turbidity measurements were performed on a Tepper turbidity photometer using a 1 mW laser at 630 – 690 nm. 2 mL of a solution of 5 mg mL^{-1} polymer in LBB was given into a QS quartz cuvette (Hellma Analytics, Müllheim, Germany). After reaching the starting temperature of 20 °C, 0.5 mL of a ConA solution (1 mg mL^{-1}) was added followed by starting the heating and cooling cycle from 20 °C up to 44 °C and back to 20 °C at a temperature rate of 1 °C min^{-1}. To determine the cloud point as an indication for the LCST of the polymers, the measurement was executed without protein (supporting information S9). From the transmission-temperature traces the onset of the transmission decrease was used to determine the cloud points.

Mannan-coating

For coating the 96-well plates mannan from *Saccaromyces cerevisiae* (Sigma-Aldrich) was used. 120 µL mannan solution (1.2 mg mL^{-1}) in carbonate buffer (pH 9.5) were filled into each well. The plates were dried at 37 °C overnight followed by washing with PBST (PBS with 0.5 wt% Tween 20, 3 x 120 µL per well).

GFP-based bacterial adhesion inhibition assay

The adhesion assay was adapted from previous protocols.[30] For blocking nonspecific binding each well was filled with 120 µL of 1 wt% polyvinylalcohol (22000 g/mol) in PBS at 37 °C followed by shaking at 120 rpm for 1 hrs. Next, the plates were washed three times with 120 µL PBST and one time with PBS. Then a dilution series of the polymers was prepared and added to the well plates. The bacteria suspension (50 µL) at a concentration of 2 mg mL^{-1} (OD = 0.4) was added to

the wells, and the plates were incubated for 1 hr at 100 rpm either 20 °C or 40 °C. Afterward the plates were washed three times with 120 µL PBS and then filled with 100 PBS µL per well. Finally, the fluorescence intensity of the adhered *E. coli* was detected at 485 nm / 535nm.

FITC-ConA adhesion inhibition assay

The mannan-coated surfaces were blocked with a 5wt% solution of bovine serum albumin in carbonate buffer (pH 9.5) by giving 120 µL of the BSA solution into each well and shaking at 120 rpm for 1 hr at ambient temperature. After blocking, the plates were washed three times with 120µL PBST and one time with 120 µL LBB. A dilution series of the polymers was prepared on the mannan-coated, BSA blocked well plates. A solution of FITC-ConA at a concentration of 0.1 mg mL^{-1} in LBB was prepared. 50 µL of the ConA solution were added to each well, and the plates were incubated for 1 hrs at 100 rpm either at 20 °C or 40 °C. After 1 hr the plates were washed three times with 120 µL LBB and filled with 100 µL LBB per well. The fluorescence intensity of the adhered FITC-ConA was determined at 485 nm / 535 nm.

3. Results and discussion

3.1 Synthesis of thermo-responsive glycopolymers

The key objective of this work is to control the sugar density and linker type in thermo-responsive glycopolymers and to test the effect of these parameters on the inhibition of ConA and *E. coli* FimH receptors. Ten different polymers with varying carbohydrate densities and different linkers were prepared. For straightforward variation of the sugar densities and linker type, a polymer analogous reaction was chosen (**Figure 1**). By post-functionalization of a poly(active ester) the NIPAM repeat units and the sugar ligands were introduced. First the succinimide-based poly(active ester) was synthesized according to a previously published protocol.[16] Via free radical polymerization (FRP) with 4,4'-azobis(4-cyanovaleric acid) as initiator the active ester polymer

poly(*N*-acryloxysuccinimide) (PNAS) was synthesized. As expected from FRP, PNAS exhibits a dispersity of 1.51 and a number average molecular weight ($\overline{M}_n$) of 15.34 kDa as determined via size exclusion chromatography (SEC) and ^{1}H-NMR. As carbohydrate ligands, varying quantities of 2-aminoethyl-α-D-mannopyranoside (**ManEL**) and 3-amino-2-hydroxypropyl-α-D-mannopyranoside (**ManHPL**) were then reacted with PNAS. The two carbohydrates have different linkers, where the hydroxypropyl (**HPL**) linker can be considered slightly more hydrophilic due to the added hydroxy group as compared to the ethyl (**EL**) linker. In a second reaction step, isopropylamine was added to quench the remaining active esters and to create the thermo-responsive NIPAM repeating units.

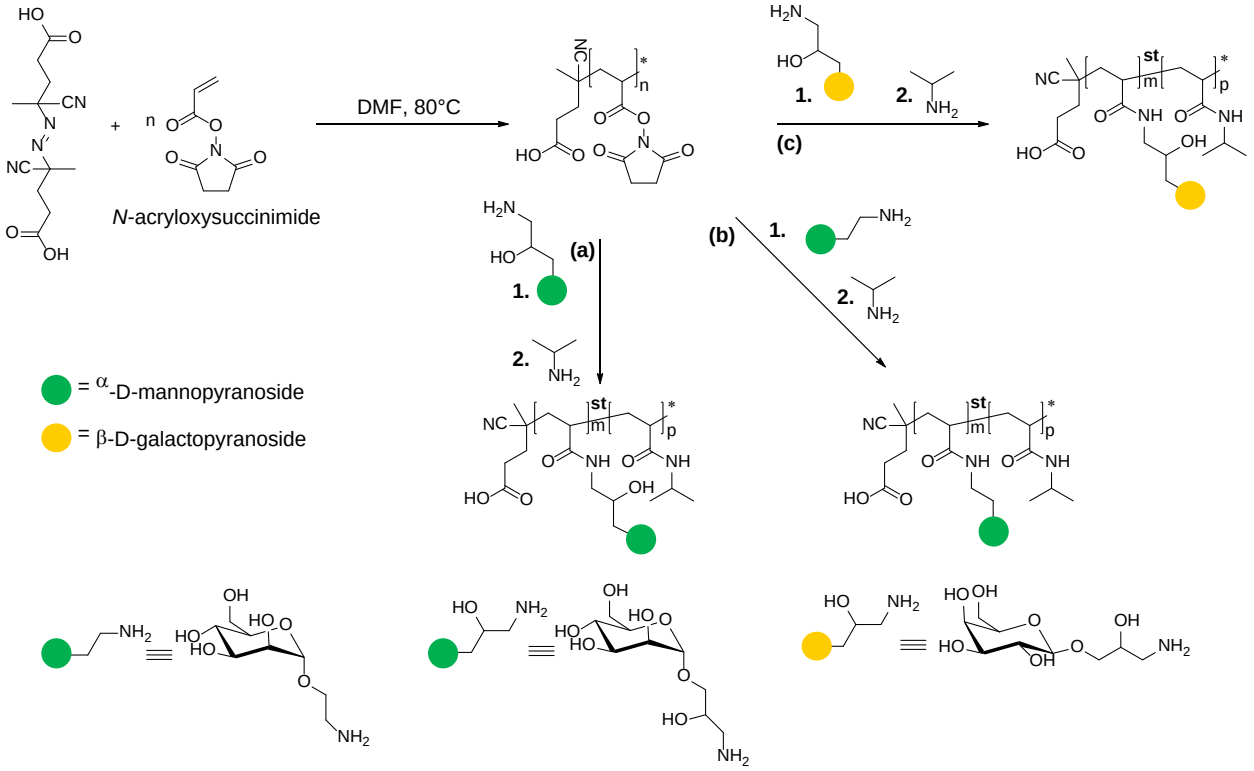

Figure 1 Schematic of the active ester synthesis and grafting of different sugars. Grafting of (a) **ManHPL**, (b) **ManEL** and (c) **GalHPL** at different carbohydrate-densities on the polymer

backbone is followed by the addition of isopropylamine to quench the remaining active esters and to form thermoresponsive NIPAM residues.

The incorporation of carbohydrates into the polymers took place with an efficiency of roughly 50% (Supporting Information S4). Due to the presence of hydroxyl groups it is possible that a fraction of sugar units was grafted to the polymer backbone via ester groups. However, as evidenced by the absence of ester groups in the IR traces (Supporting Information S7), the esters were likely substituted by isopropylamine groups in the second grafting step under basic conditions. With this synthetic route five glycopolymers bearing **ManHPL** with carbohydrate functionalization degrees from 1% to 97% and three glycopolymers bearing **ManEL** with carbohydrate functionalization degrees from 1% to 5% were produced. As negative controls two non-Man presenting polymers were prepared: pure **PNIPAM** without sugar ligands, and a galactose decorated polymer via grafting of 3-amino-2-hydroxypropyl-β-D-galactopyranoside (**GalHPL**). In **Table 1** the synthesized polymers are listed and named by the grafted carbohydrate followed by a number representing the functionalization degree, e.g. **ManHPL7** signifies 7% Man units compared to NIPAM. The polymer's carbohydrate functionalization degree was determined by a quantitative colorimetric test for sugars (PSA-test).[32, 33] In addition, the degree of carbohydrate functionalization was confirmed by AT-FTIR. The glycopolymer molecular weights were determined by considering the $\bar{M}_n$ of PNAS as determined by NMR and the degree of carbohydrate functionalization as determined by the PSA-test. Size exclusion chromatography confirmed the shifts in $\bar{M}_n$ with varying degrees of functionalization because of the higher molecular weight of the carbohydrates compared to isopropylamine. Also, the glass transition temperature (Tg) increased with a higher carbohydrate content (Supporting Information S8), in agreement with the literature.[34] The cloud point of the polymers, as determined by turbidimetry, increased at higher

carbohydrate functionalization, in line with previous studies on carbohydrate functionalized microgels.[19] For **ManHPL97** and **ManHPL34** no temperature responsive behavior could be observed owing to the high carbohydrate content.

Table 1 The glycopolymers functionalized with different quantities of Man and Gal, $\overline{M_n}$, Tg, the cloud point and the carbohydrate functionalization degree are listed. The polymers are named by the sugar type and the functionalization degree in mol%.

polymer sample	$\overline{M_n}$ [kDa][a]	Tg [°C][b]	LCST [°C] [c]	Man/Gal functionalization degree [µmol g^{-1}][d]
PNIPAM	10.30	117	32.6	0.0
ManHPL1	10.48	122	35.9	4.2 ± 0.1
ManHPL2	10.65	126	36.8	5.7 ± 0.1
ManHPL7	11.53	126	40.2	23 ± 0.1
ManHPL34	16.24	133	>45[e]	99 ± 0.3
ManHPL97	27.24	123	>45[e]	298 ± 2.2
ManEL1	10.45	125	35.6	3.5 ± 0.1
ManEL2	10.59	128	36.7	6.8 ± 0.1
ManEL5	11.04	129	40.8	14 ± 0.1
GalHPL3	10.83	132	40.8	8.3 ± 0.1

[a]The M_n of the active ester polymer (PNAS) was determined via ^{1}H-NMR, [b]DSC measurements, [c]turbidimetry measurements, [d]phenol sulfuric acid assay, [e]no sigmoidal curve, an LCST could not be determined

3.2 Aggregation of ConA with the glycopolymers

Turbidity measurements were conducted to test the temperature dependent binding of the glycopolymers to ConA. ConA exhibits a homotetrameric structure at neutral pH, with four Man binding sites and a minimum spacing of 7.2 nm.[35] All studies were carried out in lectin binding buffer (LBB) containing Mn^{2+} and Ca^{2+} to activate the ConA binding sites.[36-38] Since ConA exhibits four binding sites, it typically forms clusters with multivalent carbohydrates.[29, 39] The

formation of such ConA-glycopolymer clusters can be studied via turbidimetry in real time, due to the increase of light scattering for increased cluster sizes.[40]

The temperature dependent cluster formation was tested under a heating/cooling rate of 1 °C min^{-1} between 20 °C and 44 °C. Without adding ConA, all polymers showed an increase in turbidity above the phase transition temperature as indicated by the cloud point. This can be attributed to the formation of polymer-polymer aggregates (Supporting Information S9a-h). All measurements in the absence of ConA reach the starting turbidity value after cooling back to 20 °C. However, when ConA is added to the measurement cell, the turbidity does not return to the starting value upon cooling for all Man-bearing polymers. This hysteresis effect can be assigned to ConA-glycopolymer binding and the formation of clusters that dissolve only very slowly below the cloud point. Moreover, this hysteresis effect is stronger for the polymers bearing **ManEL**. This suggests that the more hydrophobic linker leads to slower cluster dissolution. For the non-carbohydrate bearing polymers **PNIPAm** and **GalHPL3,** the hysteresis-effect was not observed (Supporting Information S9). This indicates that the delayed cluster dissolution for Man bearing polymers is because of the specific binding to ConA. In addition, when inhibiting ConA binding using an excess of MeMan the hysteresis was significantly reduced confirming specific binding. When the cooling rate was reduced to 0.1 °C min^{-1} the starting turbidity value was reached indicating that glycoclusters with Man-functionalization degrees up to 2% as tested, are not stable below the phase transition temperature but their dissolution is merely delayed (Supporting Information S9k). Overall, these measurements confirm a statistical/additive effect on glycopolymer binding,[40] i.e. an increased number of Man-units leads to larger and more persistent clustering with ConA. Moreover, the linker chemistry affects the cluster dissolution, where the more hydrophilic linker in **ManHPL** leads to faster dissolution than the more hydrophobic linker in **ManEL**.

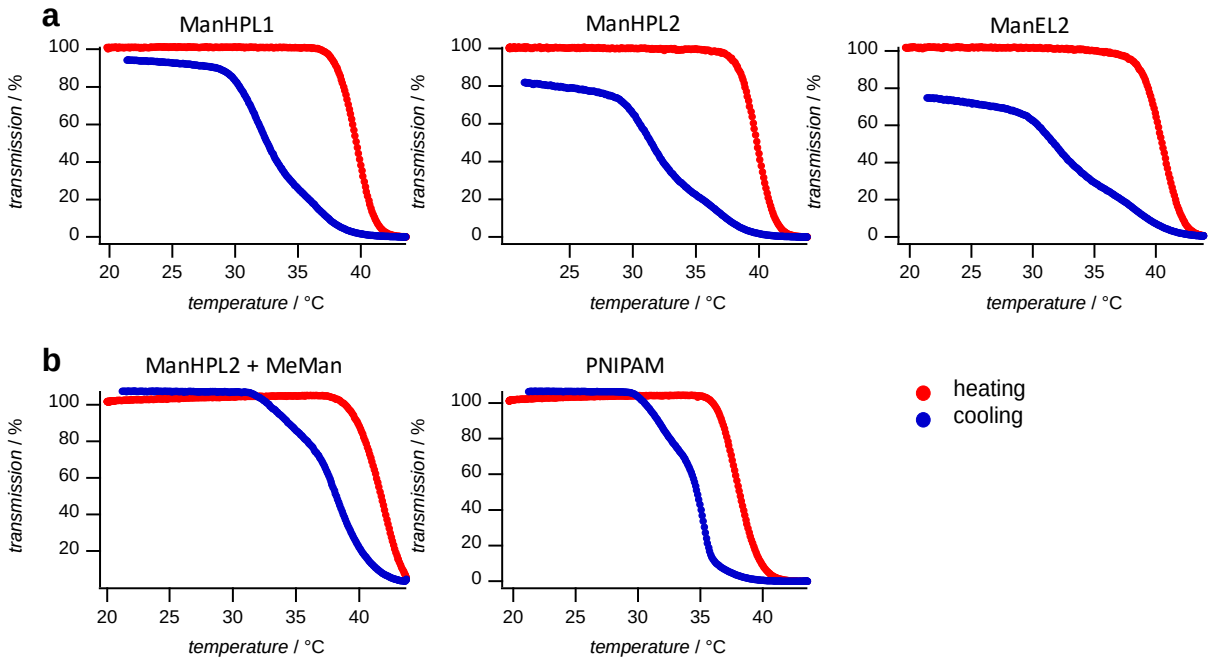

Figure 2 Turbidity under heating (red) and cooling (blue) at 1 °C min^{-1} of glycopolymer samples in the presence of 0.2 mg mL^{-1} ConA. (a) Three different Man functionalized polymers are shown. **ManEL2** showed a stronger heating/cooling hysteresis compared to **ManHPL2** as indicated by the lower transmission value after cooling. (b) Negative control samples **PNIPAm** and **GalHPL3** showed a reduced hysteresis. The inhibitor α-$_D$-mannopyranoside (MeMan) also lead to a reduced hysteresis upon cooling.

3.3 Temperature dependent adhesion inhibition of *E. coli*

The turbidity measurements confirmed the glycopolymers' phase behavior. To understand the phase transition effect on the glycopolymer affinity, a quantitative binding assay is required. Therefore, we carried out adhesion inhibition assays with *E. coli*. These bacteria have evolved hairy adhesive organelles, called pili or fimbriae, allowing the bacteria to adhere to cells via carbohydrate lectin interactions and to cause infections.[41] One of the best characterized adhesive

organelles is the type 1 fimbriae, which comprises various protein subunits and the monovalent α-D-mannopyranoside binding lectin FimH.[42, 43]

To evaluate the Man-specific adhesion of the GFP-tagged type 1-fimbriated pKL1162 strain, we use a mannan coated microtiter plate, add the bacteria, incubate/wash with the glycopolymers and then quantify the number of adhered bacteria by fluorescent readout (**Figure 3**). In this manner, glycopolymers compete with the mannan coated surface for binding to FimH. Therefore, by increasing the concentration of the Man presenting glycopolymers, a larger inhibition of the bacterial adhesion was achieved as measured by a reduction of the fluorescent signal.[30] From the changes in fluorescence intensity as a function of glycopolymer concentration, the inhibitory concentration at half maximum intensity (IC_{50}) was determined. The IC_{50} value represents the concentration of glycopolymer, where 50% of the bacterial adhesion to the surface was inhibited. If the IC_{50} is low, the inhibitory potency of the glycopolymer is high and vice versa.

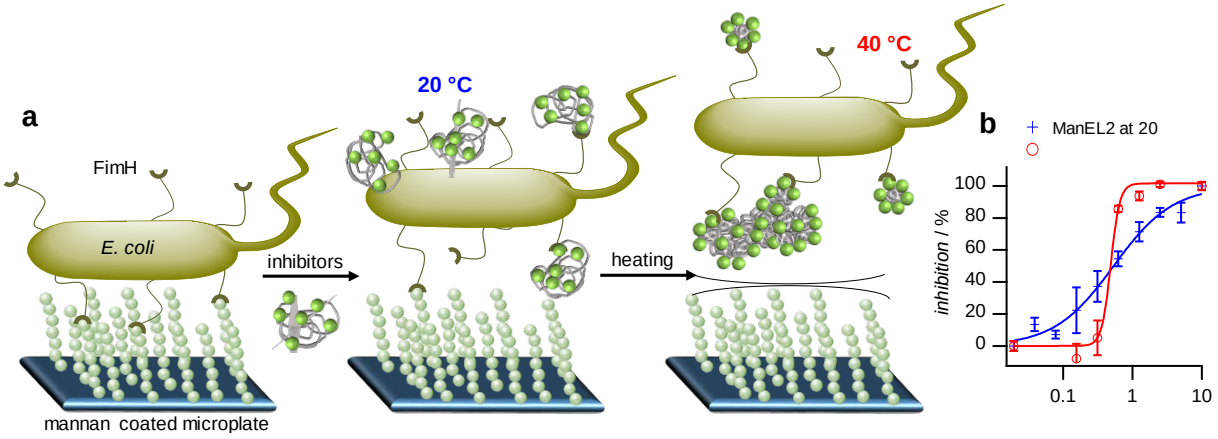

Figure 3 a) The temperature dependent inhibition assay of *E. coli* adhesion. GFP-tagged *E. coli* adhere to mannan coated surfaces. After the addition of the thermoresponsive glycopolymer the *E. coli* adhesion to the mannan coated surface is hindered. The glycopolymers form aggregates and show an increased Man surface density above the cloud point. b) A typical inhibition curve at

20 °C and 40 °C for **ManEL2**. The full set of inhibition curves is shown in Supporting Information S10.

At increased Man functionalization a stronger adhesion inhibition took place (**Figure 4**). Furthermore, **GalHPL3** showed only a small inhibition effect due to the low affinity to FimH. Comparing **ManEL** and **ManHPL** with the same Man concentration, it can be seen that the IC_{50} values for **ManEL** are lower. This is likely due to a higher lectin binding affinity of the hydrophobic linker, which was also observed by Lindhorst and coworkers.[44] For low Man functionalization degrees of less than 5%, an affinity increase is observed for both **ManEL** and **ManHPL** when heating to 40 °C, where **ManHPL** polymers show a stronger temperature response. At elevated Man content larger than 5%, the cloud point was above 40 °C for all polymers, indicating that a coil-to-globule transition and glycopolymer aggregate formation did not take place. Consequently, no clear temperature dependence on the IC_{50} was observed for these polymers.

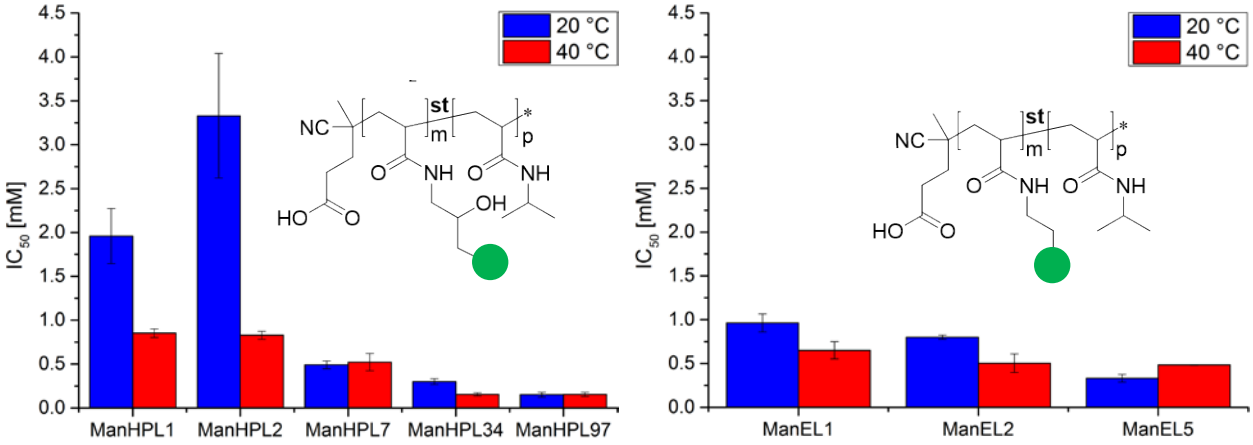

Figure 4 Left: IC_{50} values for the inhibition of *E. coli* adhesion with ManHPL glycopolymers at 20 °C (blue) and 40 °C (red). Right: IC_{50} values for ManEL glycopolymers. The IC_{50} values are

related to the polymer concentration. All measurements were performed in triplicates, averaged values and standard deviations are presented.

A comparison of the Hill coefficients shows the degree of cooperative binding. Cooperative binding is observed when ligand/receptor complex formation between multivalent structures yields higher a binding energy as compared to the sum of energies from single ligand/receptor subunits.[28] A Hill coefficient below 1 represents a negative cooperativity, a value higher than 1 stands for a positive cooperativity effect. For all polymers incorporating less than 5 mol% Man, the Hill coefficient increase above the cloud point, which was below 40°C for these polymers (**Table 2**). This could be attributed to the formation of highly multivalent glycopolymer aggregates, which likely show an increased surface presentation of hydrophilic Man units as these polymers aggregate via hydrophobic polymer-polymer contacts. This results in increased sugar surface densities, and explains the increased binding cooperativity and reduced IC_{50}. Furthermore, the glycopolymer aggregates can block additional areas on the bacteria besides the Man-FimH binding sites, i.e. steric shielding by these aggregates amplifies the inhibitory potential. The fraction of glycopolymers not incorporated into aggregates above the cloud point still attained a collapsed coil conformation since their LCST was likely exceeded, thus showing improved affinity to FimH due to the increased carbohydrate surface density. For polymers with larger Man functionalization degree (**ManHPL7**, **ManHPL34**, **ManHPL97**, **ManEL5**) the Hill coefficients tend to decrease at elevated temperature since their cloud point was not reached, i.e. a complete coil-to-globule transition and aggregate formation did not take place.

The results overall showed that the coil-to-globule transition of glycopolymers followed by aggregate formation and steric shielding increased the *E. coli* adhesion inhibition. In particular

polymers with low Man-functionalization degrees of showed a large temperature response, most likely due to the high fraction of thermoresponsive repeat units. The inhibitory potential of polymers with hydrophobic linkers (**ManEL**) did not benefit significantly from the coil-to-globule transition since they inhibit already strong in the coiled state below the LCST. In addition, owing to the linker hydrophobicity, the proposed effect of an increased surface presentation of the sugars on collapsed coils could be reduced compared to **ManHPL**.

Table 2 Hill coefficients from the *E. coli* adhesion inhibition curves.

Sample	Hill coefficient 20 °C	Hill coefficient 40 °C
ManHPL1	0.5 ± 0.2	3.1 ± 0.4
ManHPL2	0.4 ± 0.5	2.7 ± 0.2
ManHPL7	1.1 ± 0.1	2.3 ± 0.8
ManHPL34	0.6 ± 0.2	0.3 ± 1.0
ManHPL97	0.4 ± 0.2	0.1 ± 1.6
ManEL1	1.4 ± 0.2	3.3 ± 1.1
ManEL2	1.2 ± 0.4	4.0 ± 2.7
ManEL5	0.1 ± 1.4	0.7 ± 0.1

3.4 Temperature dependent adhesion inhibition of ConA

We compared *E. coli* inhibition studies to inhibition studies employing ConA as carbohydrate binding species. The microplates were again coated with mannan and the IC_{50} values were determined by incubating a glycopolymer concentration series in the presence of fluorescein labeled ConA.

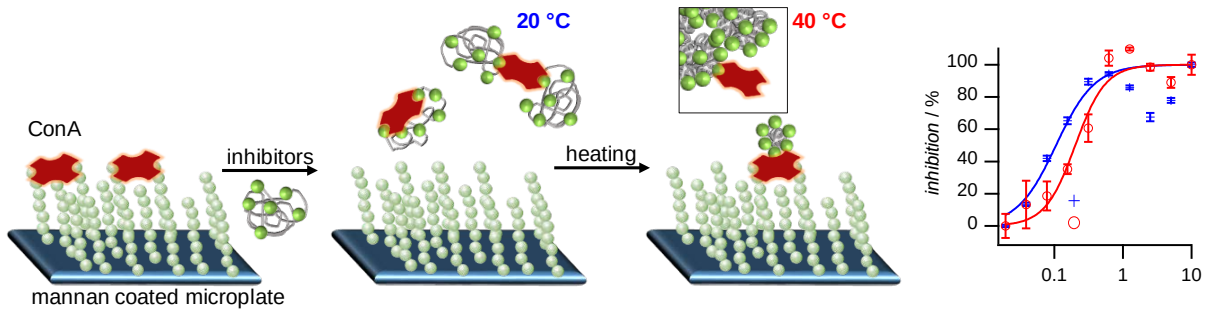

Figure 5 a) Temperature dependent inhibition of ConA binding. Below the phase transition temperature, the extended glycopolymer coil can bind to multiple ConA binding sites resulting in low IC_{50} values whereas this is not possible in the collapsed state where a large fraction of carbohydrate units is inaccessible due to aggregate formation. b) A typical inhibition curve at 20 °C and 40 °C for **ManHPL7**. The full set of inhibition curves is shown in Supporting Information S11.

As observed in the *E. coli* assay, when increasing the degree of Man functionalization reduced IC_{50} values are observed (**Figure 6**). However, the results from the inhibition assay with ConA shows two striking differences compared to the *E. coli* assay. First, the majority of compounds now shows increasing IC_{50} values (weaker inhibition) when increasing the temperature above the cloud point. Second, changing the linker type does not affect the IC_{50} values significantly. Regarding the temperature dependence of the IC_{50} values, it should be noted that ConA offers four binding sites with a minimum spacing of 7.2 nm,[29, 35] whereas *E. coli's* FimH receptor has only a single binding site and is positioned at larger spacing on the bacteria.[45] Dynamic light scattering of the glycopolymers showed that their coil-size was around 8 nm at 20 °C (Supporting Information S12). Therefore, the glycopolymers may bind to more than just one ConA binding site, whereas this is not possible when binding to FimH. At elevated temperatures where the polymer forms small globules, such multivalent binding to ConA is not possible leading to a reduced affinity and increased IC_{50} values. In addition, an extended coil conformation at 20°C

may increase the accessibility of Man-units for small receptors in solution phase (ConA), whereas a large fraction of Man units was rendered inaccessible due to aggregate formation above the cloud point temperature. The presence of microscopic aggregates thus effectively reduced the glycopolymer inhibitory potential for ConA but not for *E. coli* as the bacteria are also of microscopic size, i.e. their inhibition benefits less from an excess of low-affinity glycopolymers in solution but more from similar sized high-affinity aggregates capable of additional sterical shielding.[27] Therefore, the temperature induced phase transition upon heating resulted in weaker inhibition of ConA but stronger inhibition of *E. coli*. Nevertheless, the proposed increase in affinity in the compact-globule state at elevated temperature due to an increased surface density of Man units should also be present in case of binding to ConA. Therefore, the thermoresponsive binding of the glycooligomers to ConA is mediated by adverse effects: 1) reduced multivalent binding and lower Man accessibility above the LCST; 2) increased binding due to increased surface density of Man in the globule state. These opposing contributions may explain the less obvious temperature trend for inhibiting ConA as compared to inhibiting *E. coli*. This is also reflected in the comparatively small changes of the Hill coefficients at 20 °C and 40 °C (**Table 3**).

The reduced influence of the linker on inhibiting ConA binding when compared to *E. coli* binding, could be explained by structural differences of the binding sites. The ConA binding pocket mainly presents hydrophobic amino acids favoring hydrophobic linkers but at the binding pocket's entrance hydrophilic amino acids such as asparagine reside.[44,46] These residues may serve as hydrogen bond acceptors for the hydrophilic **HPL** linker or, alternatively, hydrogen bonding bridged by a hydration shell may increase binding to these residues.[47] In addition, such hydration layer mediated hydrogen bonding is generally less favored at increased temperature,[48] which reduces the inhibitory potential at elevated temperature, as observed.

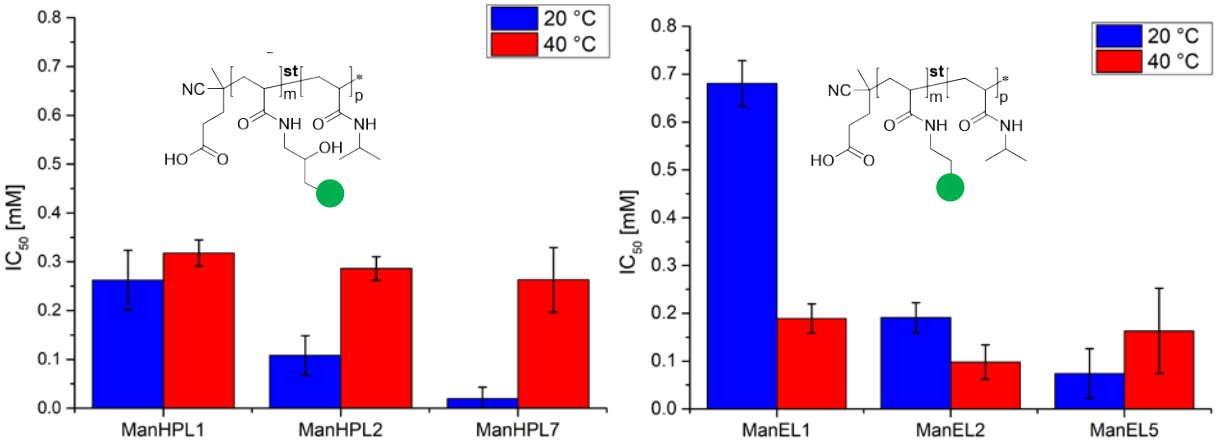

Figure 6 Left: IC_{50} values for the inhibition of ConA with ManHPL glycopolymers at 20 °C (blue) and 40 °C (red). Right: IC50 values for ManEL glycopolymers. All measurements were performed in triplicates.

Table 3 Hill coefficients from the ConA inhibition curves.

Sample	Hill coefficient at 20 °C	Hill coefficient at 40 °C
ManHPL1	5.2 ± 1.4	1.8 ± 0.4
ManHPL2	1.8 ± 0.4	3.4 ± 1.0
ManHPL7	2.0 ± 0.7	2.6 ± 1.2
ManEL1	1.7 ± 0.9	3.6 ± 0.8
ManEL2	2.5 ± 0.1	2.7 ± 1.1
ManEL5	3.4 ± 1.1	4.4 ± 0.8

Conclusion

Taken together, the straightforward synthesis of a poly(active ester) followed by grafting of carbohydrates with varying linker hydrophobicity and isopropylamine to induce thermoresponsiveness was carried out. A set of polymers was synthesized with carbohydrate functionalization degrees between 1% and 97% and tested by inhibition assays with ConA and type 1-fimbriated *E. coli* at 20°C and 40°C. For polymers with low functionalization degrees of 1% to 2% reaching the cloud point at around 40°C a large shift in affinity was observed.

Importantly, the inhibition of *E. coli* was increased under cloud point conditions, whereas the inhibition of ConA had the tendency to decrease at elevated temperatures although the results are not fully consistent in the case of ConA binding. This confirms the contradicting observations by several groups studying the effect of the temperature induced coil-to-globule transition on glycopolymer binding affinities. We propose that the enhanced inhibition of *E. coli* binding is driven by an enhanced presentation of carbohydrate units in the collapsed state where the glycopolymers form micrometer sized high-affinity aggregates capable of sterical shielding. ConA binding is reduced above the LCST due to reduced multivalent binding and reduced accessibility of the Man units compared to the extended coil state below the phase transition temperature. This suggests that small receptor targets such as single lectins in the solution phase are bound stronger by non-aggregated multivalent glycopolymers in the extended coil conformation, whereas the inhibition of large targets with surface-anchored receptors having access only to the exterior of polymer coils (bacteria, viruses) benefits from the increased surface density of ligands and steric shielding of polymer aggregates above the phase transition temperature. Overall, these results shed light on the conformation-dependent binding of glycopolymers and give the blueprint for the design of switchable ligand presenting polymers, e.g. for biomedical applications.

Acknowledgments

The authors thank Maria Breuer for performing the NMR measurements and Stephanie Scheelen for performing the SEC measurements. The authors acknowledge funding by the German Research foundation (DFG) in the project SCHM 2748/5-1.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

Tanja J. Paul and Alexander K. Strzelczyk contributed equally to this paper.

Supporting Information

Chemical analysis, polymer characterization, IC_{50} curves, DLS measurements, turbidity traces, details on instrumentation, buffers and media

References

1. Reitsma, S.; Slaaf, D. W.; Vink, H.; van Zandvoort, M. A.; oude Egbrink, M. G., The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Arch* **2007,** *454* (3), 345-59.
2. Schmidt, S.; Paul, T. J.; Strzelczyk, A. K., Interactive Polymer Gels as Biomimetic Sensors for Carbohydrate Interactions and Capture–Release Devices for Pathogens. *Macromol Chem Phys* **2019,** *220* (22), 1900323.
3. Jacobi, F.; Camaleno de la Calle, A.; Boden, S.; Grafmuller, A.; Hartmann, L.; Schmidt, S., Multivalent Binding of Precision Glycooligomers on Soft Glycocalyx Mimicking Hydrogels. *Biomacromolecules* **2018,** *19* (8), 3479-3488.
4. Ofek, I.; Hasty, D. L.; Sharon, N., Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immunol Med Microbiol* **2003,** *38* (3), 181-91.
5. Kiessling, L. L.; Grim, J. C., Glycopolymer probes of signal transduction. *Chem Soc Rev* **2013,** *42* (10), 4476-91.
6. Hudak, J. E.; Bertozzi, C. R., Glycotherapy: new advances inspire a reemergence of glycans in medicine. *Chem Biol* **2014,** *21* (1), 16-37.
7. Steinhilber, D.; Seiffert, S.; Heyman, J. A.; Paulus, F.; Weitz, D. A.; Haag, R., Hyperbranched polyglycerols on the nanometer and micrometer scale. *Biomaterials* **2011,** *32* (5), 1311-6.
8. Papp, I.; Sieben, C.; Ludwig, K.; Roskamp, M.; Bottcher, C.; Schlecht, S.; Herrmann, A.; Haag, R., Inhibition of influenza virus infection by multivalent sialic-acid-functionalized gold nanoparticles. *Small* **2010,** *6* (24), 2900-6.
9. Li, M. H.; Choi, S. K.; Leroueil, P. R.; Baker, J. R., Jr., Evaluating binding avidities of populations of heterogeneous multivalent ligand-functionalized nanoparticles. *ACS Nano* **2014,** *8* (6), 5600-9.
10. Bernardi, A.; Jimenez-Barbero, J.; Casnati, A.; De Castro, C.; Darbre, T.; Fieschi, F.; Finne, J.; Funken, H.; Jaeger, K. E.; Lahmann, M.; Lindhorst, T. K.; Marradi, M.; Messner, P.; Molinaro, A.; Murphy, P. V.; Nativi, C.; Oscarson, S.; Penades, S.; Peri, F.; Pieters, R. J.; Renaudet, O.; Reymond, J. L.; Richichi, B.; Rojo, J.; Sansone, F.; Schaffer, C.; Turnbull, W. B.; Velasco-Torrijos, T.; Vidal, S.; Vincent, S.; Wennekes, T.; Zuilhof, H.; Imberty, A., Multivalent glycoconjugates as anti-pathogenic agents. *Chem Soc Rev* **2013,** *42* (11), 4709-27.
11. Bhatia, S.; Camacho, L. C.; Haag, R., Pathogen Inhibition by Multivalent Ligand Architectures. *J Am Chem Soc* **2016,** *138* (28), 8654-66.
12. Muller, C.; Despras, G.; Lindhorst, T. K., Organizing multivalency in carbohydrate recognition. *Chem Soc Rev* **2016,** *45* (11), 3275-302.

13. Stuart, M. A.; Huck, W. T.; Genzer, J.; Muller, M.; Ober, C.; Stamm, M.; Sukhorukov, G. B.; Szleifer, I.; Tsukruk, V. V.; Urban, M.; Winnik, F.; Zauscher, S.; Luzinov, I.; Minko, S., Emerging applications of stimuli-responsive polymer materials. *Nat Mater* **2010,** *9* (2), 101-13.
14. Pasparakis, G.; Cockayne, A.; Alexander, C., Control of bacterial aggregation by thermoresponsive glycopolymers. *J Am Chem Soc* **2007,** *129* (36), 11014-5.
15. Mahon, C. S.; Wildsmith, G. C.; Haksar, D.; de Poel, E.; Beekman, J. M.; Pieters, R. J.; Webb, M. E.; Turnbull, W. B., A 'catch-and-release' receptor for the cholera toxin. *Faraday Discuss* **2019,** *219* (0), 112-127.
16. Dalier, F.; Eghiaian, F.; Scheuring, S.; Marie, E.; Tribet, C., Temperature-Switchable Control of Ligand Display on Adlayers of Mixed Poly(lysine)-g-(PEO) and Poly(lysine)-g-(ligand-modified poly-N-isopropylacrylamide). *Biomacromolecules* **2016,** *17* (5), 1727-36.
17. Tang, J. S. J.; Rosencrantz, S.; Tepper, L.; Chea, S.; Klopzig, S.; Kruger-Genge, A.; Storsberg, J.; Rosencrantz, R. R., Functional Glyco-Nanogels for Multivalent Interaction with Lectins. *Molecules* **2019,** *24* (10), 22.
18. Siirila, J.; Hietala, S.; Ekholm, F. S.; Tenhu, H., Glucose and Maltose Surface-Functionalized Thermoresponsive Poly(N-Vinylcaprolactam) Nanogels. *Biomacromolecules* **2020,** *21* (2), 955-965.
19. Paul, T. J.; Rubel, S.; Hildebrandt, M.; Strzelczyk, A. K.; Spormann, C.; Lindhorst, T. K.; Schmidt, S., Thermosensitive Display of Carbohydrate Ligands on Microgels for Switchable Binding of Proteins and Bacteria. *ACS Appl Mater Interfaces* **2019,** *11* (30), 26674-26683.
20. Vasani, R. B.; Janardanan, N.; Prieto-Simon, B.; Cifuentes-Rius, A.; Bradley, S. J.; Moore, E.; Kraus, T.; Voelcker, N. H., Microwave Heating of Poly(N-isopropylacrylamide)-Conjugated Gold Nanoparticles for Temperature-Controlled Display of Concanavalin A. *ACS Appl Mater Interfaces* **2015,** *7* (50), 27755-64.
21. Wang, Y.; Kotsuchibashi, Y.; Liu, Y.; Narain, R., Study of bacterial adhesion on biomimetic temperature responsive glycopolymer surfaces. *ACS Appl Mater Interfaces* **2015,** *7* (3), 1652-61.
22. Won, S.; Hindmarsh, S.; Gibson, M. I., Triggerable Multivalent Glyconanoparticles for Probing Carbohydrate-Carbohydrate Interactions. *ACS Macro Lett.* **2018,** *7* (2), 178-183.
23. Won, S.; Richards, S. J.; Walker, M.; Gibson, M. I., Externally controllable glycan presentation on nanoparticle surfaces to modulate lectin recognition. *Nanoscale Horiz* **2017,** *2* (2), 106-109.
24. Siirilä, J.; Hietala, S.; Ekholm, F. S.; Tenhu, H., Glucose and Maltose Surface-Functionalized Thermoresponsive Poly(N-Vinylcaprolactam) Nanogels. *Biomacromolecules* **2020,** *21* (2), 955-965.
25. Hoshino, Y.; Nakamoto, M.; Miura, Y., Control of protein-binding kinetics on synthetic polymer nanoparticles by tuning flexibility and inducing conformation changes of polymer chains. *J Am Chem Soc* **2012,** *134* (37), 15209-12.
26. Tang, J. S. J.; Rosencrantz, S.; Tepper, L.; Chea, S.; Klöpzig, S.; Krüger-Genge, A.; Storsberg, J.; Rosencrantz, R. R., Functional Glyco-Nanogels for Multivalent Interaction with Lectins. *Molecules* **2019,** *24* (10), 1865.
27. Vonnemann, J.; Liese, S.; Kuehne, C.; Ludwig, K.; Dernedde, J.; Bottcher, C.; Netz, R. R.; Haag, R., Size dependence of steric shielding and multivalency effects for globular binding inhibitors. *J Am Chem Soc* **2015,** *137* (7), 2572-9.

28. Mammen, M.; Choi, S. K.; Whitesides, G. M., Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angew Chem Int Ed* **1998,** *37* (20), 2755-2794.
29. Wittmann, V., Structural investigation of multivalent carbohydrate-protein interactions using synthetic biomolecules. *Curr Opin Chem Biol* **2013,** *17* (6), 982-9.
30. Hartmann, M.; Horst, A. K.; Klemm, P.; Lindhorst, T. K., A kit for the investigation of live Escherichia coli cell adhesion to glycosylated surfaces. *Chem Commun* **2010,** *46* (2), 330-2.
31. Gerchakov, S. M.; Hatcher, P. G., Improved Technique for Analysis of Carbohydrates in Sediments. *Limnol Oceanogr* **1972,** *17* (6), 938-943.
32. DuBois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F., Colorimetric Method for Determination of Sugars and Related Substances. *Anal Chem* **1956,** *28* (3), 350-356.
33. Boden, S.; Wagner, K. G.; Karg, M.; Hartmann, L., Presenting Precision Glycomacromolecules on Gold Nanoparticles for Increased Lectin Binding. *Polymers* **2017,** *9* (12), 716.
34. Bordegé, V.; Muñoz-Bonilla, A.; León, O.; Sánchez-Chaves, M.; Cuervo-Rodríguez, R.; Fernández-García, M., Glycopolymers with glucosamine pendant groups: Copolymerization, physico-chemical and interaction properties. *React Funct Polym* **2011,** *71* (1), 1-10.
35. Wittmann, V.; Pieters, R. J., Bridging lectin binding sites by multivalent carbohydrates. *Chem Soc Rev* **2013,** *42* (10), 4492-503.
36. Becker, J. W.; Reeke, G. N., Jr.; Cunningham, B. A.; Edelman, G. M., New evidence on the location of the saccharide-binding site of concanavalin A. *Nature* **1976,** *259* (5542), 406-9.
37. Bryce, R. A.; Hillier, I. H.; Naismith, J. H., Carbohydrate-protein recognition: molecular dynamics simulations and free energy analysis of oligosaccharide binding to concanavalin A. *Biophys J* **2001,** *81* (3), 1373-88.
38. Dam, T. K.; Brewer, C. F., Thermodynamic studies of lectin-carbohydrate interactions by isothermal titration calorimetry. *Chem Rev* **2002,** *102* (2), 387-429.
39. Lundquist, J. J.; Toone, E. J., The cluster glycoside effect. *Chem Rev* **2002,** *102* (2), 555-78.
40. Gestwicki, J. E.; Cairo, C. W.; Strong, L. E.; Oetjen, K. A.; Kiessling, L. L., Influencing receptor-ligand binding mechanisms with multivalent ligand architecture. *J Am Chem Soc* **2002,** *124* (50), 14922-33.
41. Sperling, O.; Fuchs, A.; Lindhorst, T. K., Evaluation of the carbohydrate recognition domain of the bacterial adhesin FimH: Design, synthesis and binding properties of mannoside ligands. *Org Biomol Chem* **2006,** *4* (21), 3913-22.
42. Knight, S. D.; Bouckaert, J., Structure, function, and assembly of type 1 fimbriae. *Topics curr chem* **2009,** *288*, 67-107.
43. Cecioni, S.; Imberty, A.; Vidal, S., Glycomimetics versus multivalent glycoconjugates for the design of high affinity lectin ligands. *Chem Rev* **2015,** *115* (1), 525-61.
44. Igde, S.; Roblitz, S.; Muller, A.; Kolbe, K.; Boden, S.; Fessele, C.; Lindhorst, T. K.; Weber, M.; Hartmann, L., Linear Precision Glycomacromolecules with Varying Interligand Spacing and Linker Functionalities Binding to Concanavalin A and the Bacterial Lectin FimH. *Macromol Biosci* **2017,** *17* (12), 1700198.
45. Mortezaei, N.; Singh, B.; Zakrisson, J.; Bullitt, E.; Andersson, M., Biomechanical and structural features of CS2 fimbriae of enterotoxigenic Escherichia coli. *Biophys J* **2015,** *109* (1), 49-56.

46. Sanders, D. A.; Moothoo, D. N.; Raftery, J.; Howard, A. J.; Helliwell, J. R.; Naismith, J. H., The 1.2 A resolution structure of the Con A-dimannose complex. *J Mol Biol* **2001,** *310* (4), 875-84.
47. Loris, R.; Stas, P. P.; Wyns, L., Conserved waters in legume lectin crystal structures. The importance of bound water for the sequence-structure relationship within the legume lectin family. *J Biol Chem* **1994,** *269* (43), 26722-33.
48. Lemieux, R. U., How Water Provides the Impetus for Molecular Recognition in Aqueous Solution. *Acc Chem Res* **1996,** *29* (8), 373-380.

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Temperature switchable glycopolymers and their conformation-dependent binding to receptor targets

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