Control of the Molecular Orientation of Membrane-Anchored Biomimetic Glycopolymers

Kamil Godula,‡,§,□ Marissa L. Umbel,† David Rabuka,‡,● Zsofia Botyanszki,‡ Carolyn R. Bertozzi,‡,§,¶,‖ and Raghuveer Parthasarathy*†

Department of Physics and Materials Science Institute, University of Oregon, Eugene, Oregon 97403. Departments of Chemistry, Molecular and Cell Biology and Howard Hughes Medical Institute, University of California, and Materials Sciences Division and The Molecular Foundry, Lawrence Berkeley National Laboratory, Berkeley, California 94720

Received April 23, 2009; E-mail: raghu@uoregon.edu

Abstract: Quantifying and controlling the orientation of surface-bound macromolecules is crucial to a wide range of processes in areas as diverse as biology, materials science, and nanotechnology. Methods capable of directing orientation, as well as an understanding of the underlying physical mechanisms are, however, lacking. In this paper, we describe experiments in which the conformations of structurally well-defined polymers anchored to fluid lipid membranes were probed using Fluorescence Interference Contrast Microscopy (FLIC), an optical technique that provides topographic information with few-nanometer precision. The novel rodlike polymers mimic the architecture of mucin glycoproteins and feature a phospholipid tail for membrane incorporation and a fluorescent optical probe for FLIC imaging situated at the opposite termini of the densely glycosylated polymeric backbones. We find that the orientation of the rigid, approximately 30 nm long glycopolymers depends profoundly on the properties of the optical reporter. Molecules terminated with Alexa Fluor 488 projected away from the lipid bilayer by 11 ± 1 nm, consistent with entropy-dominated sampling of the membrane-proximal space. Molecules terminated with Texas Red lie flat at the membrane (height, 0 ± 2 nm), implying that interactions between Texas Red and the bilayer dominate the polymers’ free energy. These results demonstrate the design of macromolecules with specific orientational preferences, as well as nanometer-scale measurement of their orientation. Importantly, they reveal that seemingly minute changes in molecular structure, in this case fluorophores that comprise only 2% of the total molecular weight, can significantly alter the molecule’s presentation to the surrounding environment.

Introduction

The orientation of surface-anchored macromolecules is a crucial determinant of function in contexts as diverse as molecular electronics,1 DNA2 and carbohydrate3 microarrays, and membrane biophysics.4 In the last category, interactions between cell surface proteins and their binding partners that can include soluble proteins, surface receptors of other cells, and the extracellular matrix require extension and projection away from the lipid bilayer and its proximate space. While the orientations of densely packed collections of molecules covalently linked to solid surfaces, for example alkanethiols on gold or chemically grafted DNA, can be largely determined by

the steric constraints of their packing arrangements, proteins at membranes are more sparsely linked to a two-dimensional fluid. The resulting freedom to explore their spatial and conformational neighborhood suggests that nontrivial mechanisms are required to control protein orientation. Such physical mechanisms, however, remain largely unexplored, subject so far to only a few controlled studies.5–7 Elucidating principles of macromolecular orientation at membranes requires structurally well-defined biomimetic models as well as techniques capable of probing nanometer-scale topography.

Membrane mucins are a particularly important class of large cell-surface glycoproteins, involved in vital biological processes (e.g., cell migration, adhesion, and immunogenesis), as well as many pathological events (e.g., cancer metastasis).5–10 The study of glycoprotein behavior at membranes poses considerable challenges due to the proteins’ structural and compositional heterogeneity and nanoscale dimensions, far below the resolution limits of conventional optical microscopy. To address these challenges, we have developed a new class of uniform,
synthetically tractable mucin glycoprotein mimetics and have studied their orientation at cell-free lipid membranes with nanometer precision using interferometric imaging.

Mucins are characterized by a long, rodlike architecture resulting from dense glycosylation of serine and threonine amino acid residues abundantly distributed throughout the peptide backbone.\(^9\) While structurally diverse, mucin carbohydrate epitopes are typically attached to the protein via an \(\alpha\)-O-linked core sugar, N-acetylgalactosamine (GalNAc). In our earlier work,\(^11\) we demonstrated that these native structures can be recreated in synthetic polymers by the attachment of \(\alpha\)-aminoxy GalNAc monosaccharides to a methylvinyl ketone (MVK) polymer via stable oxime linkages (Polymer P1 in Figure 1a). In addition, the glycopolymers were furnished with a small fraction (1% of monomeric units) of Texas Red (TR) fluorophores distributed along the polymer backbone for fluorescence imaging and a terminal phospholipid tail for anchoring in supported lipid bilayers (SLBs). These rodlike mucin mimics (which we refer to as “body-labeled” to characterize the fluorophore distribution) incorporated into two-dimensionally fluid, solid-supported lipid membranes and bound soluble glycan-specific lectin proteins with the same specificities as natural mucins.\(^1\) Interferometric measurements using the intrinsic fluorophores showed a supine orientation, with a mean fluorophore height roughly 1 nm above the membrane.\(^5\)

We have now expanded the structural repertoire of mucin mimetic polymers, focusing especially on modifications that direct the position and type of the polymer’s fluorescent probe. Figure 1b depicts a second-generation mucin mimetic architecture (Polymer P2), which addresses several key issues associated with our earlier design. Namely, we determined that these glycopolymers would be of narrow chain-length distributions and would feature a GalNAc-decorated poly(MVK) backbone bracketed by a lipid anchor and a single fluorescent probe at opposite ends.

In this paper, we describe the synthesis of these new molecules and their characterization at solid-supported lipid bilayers, made possible by the lipid anchor. Supported bilayers are well-established experimental models of cell membrane architecture that replicate the two-dimensional fluidity and structure of natural membranes while allowing compositional control and a variety of imaging modes.\(^12\)–\(^17\) The bilayers are supported by reflective silicon substrates with microfabricated silica terraces that make possible the use of fluorescence interference contrast microscopy (FLIC), a technique in which interference between direct and reflected paths of fluorescence light lead to height-dependent fluorescence intensity.\(^18\)–\(^22\)

Analysis of the detected intensities, accounting for the fluorophore spectra and optical setup, reveals the mean fluorophore height within a few nanometers precision.

The mucin mimetic polymers were designed to enable and exploit the topographic power of FLIC imaging. First, the precise end-localization of the fluorescent probe in molecules P2 dictates that the height determined by FLIC is the height of the molecule, rather than being the average of signals from fluorophores randomly distributed along the body of the polymer, as in P1. Second, we created polymers with different fluorescent probes, Texas Red and Alexa Fluor 488 (AF488). Simple lipids are available with conjugated Texas Red and fluorescein (FITC); the FITC excitation and emission spectra are nearly identical to those of Alexa Fluor 488. Comparison of the FLIC signal of mucin glycopolymers and lipids in separate experiments, both with the same or similar fluorescent probe, therefore provides a topographic measure that is robust with respect to potential systematic uncertainties, enhancing the already considerable appeal of FLIC.

Most importantly, comparison of differently labeled polymers, both P1 and P2, allows discrimination of the optical reporter’s effect on molecular orientation. We find that Texas Red-labeled molecules are supine, while Alexa Fluor 488-labeled molecules project away from lipid membranes, a profound difference that is striking in light of the small size of the fluorophore relative to that of the whole polymer. These experiments show that macromolecular orientation at membranes (i.e., supine or upright) can be dictated by synthetic design and controlled by small modifications of chemical structure.

**Results**

**Modular Synthesis of Dual End-Functionalized Mucin Mimetic Polymers.** The synthesis of TR and AF488-containing “end-labeled” mucin mimics P2 is shown in Scheme 1, with details provided in the Experimental Methods section. First, we prepared a poly(MVK) intermediate P3 of narrow chain length distributions using reversible addition—fragmentation chain

\(\text{(12) Boxer, S. G. \textit{Curr. Opin. Chem. Biol.} 2000, 4, 704–709.}\)
\(\text{(13) Castellana, E. T.; Cremer, P. S. \textit{Surf. Sci. Rep.} 2006, 61, 429–444.}\)
\(\text{(14) Groves, J. T.; Boxer, S. G. \textit{Acc. Chem. Res.} 2002, 35, 149–157.}\)
\(\text{(15) Tamm, L. K.; McConnell, H. M. \textit{Biophys. J.} 1985, 47, 105–113.}\)
\(\text{(16) Harland, C. W.; Rabuka, D.; Bertozzi, C. R.; Parthasarathy, R. \textit{Biophys. J.} 2008, 94, 4718–4724.}\)
\(\text{(17) von Tscharner, V.; McConnell, H. M. \textit{Biophys. J.} 1981, 36, 421–427.}\)
\(\text{(18) Lambacher, A.; Fromherz, P. \textit{J. Opt. Soc. Am. B} 2002, 19, 1455–1453.}\)
\(\text{(19) Kiessling, V.; Tamm, L. K. \textit{Biophys. J.} 2003, 84, 408–418.}\)
\(\text{(20) Groves, J. T.; Parthasarathy, R.; Forstner, M. B. \textit{Annu. Rev. Biomed. Eng.} 2008, 10, 311–338.}\)
\(\text{(21) Parthasarathy, R.; Groves, J. T. \textit{Proc. Natl. Acad. Sci. U.S.A.} 2004, 101, 12798–12803.}\)
\(\text{(22) Ajo-Franklin, C. M.; Yoshina-Ishii, C.; Boxer, S. G. \textit{Langmuir} 2005, 21, 4976–4983.}\)
Polymerization of MVK in the presence of a radical initiator and the phospholipid-containing trithiocarbonate chain transfer agent, a chain-growth mediator, allowed us to simultaneously control the polydispersity (PDI = 1.10) of the resulting polymer and to introduce an SLB anchor and a point of fluorophore attachment at each terminus of the resulting polymer chains. Condensation of intermediate P3 with α-aminoxy GalNAc furnished glycopolymer P4 with a terminal lipid tail and pendant mucin-like glycans. The free terminal sulfhydryl group released under the condensation conditions was capped with a maleimide-conjugated TR or AF488 fluorophore, thus, completing the assembly of the target fluorescent mucin mimics P2. The efficiency of glycan ligation was established by NMR analysis to be approximately 60%, which was in good agreement with the previously synthesized “body-labeled” mucin mimic P1.

The P2 polymers adopted extended mucin-like conformations with lengths of approximately 30 nm, as determined by several methods. The hydrodynamic radii of polymers P2-TR and P2-AF488 were 31 ± 7 and 25 ± 5 nm, respectively, obtained by dynamic light scattering (DLS) in PBS buffer (pH = 7.2). More direct evidence of their extended form was obtained by TEM imaging of single glycopolymer chains (Figure 2). Theoretical length values calculated for fully extended 240-monomer unit polymers P2 using MM2 force field-based molecular mechanics predictions were approximately 35 nm, consistent with the experimental measures. As reported previously, polymers P1 with similar degree of polymerization also adopted extended conformations approximately 30 nm long.

**Insertion of Lipid-Terminated Mucin Mimics into Supported Lipid Bilayers and Their Analysis by FRAP.** The lipid tail of the mucin glycopolymer allowed for their integration with solid-supported lipid membranes. Molecules P2-TR and P2-AF488 incorporated into supported lipid bilayers are illustrated schematically in Figure 3a. The bilayer preparation and polymer incubation are as described earlier and are also detailed in the Experimental Methods section. In all experiments, the use of a small fraction of fluorophore-conjugated lipids, spectrally distinct from the probes conjugated to the polymers, allowed independent examination of the bilayer and the glycopolymers.

Fluorescence imaging of the glycopolymers indicated robust membrane incorporation. Absolute quantification of molecular density from fluorescence brightness is, in general, challenging. Comparison of the brightness of the membrane-incorporated Texas Red-labeled polymer P2-TR with membranes containing controlled fractions of Texas Red conjugated lipids allows determination of the surface density of molecule P2-TR, found to be approximately 20,000 molecules/µm². No Alexa Fluor 488-labeled lipids are available, prohibiting a similar quantification of density for molecule P2-AF488. However, as its structure is nearly identical to that of molecule P2-TR, featuring the same lipid anchor, its density is likely to be similar.

Lateral mobility is an important attribute of membrane-incorporated molecules. FRAP measurements, as illustrated in Figure 3b, showed the glycopolymers to be two dimensionally fluid with diffusion coefficients $D = 1.7 ± 0.7 \mu m^2/s$ and $0.9 ± 0.2 \mu m^2/s$ for polymers P2-AF488 and P2-TR, respectively. These mobilities are similar to those of the membrane lipids, $D = 1.2 ± 0.4 \mu m^2/s$, consistent with anchoring of the polymers in the membrane via their lipid tails. We discuss the difference

---

(23) Chiefari, J.; Chong, Y. K.; Ercole, F.; Kristina, J.; Jeffery, J.; Le, T. P. T.; Mayadunne, R. T. A.; Meijjs, G. F.; Moad, C. L.; Moad, G.; Rizzardo, E.; Thang, S. H. Macromolecules 1998, 31, 5559–5562.
(24) Cheng, C.; Sun, G.; Khoshdel, E.; Wooley, K. L. J. Am. Chem. Soc. 2007, 129, 10086–10087.
between the $D$ values of P2-AF488 and P2-TR below. The lipidlike mobility and the laterally homogeneous fluorescence images imply that the glycopolymers do not aggregate.

**Determination of the Orientation of Membrane-Bound Mucin Mimics by FLIC Imaging.** To probe the orientation of the membrane-incorporated polymers, we used Fluorescence Interference Contrast Microscopy (FLIC), *vide supra*. The raw data consist of fluorescence images of the labeled molecules of interest, lipids or polymers, on reflective substrates with terraced oxide layers (Figure 3a). These provide the intensity as a function of the terrace thickness, data that are then fit to a functional form that reveals the mean height of the probe above the oxide. We show in Figure 4a a representative fluorescence image from a sample with membrane-incorporated molecule P2-AF488, the mucin mimetic glycopolymer with AlexaFluor 488 at the distal end. The intensity and terrace height values are plotted in Figure 4c, with a fit that yields a mean fluorophore height of $\Delta z_{P2-AF488} = 17 \pm 6$ nm above the oxide surface. In Figure 4b, we show the fluorescence from Texas Red-labeled lipids from the same membrane, with the corresponding data and fit (Figure 4d): $z_{lipid} = 4 \pm 4$ nm. The data from several samples ($N = 13$) reveal a polymer probe height $\Delta z_{P2-AF488} = 16.7 \pm 1.0$ nm, a lipid probe height $z_{lipid} = 5.5 \pm 0.4$ nm, and a difference between the two, $\Delta z_{P2-AF488} - z_{lipid} = 11.2 \pm 1.1$ nm. These values are also provided in Table 1. The FLIC data reveal that the Alexa Fluor 488-labeled polymers P2-TR project out from the membrane.

**Effects of Physical and Electrostatic Properties of the Terminal Label on Macromolecular Orientation.** Markedly different behavior is exhibited by molecule P2-TR, the mucin mimetic glycopolymer with Texas Red at the distal end, incorporated at supported lipid bilayers. For these, FLIC imaging reveals Texas Red probe heights $z_{lipid} = 5.1 \pm 2.1$ nm for FITC-labeled lipids from the same membranes, and a difference between the two $\Delta z_{P2-TR} = 0.2 \pm 0.1$ nm ($N = 17$); see also Table 1. The FLIC data reveal that the Texas Red-labeled polymers P2-TR, identical to P2-AF488 except for the fluorescent probe, lie flat at the membrane.

**Effects of Fluorophore Positioning within the Macromolecule on the Outcome of FLIC Analysis.** We also examined SLB-incorporated polymer P1-AF488, labeled with Alexa Fluor 488 along the MVK backbone, which showed a polymer probe height $\Delta z_{P1-AF488} = 14.4 \pm 2.1$ nm. The Texas Red lipid probe height was $z_{lipid} = 5.8 \pm 0.9$ nm; the difference between the two, $\Delta z_{P1-AF488} = 8.6 \pm 1.2$ nm ($N = 9$). Polymer P1-TR, identical except for labeling with Texas Red rather than Alexa Fluor 488, was examined in earlier work: $z_{lipid} = 5.3 \pm 1.0$ nm, $z_{lipid} = 4.4 \pm 1.6$ nm, and $\Delta z_{P1-TR} = 0.9 \pm 1.8$ nm. (In these experiments, $z_{lipid}$ was measured using Texas Red-labeled lipids in different membrane samples, so the stated $\Delta z_{P1-TR}$ is the difference of the average $z_{lipid}$ and $z_{lipid}$. Rather than the average of the differences for each sample as in the measurements of P2-TR, P2-AF488, and P1-AF488).

**Effects of Ionic Strength of the Aqueous Environment on Mucin Mimic Orientation.** Experiments conducted in deionized water (ionic strength $\approx 10 \mu$M) and in phosphate buffered saline (PBS, pH = 7.4, ionic strength 150 mM) yielded statistically identical topographic data. For P2-AF488 at lipid bilayers, $\Delta z_{P2-AF488} = 11.4 \pm 1.3$ nm in deionized water and $\Delta z_{P2-AF488} = 11.0$
approximately 15 nm, consistent with the TR
by both its lipid anchor and by the Texas Red fluorophore, is
anchoring of the Texas Red-labeled molecule
are structurally a minor component of the molecules, contribut-
identical Texas Red-labeled polymers are topographically
conformation and orientation.
contrast, is rapid, precise, and noninvasive, enabling standard
uncertainties due to the requisite staining and immobilization
Electron microscopy is susceptible to experimental artifacts and
of conventional optics renders simple microscopy inadequate.
probe with other techniques. For instance, the diffraction limit
romolecules at the nanometer scale, a regime that is difficult to
Membrane Incorporated Mucin Mimetics
E. Akande, T. Elahi, K. A. Seddon, A. S. D. R. Stuart, and R. W. O. Wouts

Discussion
Fluorescence imaging and mobility measurements reveal
membrane-incorporation of the lipid-terminated mucin mimetic
glycopolymer. Structural data demonstrate the expected ex-
tended rodlike structure of the polymers. FLIC imaging reveals
FLIC, in contrast, is rapid, precise, and noninvasive, enabling standard
fluorescent probes to convey information about molecular
formation and orientation.

Strikingly, we find that polymers labeled with Alexa Fluor 488 project out from the membrane surface, whereas otherwise identical Texas Red-labeled polymers are topographically indistinguishable from membrane lipids. The fluorescent probes are structurally a minor component of the molecules, contributi-
only about 2% of the molecular weight. Texas Red, however, is known to insert into lipid bilayers, and the molecular underpinnings of its hydrophobicity have recently been il-
minated. Despite the hydrophobic nature of the rest of the polymer, the fluorophore’s hydrophobicity appears sufficient to anchor the polymer’s distal end to the membrane. Double-
anchoring of the Texas Red-labeled molecule P2-TR, mediated by both its lipid anchor and by the Texas Red fluorophore, is the likely cause of the 2-fold lower diffusion coefficient of P2-
TR compared to that of P2-AF488 in membranes. Alexa Fluor 488 contains multiple negative charges, rendering the fluoro-
phore hydrophilic.

These polymers are uncharged and, as expected, show no
detectable ionic-strength dependence in their orientation. Their orientation should therefore be largely determined by entropic concerns, with projection from the membrane maximizing the volume available to rodlike membrane-anchored macromolecules. A rod of length L anchored at a point, freely exploring a hemisphere of allowed orientations, has a mean surface-to-
end height L/2. The expected height above the membrane, Az, for polymers P2, for which L \approx 30 nm, should therefore be approximately 15 nm, consistent with the $\Delta z_{P2-AF488} = 11.2 \pm 1.1$ nm value observed for the Alexa Fluor 488-labeled polymer.

The measured surface density of approximately 20,000 P2
molecules/μm² corresponds to a mean intermolecular spacing of roughly 8 nm, assuming 6-fold liquidlike coordination. This is greater than the width of the rodlike polymers of approximately 2 nm, again supporting the conclusion that orientational entropy, rather than dense packing, controls the P2-AF488 orientation.

Our observation that fluorophore type dramatically influences molecular orientation is not only important to the present case of membrane-bound mucin mimetic polymers but is also
generally of more consequence to the fields of chemistry, biology, and biophysics. Ubiquitous fluorescent probes are not necessarily inert readouts of molecular properties but can cause significant physical perturbations. More positively, our findings imply that control of molecular orientation, not only at membranes but also in, for example, molecular electronics and nucleic acid arrays, can be afforded by small modifications of molecular structure. Technical extensions of FLIC to probe the orientation of single molecules, though technically challenging, may offer still deeper insights into possible topographic heterogeneity of a population.

Experimental Methods

General Methods. All chemicals, unless stated otherwise, were purchased from Aldrich Chemicals. Texas Red (TR) and Alex Fluor 488 (AF488) maleimide conjugate were purchased from Invitrogen. Solvents were purified on a Glass Contour solvent purification system. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian 400 MHz Biospin Ad system. 30-MHz NMR spectrometer with multicellular CP-MAS probe. Spectra were recorded in CDCl₃ or D₂O solutions at 293 K and referenced to residual solvent peaks. Infrared spectra were collected on a Varian 3100 FT-IR spectrometer. ATR analyses were performed using Pike Technologies MR1R single reflection ATR accessory (ZnSe crystal). Size exclusion chromatography (SEC) was performed on a Viscotec TDA 302 SEC system with triple detector array. For measurements in DMF (0.2% LiBr) the instrument was equipped with two in-series Mixed Bed GMHHR-M columns, separation range 100–4 M (30 cm × 7.8 mm i.d.) at 70 °C. Transmission electron microscopy was performed on a Jeol 2100F transmission electron microscope operated at 200 kV.

Glycopolymer Synthesis. Synthesis of the Chain Transfer Agent (2). A pressure-seal tube (25 mL) equipped with a magnetic stirring bar was charged with S-dodecyl-S’-(o,α-dimethylpentfluoro-
ropheny1 acetate)trithiocarbonate (365 mg, 0.69 mmol) and 1,2-
dipalmitoyl-rac-glycero-3-phosphoethanolamine (476 mg, 0.69
mmol) under a stream of nitrogen. Mixture of chloroform and methanol (20:1, 7 mL) was added, followed by disopropylethyl-
1H NMR (CDCl₃, 500 MHz) δ: 5.28 (bm, 1H), 4.39 (bm, 1H, 1H, 1H), 3.97–3.90 (bm, 4H), 3.48 (bm, 1H). 3.27 (t, J = 72.0 Hz, 2H, 2.46–1.95 (bm, 8H, 1H, 1H, 1H), 1.75–1.50 (bm, 12H, 12H), 1.45–1.15 (m, 62H, 62H), 0.89 (m, 9H). 13C NMR (CDCl₃, 125 MHz) δ: 220.3, 173.6, 173.4, 70.4, 63.7, 57.2, 40.9, 37.1, 34.1, 34.1, 31.9, 29.8, 29.7, 29.6, 29.5, 29.5, 29.4, 29.3, 29.2, 29.1, 27.7, 25.6, 25.0, 24.9, 22.7, 14.1. FT-IR (KBr, CH₂Cl₂) ν (cm⁻¹): 3372, 2920, 2851, 1742, 1721, 1161, 1530, 1468, 1242, 1076, 814. HRMS (m/z): 1038.6684 (calculated), 1038.6677 (found).

Synthesis of Lipid-Terminated MVK Polymer P3. A flame-
dried Schlenk flask (10 mL) equipped with a magnetic stirring bar was charged with 2 (21.3 mg, 0.021 mmol, 50 mol %), ACVA (4,4’-azobis(4-cyanovinylidene acid), 1.1 mg, 0.004 mmol, 10 mol %), methylvinylketone (287.5 mg, 4.102 mmol, freshly distilled and filtered through basic alumina) and 2-butanol (377 mg, freshly distilled and filtered through basic alumina). The flask was equipped with a rubber septum and attached to a Schlenk line. The yellow solution (43 wt % in monomer) was thoroughly degassed by three freeze–pump–thaw cycles. After the final cycle, the flask was
allowed to warm to room temperature and then immersed into an oil bath preheated to 65 °C. After 16 h, the reaction mixture was diluted with CH₂Cl₂ and precipitated into hexanes. The residue was redissolved in a minimal quantity of CH₂Cl₂ and precipitated again into hexanes with vigorous stirring. This was repeated twice more. The yellow polymer was concentrated in vacuo from CH₂Cl₂ three

26 Baumgart, T.; Hunt, G.; Farkas, E. R.; Webb, W. W.; Feigenson, G. W. Biochim. Biophys. Acta - Biomembranes 2007, 1768, 2182–2194.
27 Skaug, M. J.; Longo, M. L.; Faller, R. J. Phys. Chem. B 2009, 113, 8758–8766.
times to remove residual hexanes and dried under high vacuum overnight to give polymer **P2** as a pale-yellow solid (253 mg, 82%). SEC (DMF, 2% LiBr): $M_n = 17.5$ kDa, $M_w = 15.9$ kDa, $DP \approx 240$, PDI = 1.10. $^1$H NMR (CDCl$_3$, 500 MHz) δ: 5.28–4.90 (bm, 2H), 4.45 (bm, 1H), 4.18 (bm, 1H), 4.20–3.69 (bm, 3H), 3.38 (bm, 2H), 3.21 (bm, 1H), 2.65–2.10 (bm, 716H), 2.00–1.85 (bm, 88H), 1.85–1.40 (m, 221H), 1.35–1.00 (bm, 155H), 0.89 (m, 9H). FT-IR (KBr, CH$_2$Cl$_2$) ν (cm$^{-1}$): 3406, 2926, 2848, 1647, 1554, 1375, 1117, 1051, 918.

**Synthesis of Glycopolymer P4.** A vial (4 mL) equipped with a magnetic stirring bar was charged with alkylene-terminated polymer **P3** (4.0 mg, 57.1 μmol of keto-groups) and α-aminoxy-GaINAc (14 mg, 59.3 μmol, 1.03 equiv). THF (0.2 mL) was added, and the polymer was allowed to dissolve; then 50 mM sodium phosphate buffer (0.2 mL, pH = 5.2) was added. The headspace was filled with nitrogen; the vial was capped and heated at 50 °C for 20 h. After this time, the solvents were removed, and the crude product was dialyzed against distilled water for 24 h with the water being changed periodically. The product was lyophilized to yield a fluffy, white solid (8.4 mg, 80%). Estimated from $^1$H NMR: 56% GalNAc incorporation.

**Synthesis of Glycopolymer P4.** A vial (4 mL) equipped with a magnetic stirring bar was charged with alkylene-terminated polymer **P3** (4.0 mg, 57.1 μmol of keto-groups) and α-aminoxy-GaINAc (14 mg, 59.3 μmol, 1.03 equiv). THF (0.2 mL) was added, and the polymer was allowed to dissolve; then 50 mM sodium phosphate buffer (0.2 mL, pH = 5.2) was added. The headspace was filled with nitrogen; the vial was capped and heated at 50 °C for 20 h. After this time, the solvents were removed, and the crude product was dialyzed against distilled water for 24 h with the water being changed periodically. The product was lyophilized to yield a fluffy, white solid (8.4 mg, 80%). Estimated from $^1$H NMR: 56% GalNAc incorporation.

**Synthesis of Glycopolymer P4.** A vial (4 mL) equipped with a magnetic stirring bar was charged with alkylene-terminated polymer **P3** (4.0 mg, 57.1 μmol of keto-groups) and α-aminoxy-GaINAc (14 mg, 59.3 μmol, 1.03 equiv). THF (0.2 mL) was added, and the polymer was allowed to dissolve; then 50 mM sodium phosphate buffer (0.2 mL, pH = 5.2) was added. The headspace was filled with nitrogen; the vial was capped and heated at 50 °C for 20 h. After this time, the solvents were removed, and the crude product was dialyzed against distilled water for 24 h with the water being changed periodically. The product was lyophilized to yield a fluffy, white solid (8.4 mg, 80%). Estimated from $^1$H NMR: 56% GalNAc incorporation.

**Synthesis of Glycopolymer P4.** A vial (4 mL) equipped with a magnetic stirring bar was charged with alkylene-terminated polymer **P3** (4.0 mg, 57.1 μmol of keto-groups) and α-aminoxy-GaINAc (14 mg, 59.3 μmol, 1.03 equiv). THF (0.2 mL) was added, and the polymer was allowed to dissolve; then 50 mM sodium phosphate buffer (0.2 mL, pH = 5.2) was added. The headspace was filled with nitrogen; the vial was capped and heated at 50 °C for 20 h. After this time, the solvents were removed, and the crude product was dialyzed against distilled water for 24 h with the water being changed periodically. The product was lyophilized to yield a fluffy, white solid (8.4 mg, 80%). Estimated from $^1$H NMR: 56% GalNAc incorporation.

**Synthesis of Glycopolymer P4.** A vial (4 mL) equipped with a magnetic stirring bar was charged with alkylene-terminated polymer **P3** (4.0 mg, 57.1 μmol of keto-groups) and α-aminoxy-GaINAc (14 mg, 59.3 μmol, 1.03 equiv). THF (0.2 mL) was added, and the polymer was allowed to dissolve; then 50 mM sodium phosphate buffer (0.2 mL, pH = 5.2) was added. The headspace was filled with nitrogen; the vial was capped and heated at 50 °C for 20 h. After this time, the solvents were removed, and the crude product was dialyzed against distilled water for 24 h with the water being changed periodically. The product was lyophilized to yield a fluffy, white solid (8.4 mg, 80%). Estimated from $^1$H NMR: 56% GalNAc incorporation.

**Synthesis of Glycopolymer P4.** A vial (4 mL) equipped with a magnetic stirring bar was charged with alkylene-terminated polymer **P3** (4.0 mg, 57.1 μmol of keto-groups) and α-aminoxy-GaINAc (14 mg, 59.3 μmol, 1.03 equiv). THF (0.2 mL) was added, and the polymer was allowed to dissolve; then 50 mM sodium phosphate buffer (0.2 mL, pH = 5.2) was added. The headspace was filled with nitrogen; the vial was capped and heated at 50 °C for 20 h. After this time, the solvents were removed, and the crude product was dialyzed against distilled water for 24 h with the water being changed periodically. The product was lyophilized to yield a fluffy, white solid (8.4 mg, 80%). Estimated from $^1$H NMR: 56% GalNAc incorporation.

**Synthesis of Glycopolymer P4.** A vial (4 mL) equipped with a magnetic stirring bar was charged with alkylene-terminated polymer **P3** (4.0 mg, 57.1 μmol of keto-groups) and α-aminoxy-GaINAc (14 mg, 59.3 μmol, 1.03 equiv). THF (0.2 mL) was added, and the polymer was allowed to dissolve; then 50 mM sodium phosphate buffer (0.2 mL, pH = 5.2) was added. The headspace was filled with nitrogen; the vial was capped and heated at 50 °C for 20 h. After this time, the solvents were removed, and the crude product was dialyzed against distilled water for 24 h with the water being changed periodically. The product was lyophilized to yield a fluffy, white solid (8.4 mg, 80%). Estimated from $^1$H NMR: 56% GalNAc incorporation.

**Synthesis of Glycopolymer P4.** A vial (4 mL) equipped with a magnetic stirring bar was charged with alkylene-terminated polymer **P3** (4.0 mg, 57.1 μmol of keto-groups) and α-aminoxy-GaINAc (14 mg, 59.3 μmol, 1.03 equiv). THF (0.2 mL) was added, and the polymer was allowed to dissolve; then 50 mM sodium phosphate buffer (0.2 mL, pH = 5.2) was added. The headspace was filled with nitrogen; the vial was capped and heated at 50 °C for 20 h. After this time, the solvents were removed, and the crude product was dialyzed against distilled water for 24 h with the water being changed periodically. The product was lyophilized to yield a fluffy, white solid (8.4 mg, 80%). Estimated from $^1$H NMR: 56% GalNAc incorporation.