

# Stereochemical Control Yields Mucin Mimetic Polymers

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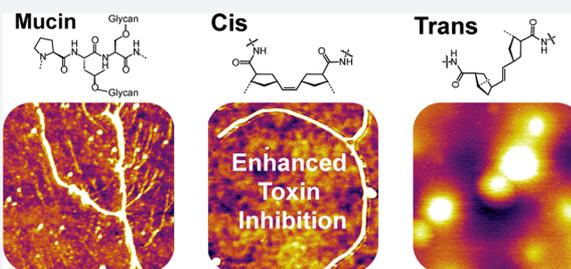


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**ABSTRACT:** All animals except sponges produce mucus. Across the animal kingdom, this hydrogel mediates surface wetting, viscosity, and protection against microbes. The primary components of mucus hydrogels are mucins—high molecular weight *O*-glycoproteins that adopt extended linear structures. Glycosylation is integral to mucin function, but other characteristics that give rise to their advantageous biological activities are unknown. We postulated that the extended conformation of mucins is critical for their ability to block microbial virulence phenotypes. To test this hypothesis, we developed synthetic mucin mimics that recapitulate the dense display of glycans and morphology of mucin. We varied the catalyst in a ring-opening metathesis polymerization (ROMP) to generate substituted norbornene-derived glycopolymers containing either *cis*- or *trans*-alkenes. Conformational analysis of the polymers based on allylic strain suggested that *cis*- rather than *trans*-poly(norbornene) glycopolymers would adopt linear structures that mimic mucins. High-resolution atomic force micrographs of our polymers and natively purified Muc2, Muc5AC, and Muc6B mucins revealed that *cis*-polymers adopt extended, mucin-like structures. The *cis*-polymers retained this structure in solution and were more water-soluble than their *trans*-analogs. Consistent with mucin's linear morphology, *cis*-glycopolymers were more potent binders of a bacterial virulence factor, cholera toxin. Our findings highlight the importance of the polymer backbone in mucin surrogate design and underscore the significance of the extended mucin backbone for inhibiting virulence.



## INTRODUCTION

Sponges lack mucus, but all other animals produce it.<sup>1</sup> Mucus coats tissue surfaces, increasing lubrication and providing spatial segregation between a host and its environment.<sup>2</sup> Barrier-forming mucus gels serve as the primary ecological niche for animal microbiomes. Accumulating evidence suggests that while providing this livable habitat mucus barriers can suppress microbial virulence traits.<sup>3–6</sup> In regulating virulence, mucus can trap microbial toxins, act as a shield against microbial invasion, and serve as a food source for symbiotic bacteria.<sup>7–12</sup> These functions are mediated by mucus's primary components, densely glycosylated polypeptides known as mucins. Uncovering the critical attributes of mucins that give rise to these different biological functions has been difficult using genetic or biochemical strategies because mucin biosynthesis is not readily programmable.<sup>13</sup> Synthetic mucin mimics can be readily varied to elucidate what features of these glycoproteins underlie their functional roles. The generation of tailored mucin mimics could lead to a suite of breakthrough technologies, including rewetting materials for eye-care and skin-care, prebiotic dietary supplements, and antibiotic alternatives.<sup>14,15</sup>

Designing mucin surrogates requires an understanding of critical mucin features. As highly *O*-glycosylated proteins, mucins adopt extended linear conformations (Figure 1) that

stretch hundreds of nanometers.<sup>16–26</sup> The resulting viscoelastic properties of mucins have previously been approximated with synthetic mimics, but those agents do not resemble the bottlebrush displays of mucin-linked glycans.<sup>27–31</sup> This glycan presentation is essential to recapitulate because perturbing mucin glycosylation *in vivo* abrogates function.<sup>32–36</sup> Indeed, studies of polymer mimics of mucins highlight the importance of length and glycan identity.<sup>37–39</sup> We postulated that another important attribute of mucins was their extended linear backbones.

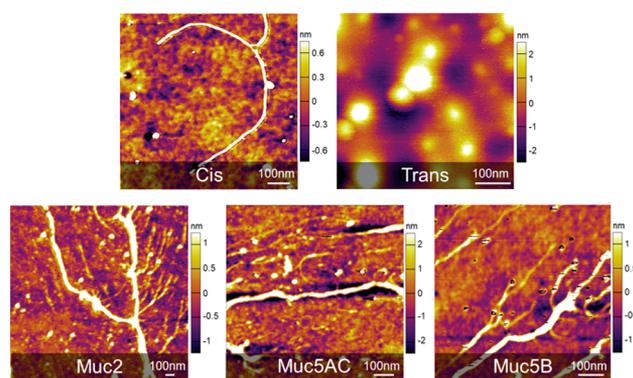
To test the role of conformation, we sought to compare the mucin mimicking properties of glycan-substituted polymers that either adopt an extended conformation or a less rigid, globular structure. We reasoned that stereocontrolled ring-opening metathesis polymerization (ROMP) reactions<sup>40–46</sup> would provide access to the target polymers. Though many studies have focused on developing *cis*- or *trans*-selective

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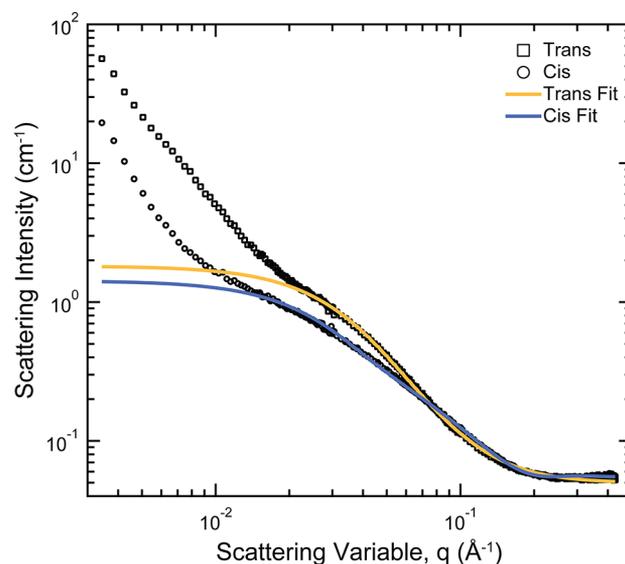
**Figure 3.** Atomic force microscopy images of 50% galactose functionalized cis- and trans-poly(norbornene) 200mers ( $\sim 10$  nM) and natively purified porcine intestinal mucin (Muc2), salivary mucin (Muc5B), and gastric mucin (Muc5AC).

Cis-200mers with 25% galactose functionalization always adopted an extended linear structure (SI Figure 3), but polymers fully substituted with galactose had the most chain entanglement (SI Figure 4). We attribute this behavior to the increased water solubility of the densely glycosylated cis-polymers. During evaporation, highly concentrated droplets form, and their final drying results in the precipitation of clusters of intertangled polymers. By contrast, the trans-polymers always generate spherical globules at both 50% and 100% functionalization densities (SI Figure 5–6). These globules ranged from 10 to 100 nm in diameter and about 4 nm in height, indicating that multiple polymers aggregated.

We next compared the morphology of our synthetic glycopolymers to native secretory mucins. We purified Muc2 (intestinal mucin) and Muc5AC (gastric mucin) from porcine tissue samples, and Muc5B (salivary mucin) from human saliva. Each had a governing morphology corresponding to an extended bottlebrush structure (Figure 3). We imaged the mucin networks over hundreds of nanometers and obtained some of the highest resolution mucin images ever observed. We ascribe these results to the high quality of the isolated mucins, which were carefully purified to ensure minimal degradation and denaturation.<sup>54,55</sup> While all three mucins formed extended, micron-scale bottlebrushes, we also observed a unique condensed morphology in Muc5B (SI Figure 7). Thus, the extended linear structures, exclusively found in the cis-polymer, mimic those of mucins.

To confirm that the morphology observed in the solid-state translated to the solution phase, we analyzed cis- and trans-50%-Gal-200mers by using SANS (Figure 4). SANS enables the determination of the bulk solution structure for polymers composed of light elements. The cis-polymers were best fit to a model of flexible cylinders with a persistence length of 10.5 nm, confirming their solution structure as extended polymer chains. In contrast, trans-polymers, which displayed a steeper slope in their scattering intensity, best fit a globular model with a persistence length of  $\sim 1.6$  nm, indicating that they were coiling in solution. These data indicate cis- and trans-polymers retain the conformations observed in a solid-state in the aqueous phase.

We postulated that the differences in the cis- and trans-poly(norbornene) structures would be relevant for their activity. We evaluated these polymers' relative affinity for the virulence factor cholera toxin (Ctx) and compared them to purified native secretory mucins. *Vibrio cholerae* produce Ctx,

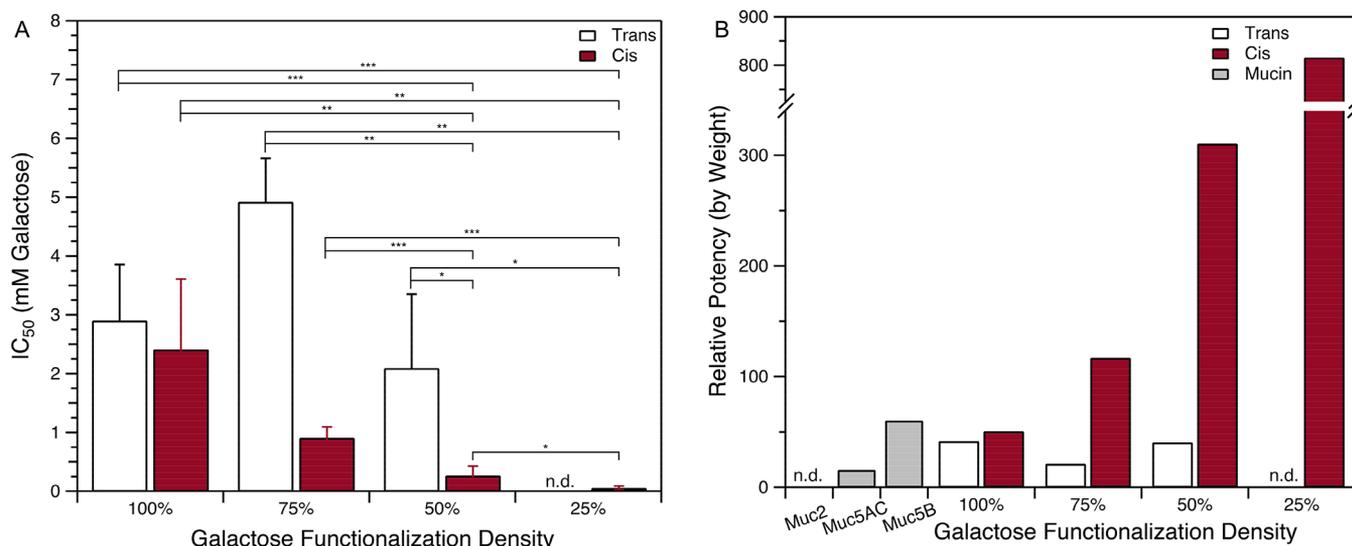


**Figure 4.** Small-angle neutron scattering (SANS) data for 50% galactose-functionalized trans-poly(norbornene) and cis-poly(norbornene) with best-fit lines in yellow and blue, respectively

and the toxin's activity promotes infection. Previous studies suggest that Ctx binding to multivalent galactose derivatives depends on their structure,<sup>51,56</sup> and well-established protocols are available for assaying Ctx inhibitors.<sup>52,56,57</sup> Finally, because Ctx must penetrate the intestinal mucus barrier before it can intoxicate cells, its interaction with mucin-linked galactose is of clinical relevance.<sup>11,58</sup>

We determined the concentration of galactose residues at which half of the toxin was bound ( $IC_{50}$  value) by each polymer (Figure 5).<sup>52</sup> The assay involved exposing fluorescein-labeled Ctx to a dilution series of polymer or mucin and adding the resulting mixtures to a plate coated with the naturally occurring ligand, GM1 ganglioside. Fluorescence was quantified, and the extent of either polymer or mucin binding was assessed in a competition assay. A comparison of the 200mers indicated that the linear cis-polymers had  $IC_{50}$  values 5- to 10-fold lower than those obtained for the corresponding globular trans-polymers. These data indicate a significant preference for cis-polymer binding (Figure 5A). The trans-polymers with galactose residue substitution below 50% were not sufficiently soluble to obtain an  $IC_{50}$  value. In contrast, the cis-analogs were not only soluble but also the best inhibitors tested. Indeed, all cis-materials proved more soluble in aqueous buffer than their trans-counterparts (SI Table 5). The observed binding enhancement for polymers with decreased functionalization is consistent with previous studies on glycan–lectin interactions; this effect presumably arises because high substitution levels result in steric occlusion of epitopes.<sup>51,59</sup> The longer 500mer polymers showed similar trends in Ctx binding and solubility (SI Figure 8).

We similarly tested samples of Muc2, Muc5AC, and Muc5B for Ctx inhibition. As all three mucins are densely glycosylated with an unknown percentage of galactose, an exact galactose concentration could not be established. Instead, we determined the  $IC_{50}$  value as a function of weight percentage and compared those values to the measured inhibition of monomeric D-galactose ( $IC_{50} = 310$  mM and 5.3 wt %) to yield a relative potency (Figure 5B). Select mucins inhibit Ctx, with Muc5B as the most potent. These data suggest that

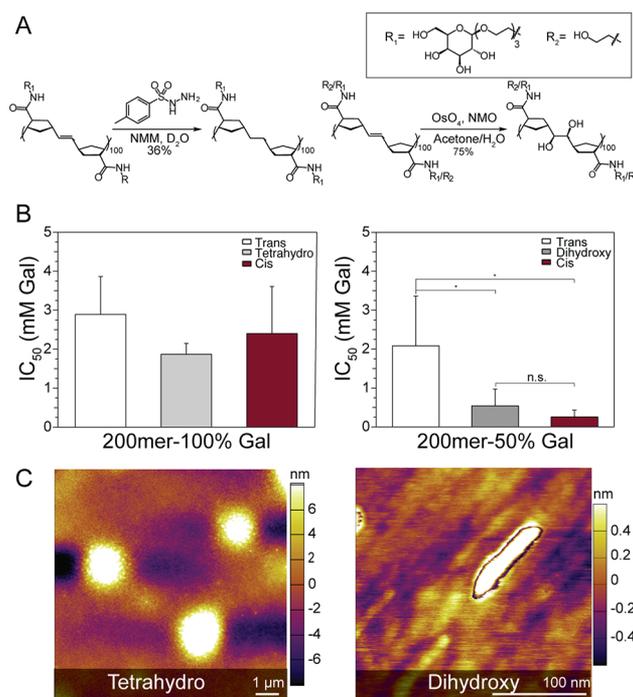


**Figure 5.** (A) Inhibition of cholera toxin binding to GM1 ganglioside by galactose-substituted cis- and trans-poly(norbornene) 200mers. Polymers are listed by their percent galactose functionalization. Inhibition data are reported as the concentration of polymer with respect to galactose at which cholera toxin binding to the GM1 ganglioside was reduced to half of its maximum value ( $IC_{50}$ ). Error bars are the standard deviation of triplicate measurements. \* $P < 0.15$ ; \*\* $P < 0.10$ ; \*\*\* $P < 0.05$ . (B) Relative inhibitory potency toward cholera toxin by weight percent for galactose-functionalized cis- and trans-poly(norbornene) 200mers, Muc2, Muc5AC, and Muc5B relative to monomeric galactose. Values were not determined (n.d.) for those polymers with  $IC_{50}$  values greater than their maximum solubility in buffer.

purified gastric mucin (Muc5AC) and salivary mucin (Muc5B) can serve as efficient decoy receptors for Ctx. Given Muc5B is also highly expressed in the gastrointestinal tract, its mitigating activity may be especially relevant as Ctx acts on intestinal barriers. However, when compared to our synthetic mucin mimics, the native mucins are far less potent inhibitors. This preferential binding of our mucin mimetic polymers is rooted in their design: they have terminal galactose residues for Ctx binding, whereas native mucins possess a diversity of glycans.

We next evaluated whether the observed Ctx binding was galactose-dependent, as we postulated that the observed differences in solubility were due to polymer architecture. To this end, we generated glucamine-functionalized poly(norbornene). Glucamine is an open-chain, reduced sugar that should exhibit no affinity for Ctx. Neither cis- nor trans-glucamine polymer bound to Ctx. As with the galactose-substituted polymers, the cis-polymer was much more water-soluble than its trans-analog (SI Table 5). Together with the Ctx inhibition results, these data indicate that the glycopolymer backbone modulates structure and solubility and can impact glycan–lectin binding in mucin-mimetic interactions.

To assess whether the backbone drives poly(norbornene) conformation and therefore influences Ctx inhibition, we used hydrogenation and syn-dihydroxylation (Figure 6A) to modify the backbone alkenes. Backbone reduction should increase chain flexibility and thereby promote aggregation and enhance globule formation. To test this idea, we subjected our polymers to transfer hydrogenation conditions using *p*-toluenesulfonylhydrazide (Figure 6A, left).<sup>60</sup> The reduction of partially functionalized materials yielded insoluble films, but a fully functionalized galactose 200mer underwent reduction with ~85% efficiency. The resulting polymer was slightly less soluble than its trans-alkene analog and proved to be an equivalent Ctx inhibitor (Figure 6B, left). This finding was unsurprising since differences in inhibition between cis- and trans-polymers are only observed at lower saccharide functionalization densities. Consistent with our structural



**Figure 6.** (A) Synthetic scheme; (B)  $IC_{50}$  values for cholera toxin; and (C) atomic force microscopy images of galactose-substituted polymers with reduced (left) or dihydroxylated (right) poly(norbornene) backbones. Error bars are the standard deviation of triplicate measurement (for reduced polymers (left) no statistically relevant differences were observed). \* $P < 0.15$ ; n.s.  $P \geq 0.15$ .

prediction, AFM revealed that the polymer with the reduced backbone adopts giant globular structures  $>1 \mu\text{m}$  in diameter (Figure 6C, left).

We compared the results with the fully reduced polymer to those with dihydroxylated polymer. We expected the latter to be less hydrophobic than the parent trans-polymer. Because

the gauche effect should restrict rotational freedom, we anticipated that syn-dihydroxylation would afford materials with a more cis-like morphology, solubility, and potency toward Ctx. Trans-200mers 50% functionalized with galactose readily underwent near quantitative dihydroxylation with osmium tetroxide and *N*-methylmorpholine *N*-oxide (Figure 6A, right). In line with our predictions, the dihydroxy-polymer was far more water-soluble than its parent polymer (SI Table 6). This material yielded an IC<sub>50</sub> value between that of the cis- and trans-polymers (Figure 6B, right) and adopted a compact cylindrical structure intermediate between the morphology of the trans- and cis-polymers (Figure 6C, right). These data suggest that trans-polymer aggregation contributed to their weak affinity for Ctx. Thus, chemical and stereochemical manipulation of poly(norbornene) backbones dramatically affects polymer structure and biological function.

In sum, we synthesized cis-, trans-, dihydroxy-, and tetrahydro-poly(norbornene) functionalized with galactose to evaluate their mucin mimetic properties. We found that the conformation and aggregation states of these poly(norbornene) systems are governed by polymer backbone allylic strain and hydrophobic interactions. Exploiting these structural control elements, we generated cis- and dihydroxy-poly(norbornene), which adopt an extended linear morphology that mimics native mucin. These extended-backbone polymers have enhanced water solubility and more effectively sequester a microbial virulence factor. Our findings outline a critical design principle for synthetic mucin mimics that will guide future studies of mucin's role in microbial symbiosis and pathogenesis and serve as a blueprint for generating mucin mimics that act as lubricants or control microbiome composition and infectious disease.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscentsci.0c01569>.

Experimental procedures, materials, instrumentation, and additional tables and figures including ligand and polymer syntheses, fluorescence inhibition assay protocol, polymer solubility data, Fourier-transform infrared spectroscopy, additional atomic force micrographs, fluorescence inhibition results of 500mers, and <sup>1</sup>H NMR spectra (PDF)

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### Author Contributions

||A.G.K. and S.D.B. contributed equally. A.G.K., S.D.B., and L.L.K. conceived of the experimental design. A.G.K., S.D.B., D.H.W., and D.R.C. synthesized and characterized all materials except catalyst 2 and performed atomic force microscopy. G.C.-O. and K.R. provided mucin. Y.W. and M.J.A.H. performed small-angle neutron scattering and interpreted the data. T.Y. and R.R.S. provided catalyst 2. L.L.K. oversaw the majority of the experimental work. A.G.K. and S.D.B. wrote the manuscript with input from all of the authors.

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### Notes

The authors declare no competing financial interest. Protocols involving samples from human participants were approved by the Massachusetts Institute of Technology's Committee on the Use of Humans as Experimental Subjects.

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## ■ ABBREVIATIONS

ROMP, ring-opening metathesis polymerization; AFM, atomic force microscopy; SANS, small-angle neutron scattering; Ctx, cholera toxin

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#### ■ NOTE ADDED AFTER ASAP PUBLICATION

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