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Double-Modified Glycopolymers from Thiolactones to Modulate Lectin Selectivity and Affinity

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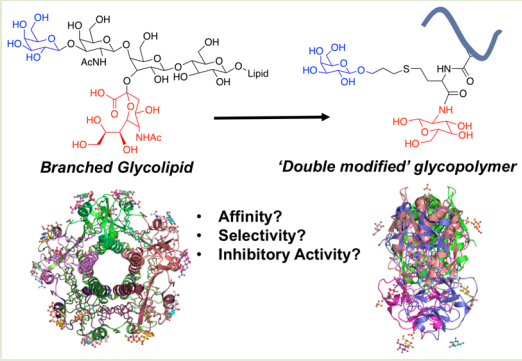
Supporting Information

ABSTRACT: Multivalent glycomaterials show high affinity toward lectins but are often nonselective as they lack the precise 3-D presentation found in native glycans. Here, thiolactone chemistry is exploited to enable the synthesis of glycopolymers with both a primary binding (galactose) and a variable secondary binding unit in close proximity to each other on the linker. These polymers are used to target the Cholera toxin B subunit, CTxB, inspired by its native branched glycan target, GM-1. The secondary, nonbinding unit was shown to dramatically modulate affinity and selectivity toward the Cholera toxin. These increasingly complex glycopolymers, assembled using accessible chemistry, can help breach the synthetic/biological divide to obtain future glycomimetics.



Many bacterial and viral pathogens exploit carbohydrate-binding proteins (lectins) as part of their infection cycle. This includes viral glycans binding to host cells,[1] bacterial lectins modulating biofilm formation[2] or cell adhesion,[3,4] or secreted toxins which are glycan-binding in their mode of action.[5] With the increase in antimicrobial resistance[6] there is an urgent need for new therapeutics and diagnostics which are not based on the traditional small-molecule approach. Glycans typically have weak affinities to lectins (carbohydrate binding proteins which are not enzymes nor antibodies) in mM range. In contrast, due to the cluster glycoside effect, multivalent presentation of glycans on, e.g., polymers, particles, or surfaces gives a nonlinear increase in affinity, such that sub nM affinities can be obtained.[7−10] Hence there are significant opportunities in the design of multivalent glycomaterials as prophylactic treatments and biosensors and for understanding the glycome.[4,11−17]

The cholera toxin (CTx) is a lectin produced as a virulence agent by *Vibrio cholerae* and binds via its adhesive subunit (CTxB) to the GM-1 glycan on the surface of human gut epithelial cells, with more than 1 million cases/year globally.[18] Galactosylated multivalent systems have been studied as decoys for CTxB, using polymers,[19−21] dendrimers,[22] protein scaffolds,[23] and a picomolar-active pentavalent calix[5]arene.[24] These examples are all homogeneous materials bearing a single glycan/functionality, but Worstell et al. found that CTxB binding to GM-1 surfaces is enhanced by the addition of fucose, which was not thought to have affinity for CTxB.[25] Dimeric fucose has also been shown to competitively inhibit CTxB binding to epithelial cells.[26] While there is significant evidence for high affinity binders to CTxB (and other lectins) selectivity still remains a challenge; most lectins have off-specific affinity for other glycans, and any glycan can bind several lectins, especially for monosaccharides which lack a complex 3-D structure required for a match.[27]

Richards et al. and Kiick et al. have demonstrated that modulation of the galactose-backbone linker length enabled modulation of the relative affinity of galactose polymers to CTx, based on accessibility of the ligands into the deep binding pocket of CTx.[19,21] Bundle and co-workers have developed galactosylated multivalent scaffolds with increased affinity by introduction of additional functionality to target the allosteric *N*-acyl-neuraminic acid binding site in CTxB[28] with aromatic residues found to enhance affinity.[20] Fieschi and co-workers have developed small-molecule and low valency compounds to selectively target DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) without cross-binding to the Langerin lectin, which is essential for HIV antiadhesives.[29,30] Hartman and co-workers and Percec and co-workers have demonstrated that heterogeneous glycopolymers (with more than one glycan) can show surprising increases in affinity due to a combination of spacing and steric blocking effects.[31−33] In short, a homoglycopolymer may not always be the most avid binder, nor the most potent inhibitor, and hence

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exploring more chemical space using heterogeneous glycopolymers may enable selectivity to be introduced.

Advances in controlled reversible-deactivation radical polymerization and bio-orthogonal[34] (click) reactions enable easy access to a wide range of architectures and hence opportunities to modulate affinity. Becer et al. have shown that changing from flexible to rigid[35] or linear to star shapes has profound effects on glycopolymer binding of DC-SIGN and dendritic cell cytokine production.[36,37] The introduction of *selectivity* toward *relevant* lectins is essential to enable translation of these exciting materials.

We report here the synthesis and lectin binding properties of glycopolymers obtained using thiolactone chemistry.[38−40] This functional group enables one-pot, two-sequence reactions on a single monomer unit, to allow the linker chemistry in proximity to the primary binding unit (galactose) to be modulated. Using a combination of inhibitory assays and biolayer interferometry we demonstrate that the selectivity and affinity of glycopolymers can be tuned through introduction of secondary functionalities and show that the total binding capacity, not affinity, correlates strongly with inhibitory activity.

To introduce variable carbohydrate density on polymer backbones, the reactive precursor, thiolactone acrylamide (TLAm), was copolymerized with *N*-hydroxyethylacrylamide (HEA) using RAFT (reversible addition−fragmentation chain-transfer) polymerization (Figure 1). The molar ratio of TLAm

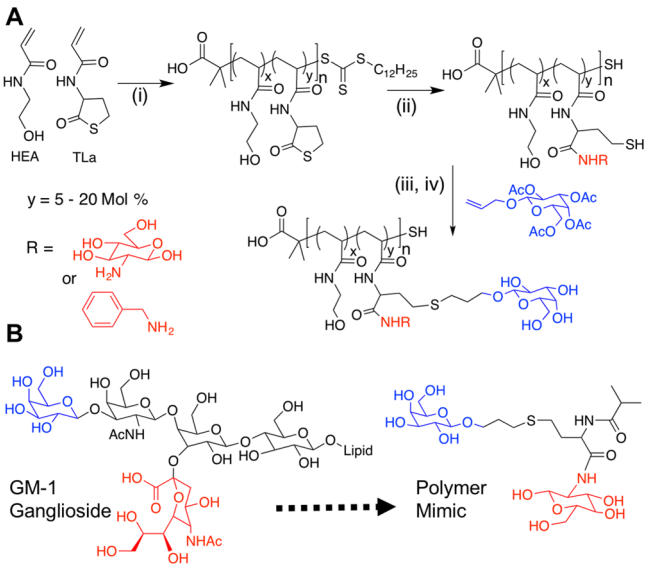

Figure 1. (A) Synthetic methodology. (i) RAFT polymerization, (ii) ring opening of thiolactone, and (iii, iv) thiol−ene click and deprotection. (B) Polymer design concept to mimic GM-1-branched structure.

was varied from 5 to 20% to ensure a soluble polymer was obtained but with sufficient valency for cluster glycoside enhancement.[8,19,41] All polymers were analyzed by SEC, ^{1}H NMR, and FTIR analysis (Table 1).

The reactive thiolactone precursor polymers were glycosylated in a one-pot, two-stage process.[38] Thiolactones were first ring-opened by addition of benzylamine or glucosamine (secondary binding units), releasing the thiol group which could then react with *β*-1-*O*-allyl galactose tetra-acetate (primary binding unit; Supporting Information for synthesis) by "thiol−ene" radical click chemistry.[42] The acetate groups were quantitatively removed by sodium methoxide, followed by dialysis, and confirmed by FTIR spectroscopy. By using this strategy, a panel of doubly modified glycopolymers intended to mimic GM-1 was established. The number of galactose/chain is in Table 1. A longer, hexyl, linked galactose was also synthesized and used (see below for discussion).

With this diverse panel of variable carbohydrate density and secondary units glycopolymers in hand, their potency to inhibit the binding of CTxB and RCA_{120} (*Ricinus communis* agglutinin which also has affinity for terminal galactose[43]) was evaluated by a fluorescence-linked sorbent assay.[19] Fluorescently labeled lectins were incubated with a serial dilution of each polymer and then tested for binding to a galactose-modified microtiter plate. Less fluorescence indicated more inhibition. Fitted binding curves and extracted MIC_{50} (minimum inhibitory concentration) values are shown in Figure 2, in terms of [Galactose] to ensure fair comparison "per binding site".

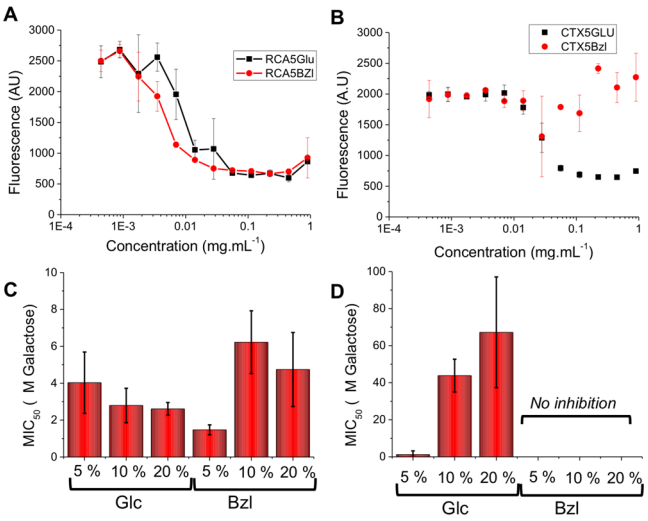

Figure 2. Inhibitory potency of glycopolymers using a fluorescence-linked sorbent assay. Inhibition curves for 5% density polymers against (A) RCA_{120} and (B) CTxB. Extrapolated MIC_{50} values (corrected to total galactose concentration) for polymers against (C) RCA and (D) CTxB.

Addition of increasingly high concentrations of the glycopolymers resulted in more inhibition of the toxins as

Table 1. Thiolactone Acrylamide Containing Polymers

code	TLAm[a] (%)	[M]:[CTA] (−)	conv.[b] (%)	composition (−)	$M_{n(theo)}$[d] (g mol^{-1})	$M_{n(SEC)}$[e] (g mol^{-1})	Đ (−)
P1	5	100	95.2, 99.5	93 (HEA), 5.0 (TLAm)	11 500	8100	1.34
P2	10	100	93.2, 98.2	86.9 (HEA), 10 (TLAm)	11 600	9700	1.29
P3	20	100	76.3, 84.0	61.1 (HEA), 16.8 (TLAm)	9700	9200	1.26

[a]mol % TLAm monomer. [b]Conversion by ^{1}H NMR (the first value shown is percentage conversion of HEA; second is for TLAm). [c]Composition of polymer based on conversion of each monomer. [d]Theoretical M_n from feed ratio. [e]M_n from SEC using PMMA standards.

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expected. Against RCA_{120} (which has a surface-accessible, relatively unhindered, galactose binding site) there was little impact of changing the secondary unit from glucose to benzyl with MIC_{50} < 0.1 mg mL^{-1} observed. In dramatic contrast, the benzyl-modified glycopolymers were very poor inhibitors of CTxB binding, with essentially no inhibition (which does not necessarily rule out binding; see below). The glucose-modified polymer was a potent inhibitor with μM MIC_{50} values in terms of [Galactose], which is nM in [polymer] (due to their relatively high molecular weight). The lower density polymers (5%) were more potent inhibitors than higher density ones (20%) on a per-galactose basis. Nonlinear relationships between galactose density and CTX inhibition have previously been reported on both rigid[21] and flexible glycopolymers,[19] and one of the most potent inhibitors reported had only five galactose units but perfect symmetry matching with CTxB.[23] Per-sugar affinity gains are obtained by matching the spacing between binding pockets and controlling accessibility as is seen here.

The lack of inhibitory activity for the benzyl modification was surprising, as introducing aromatic groups near galactose to target an allosteric site in CTxB has proven beneficial.[20,28] However, this requires precision placement of individual ligands which might not occur here, and the benzyl unit could simply be acting as a steric block. A simple docking study was conducted using the "Swissdock" server to probe if the repeat unit could access the CTx binding pocket. This suggested that the linker could extend sufficiently deep into the pocket (12.7 Å) but would be subject to significant steric requirements if on a polymer backbone and did not show any favorable interactions. To probe the interactions in more detail, biolayer interferometry (BLI) was employed. BLI enables label-free evaluation of binding interactions and is similar to surface plasmon resonance (SPR).[44] Lectins were immobilized onto the BLI sensors using conventional NHS/EDC coupling. The polymers were studied in a dose-dependent manner against the lectins, and results were fitted using a heterogeneous sites model (Figure 3).

Figure 3A and B shows example BLI binding curves for glucose and benzyl secondary substituents. In both cases there are clear association and dissociation phases, and dose-dependent responses in total mass bound are observed. The glucose-modified polymers showed a steeper association phase and overall larger Δ_{max} (mass bound). Figure 3C shows the total mass captured (Δ_{max}) by the surface-immobilized CTxB per polymer. For all galactose densities, polymers with glucose as the secondary unit showed significantly increased binding compared to the benzyl. Our postpolymerization synthetic strategy ensures these differences are not biased by M_w differences and hence are due to the ability of the polymers to bind to the CTxB. Interestingly, the Δ_{max} values correlated with the MIC_{50} values (Figure 2), suggesting that the total *extent* of binding is the most important descriptor of inhibitory potency rather than just affinity, along with the ability to bind multiple sites simultaneously.[45,46] Drug residence time, rather than affinity, has emerged as a key target in small-molecule drug discovery, supporting this design approach.[47] Estimates of the dissociation constant were made from the fitted BLI curves (to a heterogeneous site model) which suggests that the glucose polymers had lower overall affinity, but this is biased by the plateauing effect at low concentrations and low mass captured, so is only an estimate. Extracted values are tabulated for completeness in the Supporting Information but due to the

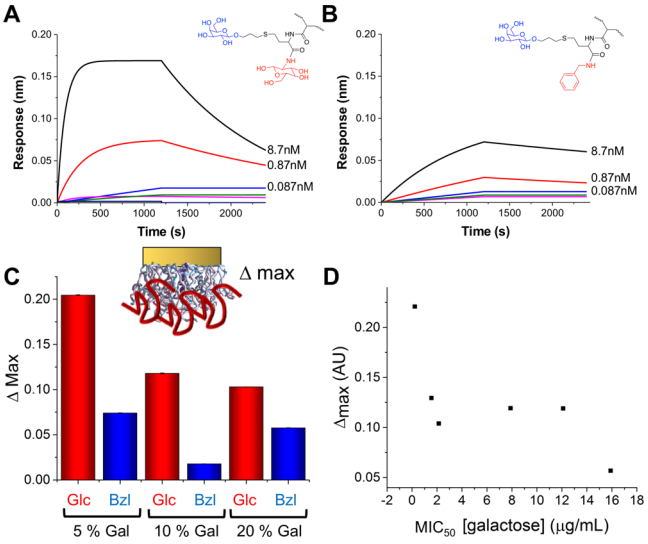

Figure 3. Biolayer interferometry analysis of glycopolymers binding to CTx. Example binding curves for Glc (A) and Bzl (B) side chains (total polymer concentration). (C) Maximum mass of glycopolymer bound (Δ_{max}) in biolayer interferometry assay. (D) MIC_{50} [galactose] from fluorescence-linked inhibitory assays versus Δ_{max}.

heterogeneity and incomplete fitting were not considered further. To reiterate, the aim here was to develop inhibitors, and the BLI enabled a link to the observed MIC testing.

Analysis of the CTxB binding site depth showed that the allyl linker was only just long enough to probe into it, which might explain the observations above. Therefore, a longer linker, β-D-1-*O*-hexyl-galactose tetra-acetate, was synthesized and incorporated into the same precursor polymers, with both benzyl and glucose secondary groups to give a linker that can probe up to 16.5 Å, rather than 12.7 Å. BLI was again conducted, and the total mass captured is plotted in Figure 4. This increase in linker length did not modulate the total mass captured significantly, with the same trends seen as for the allyl linker, suggesting both had equal access. This suggests the secondary units are modulating the overall sterics, rather than binding to any defined allosteric sites (as the change in spacing would change this). These inhibitory assays and BLI results

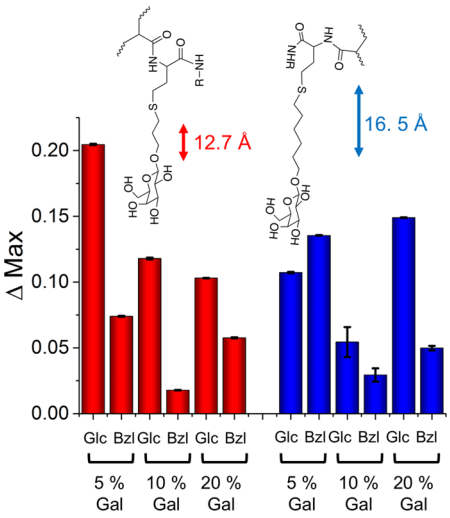

Figure 4. Effect of linker length on total mass captured as a function of linker length for polymer library against CTxB.

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together demonstrate that by using accessible and modular chemistries the affinity and selectivity of multivalent glycopolymer antiadhesives can be tuned by proximal modification of the key binding motifs, in addition to the well-explored impact of polymer molecular weight and branching.

In conclusion, we have taken advantage of the modular and versatile thiolactone functionality to develop more complex glycopolymers bearing both primary glycan ligands and also secondary units to modulate the selectivity of these materials toward lectins implicated in disease. A library of polymers were synthesized with systematic variation of their glycan density and secondary group, and these were evaluated in a competitive binding assay. This analysis revealed that low density glycopolymers were the most active (lowest MIC_{50}). Furthermore, addition of secondary groups proximal to the galactose enabled complete switching off of the activity versus the cholera toxin, while retaining all activity against RCA_{120}, and is a rare example of a glycopolymer with selectivity. Biolayer interferometry revealed that the total mass of glycopolymers bound by the lectin correlated strongly with the observed inhibitory activity which will help the design of new inhibitors for application in biosensing or antiadhesion therapy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.8b00825. The research data supporting this publication can be found at http://wrap.warwick.ac.uk.

Full experimental details, including synthesis/characterization and additional BLI data (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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