# Effects of Saccharide Spacing and Chain Extension on Toxin Inhibition by Glycopolypeptides of Well-Defined Architecture

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ABSTRACT: Many recognition events important in biology are mediated via multivalent interactions between relevant oligosaccharides and multiple saccharide receptors present on lectins, viruses, toxins, and cell surfaces. Because of the important role played by protein−carbohydrate interactions in these pathogenic recognition events and in other human diseases, considerable effort has been devoted toward the development of multivalent polymeric ligands for carbohydrate-binding proteins. In this work, we report the synthesis of new polypeptide-based glycopolymers produced via a combination of protein engineering and chemical methods. These methodologies permit control over the number and the spacing of saccharides on the scaffold, as well as the conformation of the polymer backbone, and allow a more purposeful design of polymers for manipulation of multivalent binding events. Two families of galactose-bearing glycopolypeptides with random coil conformations, $[(AG)_3PEG]_y$ ($y$ = 10 and 16) and $\{[(AG)_2PSG]_2[(AG)_2PEG][(AG)_2PSG]_2\}_y$ ($y$ = 6), have been synthesized. The carboxylic acid functionality of the glutamic acid residues allowed subsequent modification with amino-saccharides to yield the desired glycopolypeptides; selective placement of the glutamic acid group permitted investigation of the effects of multivalency and saccharide spacing on toxin inhibition. In addition, a family of galactose-functionalized PGA-based glycopolymers of varying molecular weights was also synthesized to compare the effects of backbone flexibility and hydrodynamic volume, relative to the recombinant glycopolypeptides, on toxin inhibition. Glycopolypeptides were characterized via $^1$H NMR, MALDI-TOF mass spectrometry, SDS-PAGE analysis, and spectrophotometric assays. They were tested as inhibitors of the binding of the cholera toxin B subunit via direct enzyme-linked assays. The data from these experiments confirm the relevance of appropriate saccharide spacing on controlling the binding event and also indicate the influence of chain extension in improving inhibition.

## Introduction

Multivalent interactions are known to occur throughout nature and are characterized by the simultaneous binding of multiple ligands on one biological entity to multiple receptors on another.[1] Such phenomena are typically observed in cell−protein, cell−cell, protein−protein, and protein−carbohydrate interactions and are known to play a key role in mediating a variety of processes relevant to human biology. Of particular interest are those interactions that occur between proteins and carbohydrates, as they are known to participate in the initial stages of infection by a variety of pathogens. For example, the $AB_5$ bacterial toxins (which include the cholera toxin, shiga-like toxins, and the heat labile enterotoxin) gain entry into the host cell via interactions between five identical receptor-binding sites on the toxin's B pentamer with specific gangliosides displayed on the epithelial cell surface of the human host. Other examples of receptor-mediated binding events include the adhesion of the influenza virus to the surface of bronchial epithelial cells,[1,2] the recruitment of neutrophils to a site of inflammation,[3−5] and cell signaling, organogenesis, and fertilization.[6−8]

Recent efforts by a variety of researchers have yielded many synthetic small-molecule and polymer- and polypeptide-derived multivalent ligands designed to act as inhibitors and effectors of various protein−carbohydrate recognition events.[9−29] In particular, these investigations have probed the effects of varying the saccharide density (which influences distance between adjacent saccharides), valency (number of saccharides), as well as the length and hydrophobicity of the tether group on the ability of these materials to control the multivalent binding event. For example, Cairo et al. used ROMP methods to study the influence of multivalent ligand binding epitope density on the clustering of a model receptor, concanavalin A (Con A).[20] Woller et al. demonstrated changes in the binding to Con A of PAMAM dendrimers (generations three through six), in which the loading of mannose surface residues was varied.[19] Iyer et al. have produced HA (hyaluronan)-functionalized norbornene glycopolymers via ROMP methods to facilitate the events mediated by HA binding receptors,[21] and Baessler et al. have also employed ROMP to produce peptide-modified polymers for the investigation of the interaction of sperm protein fertilin $\beta$ with its egg receptor.[22] Other controlled radical polymerization methods, such as ATRP, have been used in the synthesis of biotinylated glycopolymers with high affinity to streptavidin via the synthesis of polymers decorated with linear and cyclic carbohydrate moieties.[23] Glycopolymers equipped with globotriose and/or lactose have shown inhibitory effects on the cytotoxity of certain toxins.[24] These previous investigations of glycopolymeric materials have suggested the importance of architectural variables in the binding event, and have contributed significantly to the general understanding of architectural guidelines that govern multivalent interactions in chemically derived glycopolymers. The lack of precise control in the polydispersity and placement of saccharides on polymer backbones, however, has limited the detailed understanding and manipulation of the multivalent binding event on the basis of polymer architectural variables.

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In a related area of research, the development of multivalent antagonists for disrupting bacterial adhesion to cell surfaces has been of significant interest, and multiple groups have investigated inhibitors of various $AB_5$ toxins, including cholera toxin (CT), shiga-like toxins (SLT-I and SLT-II), and the heat-labile enterotoxin (LT).[30−40] Infection by these pathogens is initiated via binding of the $B_5$ subunit of the $AB_5$ toxin with multiple gangliosides on the surface of human intestinal epithelial cells; inhibition of the binding of the $B_5$ subunit therefore serves as a therapeutic target as well as model system for the development of improved oligo- and multivalent inhibitors. For example, Kitov et al. reported optimization of the tether length in linear, non-glycosidically linked bivalent ligands that target sites 2 and 1 of the Shiga-like toxin,[34] and Pickens et al. demonstrated inhibition of the cholera toxin $B_5$ subunit (CT $B_5$) with linear bivalent inhibitors.[39] Fan et al. have reported the synthesis of a highly potent, branched pentavalent ligand with varying linker arm lengths designed to inhibit heat-labile enterotoxin[38] and cholera toxin.[31,35] These investigations of the small-molecule inhibitors underscore the ability to tailor activity on the basis of controlling molecular architecture and saccharide spacing, which remains elusive for synthetic glycopolymeric systems. Investigations of CT inhibitors, in particular, have been facilitated by the fact that inhibition of the binding of CT $B_5$ and its ganglioside target GM1 (Gal$\beta$1-3GalNAc$\beta$1-4(Neu5Ac$\alpha$2-3)-Gal-$\beta$1-4Glc ceramide) can be mediated by oligo- and multivalent molecules derivatized with various simple galactopyranosides.[38,40] The CT $B_5$ therefore also serves as a suitable model system for the development of glycopolymeric inhibitors and glycopolymer design principles that may be applied to a broader range of targets (e.g., larger-scale receptor assemblies)[41] than small molecule approaches.

Accordingly, we have been developing strategies for the production of polypeptide-based glycopolymeric materials that can be used to study and manipulate interactions between saccharides and their receptors. Polypeptides offer advantages such as manipulation of backbone conformation and functional group placement, as well as opportunities for additional, prescribed secondary interactions with protein targets. We recently reported the synthesis of select galactose-functionalized glycopolypeptides with random coil and helical conformations (polypeptides generated via chemical and recombinant methods, respectively), in which the density and linker length of the pendant carbohydrate moiety could be varied.[42,43] The ability of the polypeptide-based glycopolymers to act as inhibitors of the CT $B_5$ subunit was tested, and our preliminary results suggested that these polypeptides would be useful for gauging variations in binding affinity with variations in glycopolymer architecture.

In this work, we describe the synthesis, via a combination of protein engineering and chemical approaches, of an expanded set of random-coil glycopolypeptides of precise sequence and controlled saccharide placement, for comparison to previously reported glycopolypeptides. Two families of galactose-bearing glycopolypeptides with random coil conformations, $[(AG)_3PEG]_y$ ($y = 10$ and 16) and $\{[(AG)_2PSG]_2[(AG)_2PEG][(AG)_2PSG]_2\}_y$ ($y = 6$), have been synthesized. The carboxylic acid functionality of the glutamic acid residue allows the coupling of various amine-functionalized saccharides, and in these sequences, the distance between adjacent glutamic acid residues has been varied in order to monitor the effects of saccharide spacing on toxin inhibition with greater precision than previously possible. A family of galactose-functionalized PGA-based glycopolymers of varying molecular weights was also synthesized, for comparison of the effects of backbone charge and chain flexibility (relative to the recombinant glycopolypeptides) on toxin inhibition. Comparisons of the inhibition of CT $B_5$ by these random-coil polypeptide-based glycopolymers were made via direct enzyme-linked assays.

## Experimental Section

**Materials and Methods.** *Protein Engineering.* Stab cultures of pET3b (BL21(DE3)pLysS) hosts equipped with plasmids encoding the $[(AG)_xPEG]_y$ polypeptides were donated by Jill Sakata and David Tirrell.[44] The cloning plasmid pUC-19 and expression plasmids pET-28b and pET-21b were purchased from Novagen (San Diego, CA) and EMD Biosciences (San Diego, CA), respectively. Expression plasmid pET-28b was further modified via insertion of an oligonucleotide containing two internal *Bsa* I restriction sites for the insertion of the oligonucleotides that encode target protein polymers (pET28b-JS1). All restriction endonucleases and DNA mass ladders were purchased from New England Biolabs (Beverly, MA). Synthetic oligonucleotides were obtained from Sigma Genosys (The Woodlands, TX). Mini prep kits, gel extraction kits, plasmid purification kits, and nickel-chelated sepharose resin were obtained from Qiagen (Valencia, CA). General reagents for protein expression and purification were obtained from Sigma (St. Louis, MO) and Fisher Scientific (Fairlawn, NJ).

*Glycopolypeptide Synthesis and Characterization.* 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Fmoc-6-aminohexanoic acid (Fmoc-Ahx-OH) were obtained from EMD Biosciences Inc. (San Diego, CA). The cholera toxin $B_5$ subunit horseradish peroxidase conjugate (CT $B_5$-HRP) was obtained from List Biological Laboratories (Campbell, CA). Ganglioside $GD_{1b}$ was obtained from Matreya (Pleasant Gap, PA). C96 Maxisorp microtiter plates were obtained from Fisher Scientific (Pittsburgh, PA). A glycoprotein carbohydrate estimation kit and a GelCode glycoprotein staining kit were purchased from Pierce (Rockford, IL). Poly(L-glutamic acid) (PGA, DP = 113, 86 and 30), $\beta$-D-galactosylamine, dimethyl sulfoxide (DMSO), diisopropylethylamine (DIPEA), and all other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO) and used as received without any further purification.

*Instrumental Methods.* $^1$H NMR spectra were acquired on a Bruker DRX-400 NMR spectrometer under standard quantitative conditions at ambient temperature. MALDI-TOF analysis of purified polypeptides and glycopolypeptides was performed at the Mass Spectrometry Facility in the Department of Chemistry and Biochemistry at the University of Delaware on a Biflex III (Bruker, Billerica, MA). The samples were prepared in a 3,5-dimethoxy-4-hydroxycinnamic acid matrix with the calibration mixture: insulin from bovine $(M + H)^+ = 5734.59$, thioredoxin from *E. coli* $(M + H)^+ = 11\,674.48$, and apomyoglobin from horse $(M + H)^+ = 16\,952.56$. Data were recorded with the OmniFLEX program and subsequently analyzed in the XmassOmni program.

**Protein Expression and Purification.** Expression of $[(AG)_3PEG]_y$ (where $y = 10$ and 16) from expression plasmid pET-21b was conducted via similar methods as previously described,[44] with purification via metal chelate affinity chromatography (Supporting Information). These polypeptides are designated as **1** and **2**, respectively. A similar protocol was employed for the expression and purification of $\{[(AG)_2PSG]_2[(AG)_2PEG][(AG)_2PSG]_2\}_y$ (**3**). The cloning plasmid pUC19-35-RC and expression plasmid pET-28b-JS1-35-RC-6, encoding genes for the synthesis of $\{[(AG)_2PSG]_2[(AG)_2PEG][(AG)_2PSG]_2\}_y$, were produced via standard molecular biology protocols, and the polypeptide expression and purification were also conducted via standard methods (Supporting Information). The purity of the polypeptides was confirmed via reverse-phase HPLC, MALDI-TOF MS, SDS-PAGE, $^1$H NMR, and amino acid analysis (Supporting Information).

**Preparation of Glycopolypeptides.** The general procedure for the preparation of glycopolypeptides modified with *N*-($\epsilon$-aminocaproyl)-$\beta$-D-galactosylamine has been previously reported.[42,45] The synthesis of *N*-($\epsilon$-aminocaproyl)-$\beta$-D-galactosylamine has also been

**Table 1. Composition of Synthesized Glycopolypeptides**

| ID | sequence | DS (mol %)[a] | approx dist between adjacent saccharides (Å)[b] | mol mass (kDa)[e] |
|---|---|---|---|---|
| **1** | 17-RC-10[c] | N/A | N/A | 7.1 |
| **2** | 17-RC-16[c] | N/A | N/A | 11.1 |
| **3** | 35-RC-6[c] | N/A | N/A | 18.1 |
| **4** | Cap-17-RC-10[d] | 85 ± 0.9 | 17 | 9.7 |
| **5** | Cap-17-RC-16 | 71 ± 2.1 | 17 | 14.3 |
| **6** | Cap-35-RC-6 | 72 ± 1 | 32 | 19.5 |
| **7** | Cap-poly(Glu)$_{30}$ | 25 ± 1.1 | 15.2 | 5.9 |
| **8** | Cap-poly(Glu)$_{86}$ | 45 ± 4.0 | 8.4 | 17.9 |
| **9** | Cap-poly(Glu)$_{113}$ | 11.1 ± 2.2 | 34.2 | 21.8 |

[a] DS for all samples was determined via two or more of the following techniques: $^1$H NMR spectroscopy, MALDI-TOF mass spectrometry, and a spectrophotometeric assay. The reported value is the average degree of substitution as determined by the multiple techniques, and the reported error is the standard deviation. These results were reproducibly obtained from multiple syntheses of glycopolypeptides with the targeted degrees of substitution. [b] The distance between adjacent functional groups for **1**−**3** (and therefore saccharides for compounds **4**−**6**) was determined via molecular dynamics simulations (Supporting Information), whereas for compounds **7**−**9**, the distance between adjacent saccharide moieties was estimated by calculating the distance assuming a uniform density of saccharides on a polyglutamic acid chain in an extended conformation. [c] 17-RC-10 = $[(AG)_3PEG]_{10}$; 17-RC-16 = $[(AG)_3PEG]_{16}$; 35-RC-6 = $\{[(AG)_2PSG]_2[(AG)_2$-PEG] $[(AG)_2PSG]_2\}_6$. [d] Cap = *N*-($\epsilon$-aminocaproyl)-$\beta$-D-galactosylamine. [e] The experimental molecular masses listed were obtained from the MALDI experiments and represent the mass of the most intense species. The numbers are also in agreement with the calculated DS reported for each glycopolypeptide. There is some breadth in the signal resulting from slight heterogeneity in glycosylation as well as from the very high laser power needed to ionize the samples in the MALDI experiments.

reported previously.[42,45,46] Representative characterization data for the glycopolypeptides are given below.

Mass spectroscopy: **4**: calculated (expected molecular weight for 100% glycosylation) = 10 094, observed $(M + H)^+ = 9646.26$; **5**: calculated = 15 744, observed $(M + H)^+ = 14\,253.38$; **6**: calculated = 20 073, observed $(M + H)^+ = 19\,497.2$. Calculated values are those based on a fully glycosylated polypeptide. Observed values differ from the calculated values by different extents depending on and in agreement with the actual degree of substitution of the glycopolypeptide (Table 1).

$^1$H NMR data of **6**: ($D_2O$, 25 °C, 400 MHz): $\delta$ 4.29−4.70 and $\delta$ 3.97 (237H, polypeptide α-methine NHC*H*CO), $\delta$ 3.88 (52H, Ser $\beta$H's), $\delta$ 3.55−3.74 (60H, Pro $\delta$H's and 30 galactose ring H's), $\delta$ 3.2−3.26 (24H, His $\beta$ H's and 12H, methylene groups $-NHCOCH_2CH_2CH_2CH_2CH_2NH_2$), $\delta$ 2.25−2.35 (42H, Glu $\gamma$ H's and Pro $\beta$ H's), $\delta$ 2.18 (12H, methylene-$NHCOCH_2CH_2CH_2CH_2CH_2NH_2$), $\delta$ 1.97−2.09 (102H, Glu $\beta$ H's and Pro $\beta$ and $\gamma$H's), $\delta$ 1.39 (183H, Ala $\beta$ H's), $\delta$ 1.29−1.57 (36H, methylene groups $-NHCOCH_2CH_2CH_2CH_2CH_2NH_2$).

$^1$H NMR data of **7** ($D_2O$, 25 °C, 400 MHz): $\delta$ 4.16 (30H, polypeptide α-methine NHC*H*CO), $\delta$ 3.98 (7H, Gal H-2 proton), $\delta$ 3.64−3.80 (35H, ring protons from Gal unit), $\delta$ 3.21 (14H, methylene-$NHCOCH_2CH_2CH_2CH_2CH_2NH_2$), $\delta$ 2.51 (60H, polypeptide $\gamma$-methylene $CHCH_2CH_2$), $\delta$ 2.19 (60H, polypeptide $\beta$-methylene $CHCH_2CH_2$), $\delta$ 1.34−1.63 (56H, methylene groups $-NHCOCH_2CH_2CH_2CH_2CH_2NH_2$).

**SDS-PAGE Analysis with Periodate Staining**. Successful synthesis of glycopolypeptides was monitored via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE, 15%). Visualization of the glycoproteins was achieved via oxidation of the carbohydrate residues with periodic acid to the corresponding aldehydes followed by subsequent treatment with GelCode Glycoprotein Staining solution (Pierce).

**Spectrophotometric Assay.** The carbohydrate content of the synthesized glycopolypeptides was determined via a Glycoprotein Carbohydrate Estimation Kit (Pierce). Initial oxidation of the carbohydrate via treatment with periodic acid results in the formation of aldehydes that are subsequently reacted with a glycoprotein detection reagent (Pierce), which produces a colored solution with an absorption maximum at 550 nm. Extrapolation from a standard curve (from a series of glycoproteins of known carbohydrate content) provided an estimation of the degree of substitution.

**Circular Dichroic Spectroscopy**. Circular dichroic spectra were recorded on a JASCO 810 spectrophotometer (Jasco, Inc., Easton, MD) in a 1 mm path length quartz cuvette in the single-cell mount setup. Background scans of buffer (10 mM phosphate, 150 mM NaCl (PBS) pH 7.3) were recorded and automatically subtracted from the sample scans. Samples were made with the appropriate buffer via serial dilution at a concentration of ~0.3 mg/mL from a 1 mg/mL stock solution of protein in PBS buffer pH 7.3. The samples (400 $\mu$L) were loaded into a 1 mm path length quartz cuvette. Data points for the wavelength-dependent CD spectra were recorded with 1 nm bandwidth.

**Direct Enzyme Linked Assay (DELA)**. The GD1b competitive DELA assays were carried out in a 96-well format as previously reported.[40,42] Samples consisted of 6 ng/mL cholera toxin B subunit conjugated to horseradish peroxidase (CT $B_5$-HRP) incubated for 2 h in the presence of ligand at varying concentrations prior to exposure to the wells and subsequent analysis of bound CT $B_5$. $IC_{50}$ values were calculated from at least five different concentrations of competitive ligand via nonlinear regression, as described previously,[47] with the statistical package Microcal Origin.

**Gel Permeation Chromatography**. The relative hydrodynamic volume of select polypeptides and glycopolypeptides was determined via gel permeation chromatography (GPC). Samples were dissolved at 1 mg/mL in phosphate-buffered saline, pH 7.3, and filtered through a 0.22 $\mu$m filter prior to injection. The samples were separated using a Waters Ultrahydrogel Linear column (7.8 × 300 mm) followed by a Ultrahydrogel 250 (7.8 × 300 mm) column. Detection was achieved via the use of a Waters 2996 photodiode array detector and a Waters 2414 refractive index detector. Additional experiments were conducted for polypeptides dissolved in 0.1 M $NaNO_3$ or in buffer (PBS or $NaNO_3$) containing 20% acetonitrile, to confirm that neither electrostatic interactions nor hydrophobic interactions significantly affected the observed trends in retention. Identical trends in elution time with polymer identity were observed under all conditions, confirming the lack of interference from significant enthalpic effects during the GPC separation; results for the samples in PBS are given below.

## Results

**Synthesis of Glycopolypeptides.** A series of well-defined glycopolypeptides of random-coil conformation were produced via a combination of protein engineering and chemical strategies in order to study with more precision the role of specific glycopolymer architectural variables on multivalent binding events. The expression and purification of polypeptides **1** and **2** had been previously reported,[44] and similar protocols were employed in these studies, yielding polypeptides with the expected molecular weight, composition, and homogeneity. Polypeptide **3** was designed for these investigations for presentation of glutamic acid residues at distances commensurate with the receptor spacing of the cholera toxin $B_5$ subunit. This new polypeptide was produced via standard protein engineering methods (Supporting Information) in good yields (25 mg/L) and high purity and homogeneity.

The synthesis of the N-linked glycopolypeptides was accomplished via amide bond formation between an amine functionality of the desired saccharide and the glutamic acid side chains in the presence of HBTU as a coupling agent. The glycosylation was verified via SDS-PAGE analysis visualized with a modified periodic acid assay (Supporting Information). The degree of substitution (DS) for **4**−**9** (Table 1) was determined via a combination of MALDI-TOF mass spectrometry, a spectrophotometric assay (Pierce), and $^1$H NMR spectroscopy. $^1$H NMR data were essentially identical to that

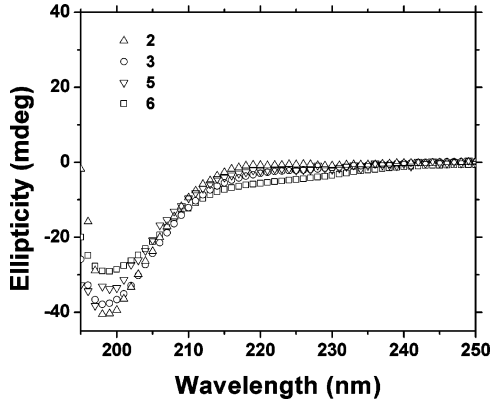


**Figure 1.** CD spectra of compounds **2**, **3**, **5**, and **6** (25 °C, PBS, 0.3 mg/mL).

previously reported (Supporting Information) and confirmed maintenance of the $\beta$-linkage of the saccharides.[42] The DS values listed in Table 1 indicate the average fraction of residues modified, based on multiple measurements of a glycopolypeptide via at least two of the above techniques. Although the DS values listed below are in some cases significantly lower than 100%, the obtained values indicated that a sufficient number of glutamic acids (e.g., 9/11 for 17-RC-10, 12/17 for 17-RC-16, and 5/7 for 35-RC-6) were modified to yield a well-defined average distance between pendant saccharides in the recombinantly derived glycopolypeptides.

**Circular Dichroic (CD) Spectroscopy.** Circular dichroism experiments were conducted on compounds **2**, **3**, **5**, and **6**, at similar polypeptide concentration ranges employed in the DELA experiments, to confirm that glycosylation did not alter the conformation of the polypeptide. The mean residue ellipticity as a function of wavelength was recorded for solutions of the polypeptides (0.3 mg/mL) in 10 mM phosphate buffer at a pH of 7.3 at 25 °C. The data from these experiments are shown in Figure 1. As is indicated in the figure, the spectra show a minimum at 198 nm, which is consistent with that reported for random coil polypeptides;[48] reliable data could only be collected to 195 nm due the sodium chloride in the PBS buffer. These results confirm that chemical modification of **2** and **3** with the amino-functionalized galactopyranoside does not alter the secondary structure of the recombinant polypeptides, consistent with our previous observations for modified PGAs.[42]

**Binding Assays.** The potential for these glycopolypeptides to act as efficient inhibitors of the cholera toxin was tested using a competitive DELA format developed by Minke et al.[40] Inhibition curves from the DELA experiment for galactose and for the polypeptides modified with *N*-($\epsilon$-aminocaproyl)-$\beta$-D-galactosylamine) (**4**−**6**) are shown in Figure 2. Inhibition curves for compounds **7**−**9** are shown in the Supporting Information. The concentration of samples **1**−**3** in the DELA assay ranged from 0 to 2000 $\mu$M (polypeptide concentration). Similarly, the concentration of glycopolypeptides **4**−**9** also ranged from 0 to 2000 $\mu$M (saccharide concentration), whereas the concentration of monovalent galactose was varied from 0 to 100 mM. No visible aggregation was observed upon the addition of the glycopolypeptides to the CT $B_5$. Nonlinear regression analysis of the inhibition curves provided $IC_{50}$ values of 1150, 1400, 250, 150, 80, and 55 $\mu$M for compounds **4**−**9,** respectively (Table 2), which are statistically different as confirmed via Fisher's pairwise comparison[49] (Minitab Stat Guide, version 3). Monovalent galactose exhibited an $IC_{50}$ of ∼35 mM in these assays, which is consistent with previously reported values.[37,40] These results clearly indicate that glycopolypeptides **4**−**9** showed increased inhibitory activity relative to the monovalent galactose. Unmodified control polypeptides **1**−**3** did not show any significant inhibition up to a polypeptide concentration of 1 mM, confirming the lack of nonspecific inhibition by the polypeptides at $IC_{50}$ concentrations. Unmodified polypeptides at concentrations greater than 1 mM exhibited a small degree of nonspecific binding (scattered values, 10−15%).

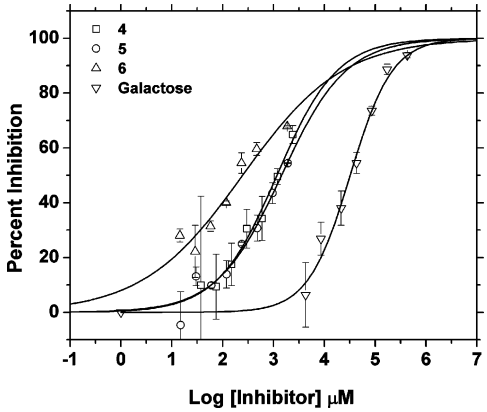


**Figure 2.** Inhibition of CT $B_5$ binding by compounds **4**−**6** and galactose as determined via competitive DELA. The solid line represents the best curve fit obtained by fitting the data to a dose−response curve via nonlinear regression analysis. All experiments were run in triplicate; the error bars represent the standard deviation among the three trials. Similar $IC_{50}$ values were obtained for multiple batches of glycopolypeptides of similar degrees of substitution.

**Table 2. DELA and GPC Results for Glycopolypeptides**

| compound | $IC_{50}$ ($\mu$M)[a] | improvement over galactose[b] | retention times (min) |
|---|---|---|---|
| **1** | | | ND[c] |
| **2** | | | ND |
| **3** | | | ND |
| **4** | 1150 ± 10 | 29 ± 19 | ND |
| **5** | 1400 ± 20 | 27 ± 10 | 15.3 |
| **6** | 250 ± 50 | 140 ± 20 | 15.3 |
| **7** | 150 ± 20 | 230 ± 20 | 14.7 |
| **8** | 80 ± 5 | 440 ± 40 | 14.4 |
| **9** | 55 ± 5 | 550 ± 120 | 14.3 |

[a] Errors are reported as the standard deviations of the average value obtained from triplicate assays of a given inhibitor. [b] Errors are reported as the absolute uncertainty as determined via standard propagation of error. [c] Not determined.

**Gel Permeation Chromatography (GPC).** GPC experiments were conducted to compare the relative hydrodynamic volume of polypeptides, glycopolypeptides, and poly(L-glutamic acid) polymers of varying degrees of polymerization (DP = 30, 86, and 113). The absorbance at 214 nm was monitored as a function of time for solutions of polypeptides and glycopolypeptides (∼1 mg/mL, a similar concentration range as in DELA experiments) in 10 mM phosphate buffer, 150 mM NaCl at a pH of 7.3. The data for the PGA polymers and compounds **5**−**7** are shown in Figure 3; the retention of the PGA-based polypeptides was unchanged after glycosylation. In these experiments, PGA113 (14.3 min) and PGA86 (14.4 min) exhibited the shortest retention times, indicating the largest hydrodynamic volumes, followed by PGA30/Cap-PGA30 (14.7 min) and **5** and **6** (15.3 min). The relative retention times of the glycopolypeptides generally decreased slightly with increasing molecular weight and charge density, as expected, although **5** and **6** eluted at the same retention time despite the differences in their molecular masses. This is likely an effect of the high degree of glycine present in both sequences, which in the absence of any significant electrostatic repulsion, may cause the glycopolypeptides to adopt compact structures with insignificant differences

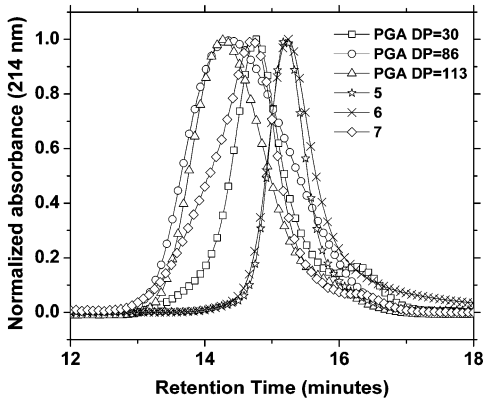


**Figure 3.** Retention times of compounds **5**−**7** relative to a series of poly(L-glutamic acid) polymers of varying degrees of polymerization, as determined via gel permeation chromatography. Samples were analyzed in PBS buffer and separated using a Waters Hydrogel column (7.8 × 300 mm), with photometric detection at 214 nm.

in elution times under these experimental conditions. All of the PGA-based glycopolypeptides eluted at shorter elution times than did the recombinantly derived glycopolypeptides, suggesting the higher hydrodynamic volume of the PGA-based glycopolypeptides (**7**−**9**) despite their lower molecular masses.

The impact of side-chain charge on the hydrodynamic volume of the PGA30, and its impact on inhibitory efficiency, was explored in an additional set of experiments. A Cap-functionalized PGA30 was produced via the same chemical modification protocols described above. The degree of substitution of this PGA was targeted for a density that would present a similar number of saccharides as **6**.[42] After chemical modification, a fraction of the glycosylated sample was then treated with (trimethylsilyl)diazomethane to form methyl esters at ∼75% of the unglycosylated glutamic acid sites, according to previously reported methods;[50] the resulting molecule is designated as Met Cap PGA30. The elution profiles and inhibition efficiencies demonstrated by these macromolecules are shown in Figure 4 (a and b) and Table 3. As shown in the data, methylation of the Cap PGA30 results in an increase in the elution time from 14.7 to 15.1 min, indicating a slight reduction in hydrodynamic volume, as expected (Figure 4a). As indicated in Figure 4b and Table 3, the Cap PGA30 from this set of experiments showed a 230-fold improvement in inhibition over monovalent galactose, while the Met Cap PGA30 showed only a 50-fold improvement in inhibition.

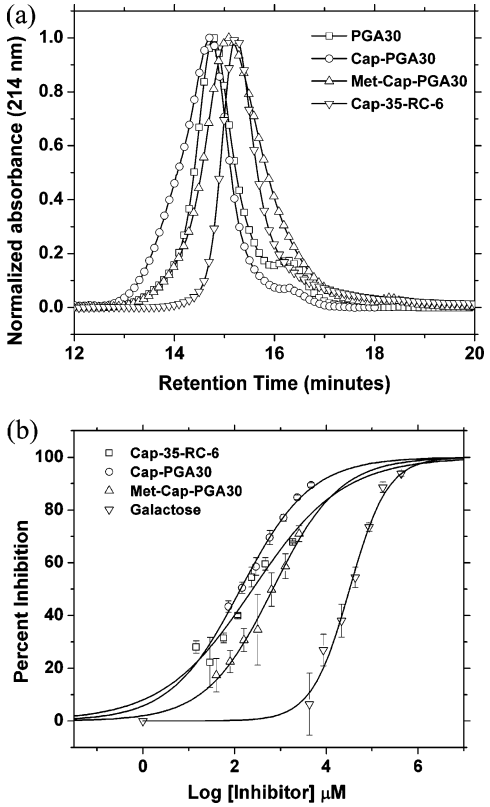


**Figure 4.** (a) Gel permeation chromatography results for select glycopolypeptides. Representative results for the PGA30-derived and Cap 35-RC-6 glycopolypeptides are shown. (b) Inhibition of CT $B_5$ binding by the glycopolypeptides, as determined via DELA.

**Table 3. $IC_{50}$ Values for Select Glycopolypeptides**

| molecule | retention time (min) | DS (mol %) | $IC_{50}$ (DELA) ($\mu$M) | improvement vs galactose[b] |
|---|---|---|---|---|
| galactose | N/A | N/A | 35000−70000[a] | 1 |
| Cap 35-RC-6 | 15.3 | 72 ± 1 | 250 ± 50 | 140 ± 20 |
| Cap PGA30 | 14.7 | 25 ± 5 | 150 ± 20 | 230 ± 20 |
| Met Cap PGA30 (DM = 80%)[c] | 15.1 | 25 ± 5 | 700 ± 180 | 50 ± 10 |

[a] Values vary slightly depending on the specific DELA trial. [b] Values relative to the monovalent galactose are given for the approximate ratio of the average $IC_{50}$ value for the multivalent inhibitor relative to the average value for the monovalent inhibitor for the specific trials conducted. Errors are calculated as the standard deviation of at least three measurements. [c] DM = degree of methylation.

## Discussion

**Design of Glycopolypeptides.** The solved crystal structure of the complex between cholera toxin $B_5$ subunit and the GM1 ganglioside (Gal$\beta$1-3GalNAc$\beta$1-4(Neu5Ac$\alpha$2-3)Gal-$\beta$1-4Glc-ceramide) reveals 5 GM1 binding sites per $B_5$ subunit, with a distance between adjacent binding sites of 35−37 Å.[51] The path between sites is not severely obstructed by any amino acid residue side chains. Saccharides presented on oligomeric and polymeric backbones may therefore be accessible to the binding sites; indeed, the use of linear bivalent ligands to inhibit pentameric toxins has been reported.[34,39] We have therefore employed a combination of protein engineering and chemical strategies to produce random coil glycopolypeptides with controlled distances between pendant carbohydrate groups. The use of polypeptide-based inhibitors of recombinant origin allows control over the number and placement of the pendant carbohydrate moieties via strategic placement of chemically reactive amino acids. Molecular dynamics calculations (Supporting Information) of the polypeptide backbones yielded distances between glutamic acid residues of ∼17 Å for polypeptides **1** and **2** (and therefore between saccharides in **4** and **5**). In addition, the rms distance was theoretically estimated for polypeptides **1**−**3** by assuming a random-flight model, employing a $C_n$ (characteristic ratio) value of 2.0, which accounts for the lower inherent flexibility of a glycine-containing polypeptide chain relative to the random flight chain (Supporting Information).[52,53] These calculations yielded distances of 17 Å for **1**, **2**, **4,** and **5** and 32 Å for **3** and **6**, consistent with the molecular dynamics simulations and indicating the relevance of these functional group distances for testing inhibition of CT $B_5$.

Modification of the glutamic acid residues with amino-modified saccharides, via standard solution-phase coupling chemistries, provides glycopolypeptides of well-defined structure. In these investigations, polypeptide scaffolds were modified with *N*-($\epsilon$-aminocaproyl)-$\beta$-D-galactosylamine, given the previous reports of the binding of this saccharide to CT $B_5$.[40,54] The use of this galactopyranoside causes the final length of the linker

arm between the saccharide and polypeptide backbone (after conjugation) to better mimic the length of the native GM1 ligand for the CT $B_5$[51] and has been shown to improve inhibition,[42,55] likely via a combination of both improved accessibility of the terminal galactose in the binding pocket, as well as by providing favorable hydrophobic interactions between the linker arm and hydrophobic amino acids in the pocket.[37,42,56] Although the inhibition by such modified glycopolypeptides is likely to be poorer than the inhibition by the native GM1 ligand, the Cap-modified glycopolypeptides serve as synthetically facile targets for investigating polymer architectural variables that can enhance multivalent interactions. A comparison of the relative binding activity of the three glycopolypeptide scaffolds (**4**−**6**) therefore provides information about the role of valency and controlled saccharide spacing in the design of linear polymeric antagonists of CT binding. In addition, comparison of the relative binding activity of the recombinantly derived glycopolypeptides (**4**−**6**) versus the PGA-based glycopolypeptides (**7**−**9**) affords insight into the role of backbone extension and hydrodynamic volume on the binding event.

**Effect of Saccharide Spacing.** Three different polypeptides, $[(AG)_3PEG]_{10}$ (**1**), $[(AG)_3PEG]_{16}$ (**2**), and $\{[(AG)_2PSG]_2$-$[(AG)_2PEG][(AG)_2PSG]_2\}_6$ (**3**), were produced and modified with *N*-($\epsilon$-aminocaproyl)-$\beta$-D-galactosylamine and tested as inhibitors of the CT $B_5$ subunit. Results obtained from binding experiments revealed that **6** exhibited the greatest inhibition of the recombinant glycopolypeptides, with a 140-fold improvement over the monovalent galactose (Table 2). This compares favorably to the 30-fold improvement exhibited by **4** and **5** despite the lower valency of **6**, confirming the importance of appropriate saccharide spacing over increased valency in inhibition by these glycopolypeptides. Although it has not been possible to directly measure the structures of the polypeptides while bound to the CT $B_5$, their random-coil character is most likely retained, based on their amino acid composition and the lack of significant non-saccharide, complementary interactions between the glycopolypeptides and the CT $B_5$ surface. The lack of significant interactions between the polypeptide and the CT $B_5$ are also indicated by the lack of inhibition of the unmodified polypeptide. In addition, aggregation of the glycopolypeptides is not indicated to mediate the inhibition event, given the lack of aggregation suggested by the GPC results and by nondenaturing poly(acrylamide) gel electrophoresis experiments (Supporting Information). There was no visible aggregation upon mixing of the glycopolypeptides with the CT $B_5$ subunit during the DELA assays, although observed differences in the Hill slopes of the inhibition curves may suggest differences in the valency and/or association state during binding;[57] additional characterization via light scattering will assist in the interpretation of the differences in Hill slope.

These avidity improvements represent a relatively small difference in free binding energies, but the results nevertheless point to the potential for controllably manipulating the avidity of the binding event via controlled and small changes in polymer architecture at a level of structural detail not previously accessible. In addition, given the control of molecular weight and functional group position on the polymer chain, the inhibition by these glycopolypeptides may also be attributed to more specific origins (e.g., statistical effects versus multivalent binding). Since **4** and **5** have a higher density and number of saccharides than **6**, at a shorter span than the adjacent binding sites on the CT $B_5$, the observed gains in avidity are likely to arise from mainly statistical effects that occur as a consequence of the high local concentration of the multivalent ligand, as has commonly been observed in glycopolymeric inhibitors or effectors.[41,58] In contrast, the architecture of **6** is likely to permit the engagement of multivalent interactions with minimal statistical effects, given the match of the saccharide spacing with receptor structure and the low valency of the glycopolypeptide. These results therefore suggest the potential for binding improvements for linear glycopolymers that do not require statistical effects for improved binding and are instead mediated by primarily a multivalent mechanism.[19,59−61] More detailed investigation of the binding via isothermal titration calorimetry will permit determination of the thermodynamic differences between these two modes of binding, quantitation of the number of saccharides engaged in binding, and the design of more potent glycopolymeric inhibitors.

**Effects of Backbone Composition and Charge.** Inspection of Figure 2 and Table 2 reveals that heterogeneous compounds **7**−**9** exhibited generally lower $IC_{50}$ values (i.e., increased potency) than **4**−**6**, in contrast to our expectations. These PGA-based glycopolypeptides show improved inhibition (Table 2) despite their more heterogeneous structure, similar (to lower) molecular weights, and similar "average" distances between adjacent saccharide residues (Table 1). The improvements in binding of **7**−**9** over **4**−**6** are not a result of the negative charge of **7**−**9**, as control experiments show that the unmodified PGAs do not interact with the CT $B_5$ subunit up to a polypeptide concentration of 1 mM. The improvements are therefore likely due to the differences in backbone flexibility between the two families of glycopolypeptides.

Gel permeation chromatography (GPC) experiments were therefore conducted to examine the differences in hydrodynamic volume of the glycopolypeptides and how these differences may impact inhibition of CT $B_5$. Given the lack of glycine residues and the high number of negatively charged glutamic acid residues in the PGA-based glycopolypeptides **7**−**9** (versus **4**−**6**), we anticipated that the PGA-based glycopolymers should have a more extended chain conformation which would lead to a greater hydrodynamic volume ($V_h$). The GPC results in Figure 3 illustrate that all of the polypeptides elute over a somewhat narrow range of elution times between approximately 14 and 16 min. The retention times of PGA113, PGA86, and PGA30 were shown to be roughly dependent on the degree of polymerization of the polymers, as expected, and partial glycosylation of the PGA-based polypeptides had no effect on the retention time of the glycopolypeptides.

The improvements in inhibition of CT $B_5$ by the glycopolypeptides **4**−**9**, normalized on a saccharide basis, ranged from approximately 30- to 550-fold. The retention times determined via GPC under aqueous experimental conditions correlate well with observed improvements in inhibition (Table 2), with glycopolypeptides of increasing $V_h$ (lower retention times) showing improved inhibition (lower $IC_{50}$ values). To further confirm the role of hydrodynamic volume in inhibition, **7** was methylated in order to reduce the electrostatic repulsion that is in large part responsible for the larger chain dimensions of the PGA-based glycopolypeptides. As shown in Figure 4a and Table 3, methylation of **7** results in a reduction in hydrodynamic volume so that the retention time of the Met Cap PGA30 is highly similar to that of **6**. Importantly, this reduction in size results in a concomitant reduction in inhibition (increased $IC_{50}$ value) relative to that observed for both **6** and **7**.

Although the differences in inhibition and $V_h$ between the various glycopolypeptides are generally moderate and in some cases small, the $V_h$ (as assessed via retention time in these GPC experiments) and inhibition correlate very well with one another

(Supporting Information), indicating that the small observed differences in $V_h$ have measurable and reliable impact on the inhibition properties of the glycopolypeptides of this size and composition. The observed improvements in inhibition with increased hydrodynamic volume are not indicated to result from steric stabilization effects that have been observed in other inhibition studies,[2,62] as both DELA (inhibition of CT $B_5$ binding to a surface) and fluorescence titration assays (direct binding in the solution phase) of the glycosylated PGAs show similar inhibition results.[42] They likely therefore result from inherent changes in saccharide accessibility as a function of backbone composition. In addition, despite previous reports of the positive correlation of polymer molecular mass with inhibition,[61−64] our results indicate that chain dimension comparisons, rather than molecular mass comparisons, are necessary for predicting inhibitory potency in the glycopolypeptides of the molecular weights and compositions described here.

These results illustrate that the larger $V_h$ of the PGA-based glycopolypeptides, even for molecules with lower molecular weights, improves toxin inhibition most likely as a result of improved accessibility of pendant saccharides. In contrast, the higher molecular weight, uncharged, and flexible glycopolypeptides **4**−**6** must adopt a more compact conformation, which must significantly decrease the accessibility of the saccharides even when they are appropriately spaced along the polypeptide backbone, thereby reducing inhibition. The poorer inhibition exhibited by the heterogeneous Met Cap PGA30 versus that of the recombinant glycopolypeptide **6**, despite their similar $V_h$ values and the more compact conformation of **6**, suggests the benefits of controlled saccharide presentation in improving inhibition. These results further suggest the potential of high molecular weight, well-defined recombinant glycopolypeptides with greater chain extension to act as improved inhibitors; such macromolecules are currently under investigation.

It is important to note that the enhancements in saccharide binding to the CT $B_5$ subunit by the well-defined glycopolypeptides are of the range normally observed for large linear synthetic glycopolymers and dendrimers of high valency ($10^1-10^3$-fold improvements),[25,60,65−68] despite the relatively small size of the glycopolypeptides. Additionally, the enhancements compare quite favorably to inhibition shown by saccharide-modified globular proteins and linear glycopolymers of similar valencies ($10^0-10^1$-fold improvements).[60,65] Although the inhibition enhancements are far smaller than those of the best pentavalent small molecule inhibitors reported by Fan and co-workers,[54] the variation in inhibition by these well-defined glycopolypeptides nevertheless allows assessments of the origins of the binding improvements based on known polymer architectural features. Furthermore, given the known and varied placement of the saccharides on the polypeptide backbone, such assessments can serve as the basis for designing new glycopolypeptide constructs of greater binding avidity via appropriate architectural design and the inclusion of complementary secondary interactions. The expansion of these glycopolymeric design principles will also be of general importance in the manipulation of binding events over larger length scales[41] for which small molecule inhibitors may not be as well-suited.

## Conclusions

A combination of genetic and chemical methods has been successful for the production of well-defined glycopolypeptides based on the sequences $[(AG)_3PEG]_y$ and $\{[(AG)_2PSG]_2$-$[(AG)_2PEG][(AG)_2PSG]_2\}_6$. Variations in the number and spacing of the pendant carbohydrate moieties were easily achieved via these methods. Competitive direct enzyme-linked assays of the recombinantly derived glycopolypeptides suggest that multivalent ligands with saccharides spaced ∼35 Å apart exhibit greater inhibitory ability toward CT $B_5$ than glycopolypeptides with a different presentation of saccharides. The combination of gel permeation chromatography experiments and enzyme-linked assays of inhibition indicates that polypeptide chains of greater hydrodynamic volume, independent of molecular weight comparisons, exhibit improved inhibition, and that homogeneous glycopolypeptides offer inhibition improvements even when the saccharides are of lower accessibility. Therefore, the synthesis of polypeptide-based glycopolymers with a more rigid backbone and appropriately spaced pendant saccharide residues may yield materials with significantly enhanced inhibitory activity; increasing the lengths of these chains is likely to afford additional benefits in inhibition as well.

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**Supporting Information Available:** Detailed explanations of expression plasmid construction for pET21b-17-RC-10 and pET21b-17-RC-16, protein expression and purification, cyanogen bromide cleavage for compounds **1** and **2**, cloning plasmid construction for pUC19-35-RC, expression plasmid construction of pET28b-JS1-35-RC-6, purification and characterization (HPLC, MALDI-TOF, amino acid analysis, $^1$H NMR) of **3**, preparation of glycopolypeptides, characterization (SDS-PAGE analysis with periodate staining, amino acid analysis, $^1$H NMR) of glycopolypeptide (**6**), molecular dynamic simulation studies, theoretical calculations of radius of gyration ($R_G$), and the correlation between inhibition and hydrodynamic volume of glycopolypeptides. This material is available free of charge via Internet at http://pubs.acs.org.

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