

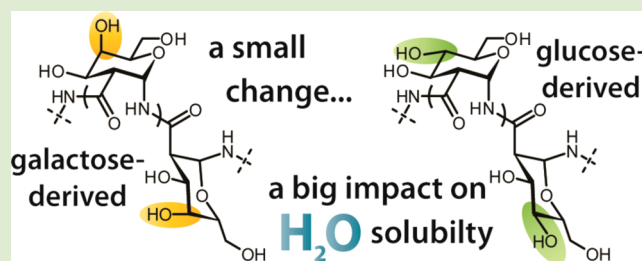
# Synthetic Enantiopure Carbohydrate Polymers That Are Highly Soluble in Water and Noncytotoxic

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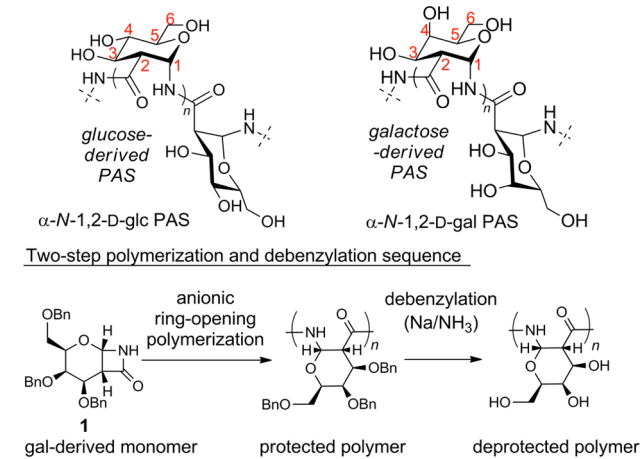
## Supporting Information

**ABSTRACT:** The first synthesis of polyamido-saccharides (PASs) from a galactose (gal)-derived  $\beta$ -lactam sugar monomer is reported. The polymers are prepared using a controlled anionic ring-opening polymerization and characterized by NMR, optical rotation, IR, and GPC. Galactose-derived PASs display high solubility in aqueous solutions and are non-cytotoxic to HepG2, CHO, and HeLa cell lines. To evaluate whether gal-derived PASs are recognized by the gal-specific lectin present on human hepatocytes, cellular uptake of rhodamine-labeled polymers is assessed using flow cytometry and fluorescence microscopy. On the basis of these results, the polymers are taken into cells via endocytosis that is not dependent on the gal-specific receptor on hepatocytes. Neutral, hydrophilic polymers, such as gal-derived PASs, are desirable materials for a range of biomedical applications, such as drug delivery, surface passivation, and hydrogel formation.



Synthetic neutral hydrophilic polymers, such as polyethylene glycols (PEGs),<sup>1</sup> polyglycerols,<sup>2</sup> poly(2-oxazoline)s (POx's),<sup>3</sup> and polyphosphoesters,<sup>4</sup> are used in a wide range of applications. Within this family, water-soluble polymers inspired by polysaccharides<sup>5</sup> are employed in a variety of important biomedical contexts, for example as drug-delivery vehicles,<sup>6</sup> as nonfouling surface coatings,<sup>7</sup> and as hydrogel<sup>8</sup> components. We are interested in preparing carbohydrate polymers that retain the stereochemically defined, cyclic backbone of natural polysaccharides to understand how these structural aspects influence polymer properties. In general, such polymers are considered difficult to access synthetically.<sup>9</sup> Recently, we reported a new approach that replaces the ether linkage found in natural polysaccharides with an amide linkage and termed these polymers polyamido-saccharides (PASs) (Scheme 1).<sup>10</sup> In the initial investigation, we used a glucose-derived monomer to prepare  $\alpha$ -N-1,2-D-glucose polyamido-saccharides ( $\alpha$ -N-1,2-D-glc PASs) using a two-step polymerization/deprotection sequence. Our approach is notable for allowing the controlled synthesis of enantiopure carbohydrate polymers of low dispersity ( $\bar{D}$ ). Additionally, the PASs obtained have the advantage of containing hydroxyl groups for facile covalent modification. Herein, we extend this methodology to the synthesis of galactose-derived PASs, specifically  $\alpha$ -N-1,2-D-galactose polyamido-saccharides ( $\alpha$ -N-1,2-D-gal PASs). Compared to the glc-derived PASs, gal-derived PASs differ only in that the hydroxyl group at the C<sub>4</sub> position is axial rather than equatorial (Scheme 1, top). This minor structural change results in polymers significantly more water soluble than the glucose derivatives. Specifically, we report: (1) the synthesis of  $\alpha$ -N-1,2-D-gal PASs with molecular weights as high as 35 kDa, (2) the preparation of amine-terminated PASs for subsequent

## Scheme 1. Synthesis of Polyamido-saccharides (PASs)



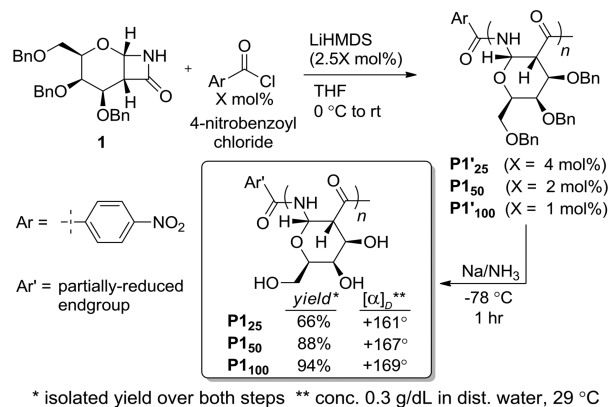
functionalization, (3) cytotoxicity studies, and (4) cellular uptake studies.

Using *para*-nitrobenzoyl chloride as the initiator, the anionic ring-opening polymerization<sup>11</sup> of monomer **1**<sup>12</sup> was performed at three different initiator loadings (4, 2, and 1 mol %) to prepare a series of polymers of increasing degrees of polymerization, **P1**'<sub>25</sub>, **P1**'<sub>50</sub>, and **P1**'<sub>100</sub> ( $DP_{th} = 25, 50, 100$ , respectively) (Scheme 2). Complete consumption of the monomer was observed with initiator loadings of 4 and 2 mol %. In contrast to our previous observations using the glc-

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Scheme 2. Polymerization of **1** with Acyl Chloride Initiator

derived monomer, monomer **1** was not completely consumed at an initiator loading of 1 mol %. On the basis of proton NMR integration of the reaction mixture, more than 90% of the monomer was consumed. The reason for polymer termination is unknown. Possible explanations include the longer polymer chains becoming unreactive due to steric bulk or the acylated  $\beta$ -lactam at the growing polymer chain end undergoing a side reaction. Polymerizations of noncarbohydrate-derived  $\beta$ -lactam monomers have also shown a trend of incomplete conversion at low initiator loadings.<sup>11a</sup>

The resulting polymers, P1'25, P1'50, and P1'100, were characterized by <sup>1</sup>H NMR, with all samples having broadened peaks. A representative <sup>13</sup>C NMR spectrum was obtained of P1'25 (see Supporting Information). The polymers were also characterized by IR (see Figure S1, Supporting Information, for spectra). Polymer molecular weights were determined using GPC with THF as the eluent and polystyrene standards (Table 1). The  $M_n$ -values showed the anticipated trend of increasing

Table 1. Polymer Characterization Using GPC

	$M_{n(th)}/M_{n(GPC)}^a$ (kDa)	$M_{w(GPC)}^a$ (kDa)	$\bar{D}^b$	DP <sub>th</sub> /DP <sub>GPC</sub>
P1'25	11.6/15.4	18.2	1.2	25/34
P1'50	23.1/28.8	31.5	1.1	50/63
P1'100	46.1/81.0	98.4	1.2	100/176
P125	4.8/9.0	10.3	1.1	25/48
P150	9.6/15.6	19.0	1.2	50/82
P1100	19.0/35.2	54.9	1.6	100/186
P2'25	11.7/7.6	9.4	1.2	25/17
P2'50	23.2/17.2	28.2	1.6	50/37
P2'100	46.2/49.7	77.2	1.6	100/108
P225	4.8/5.9	6.8	1.2	25/31
P250	9.6/17.7	23.2	1.3	50/94
P2100	19.0/23.2	47.0	2.3	100/123

<sup>a</sup>Determined by THF GPC with polystyrene standards (P1', P2') or aqueous GPC with dextran standards (P1, P2). <sup>b</sup> $M_w/M_n$ .

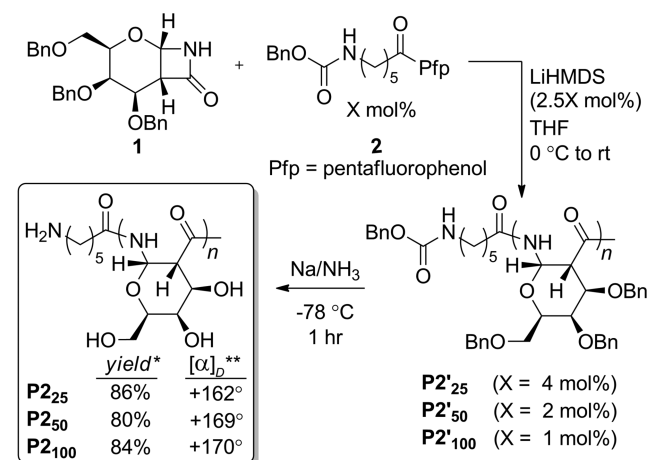
$M_n$  with decreasing initiator loading. The dispersities were low, 1.1 or 1.2, as was previously found with the glucose-derived PASs.

Polymers P1'25, P1'50, and P1'100 were debenzylated using sodium metal in liquid ammonia at -78 °C. Following dialysis and lyophilization, the resulting polymers (P125, P150, and P1100) were isolated as white powders. For P1100, unreacted monomer was removed during dialysis. The isolated yields over both steps, polymerization and debenylation, ranged from 66 to 94%. The <sup>1</sup>H NMR spectra were collected in D<sub>2</sub>O and

showed well-resolved signals and couplings. A representative <sup>13</sup>C NMR spectrum of P125 showed the expected seven signals (see Supporting Information). On the basis of the <sup>1</sup>H NMR spectrum of P125, the end group was partially reduced, as has been previously observed with end groups containing aromatic rings.<sup>10</sup> Specific rotations measured in water ranged from +161° for P125 to +169° for P1100 (Scheme 2, box). The molecular weights of the deprotected polymers were characterized using aqueous GPC with dextran standards. The  $M_n$ -values were larger than expected. Computational modeling<sup>10</sup> suggests that PASs form rod-like, rather than globular, structures, and this rod-like structure may result in an overestimation of molecular weight, as has been previously noted for other polymers.<sup>13</sup> The dispersities of P125 (1.1) and P150 (1.2) were similar to the values observed for the protected polymers. However, the dispersity of P1100 was significantly higher. This increase in dispersity does not appear to be a result of bond cleavage, as the DP<sub>GPC</sub>-values before and after reduction were comparable (176 and 186, respectively) and therefore may be related to the difference in the solution structure of the more rod-like, linear PAS compared to the more compact, branched dextran.

To perform the cellular uptake studies, we required a PAS possessing a terminal amine for subsequent functionalization with rhodamine. Thus, a Z-6-aminohexanoic acid derivative was selected because it allowed for the introduction of a benzyloxy-protected primary amine that would be deprotected under the debenylation conditions. However, preparation and purification of the corresponding acid chloride, Z-6-aminohexanoyl chloride, was expected to be challenging because of the species' high reactivity and the fact that it contains an acid-sensitive benzyl carbamate. Knowing that acylating agents less reactive than acyl chlorides, such as anhydrides,<sup>11a</sup> can successfully initiate the controlled polymerization of  $\beta$ -lactams, we explored the use of the pentafluorophenol ester (Pfp) of Z-6-aminohexanoic acid.

As shown in Scheme 3, we prepared three PAS polymers (P2'25, P2'50, and P2'100) using monomer **1** and initiator **2**

Scheme 3. Polymerization of **1** with Pfp Ester **2**

loadings of 4, 2, and 1 mol %, respectively. At initiator loadings of 4 and 2 mol %, complete consumption of monomer was observed. As with P1'100, the polymerization to form P2'100 did not reach full conversion. On the basis of proton NMR integration of the reaction mixture, more than 80% of the

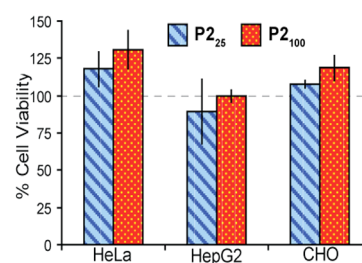
monomer was consumed. The  $^1\text{H}$  NMR spectra of the  $\text{P2}'$ -polymers were similar to those of the  $\text{P1}$ -series, as expected. For  $\text{P2}'_{25}$  and  $\text{P2}'_{50}$ , the benzylic protons of the benzyloxy carbamate protecting group at 5.05 ppm were distinguishable from the polymer signals, confirming incorporation of the end group. The dispersities of  $\text{P2}'_{50}$  and  $\text{P2}'_{100}$  were larger (1.6) than those observed for  $\text{P1}'_{50}$  and  $\text{P1}'_{100}$  (1.1 and 1.2, respectively). Therefore, compared to the acyl chloride initiator, initiator 2 provides similar results at a loading of 4 mol % but shows an increase in dispersities at 2 and 1 mol %. Additionally, use of initiator 2 afforded lower monomer consumption when 1 mol % of initiator was used. The differences in polymerization results at lower initiator loadings were likely the result of the lower reactivity of Pfp esters in comparison to acyl chlorides leading to a slower rate of initiation relative to the rate of polymerization.

The  $\text{P2}'$  polymer series was protected and isolated in the same manner as  $\text{P1}'$ , and isolated yields over both steps (80–86%) were comparable to those obtained for the  $\text{P1}$ -series. The  $^1\text{H}$  NMR spectra in  $\text{D}_2\text{O}$  were similar to those of  $\text{P1}$ . For  $\text{P2}_{25}$  and  $\text{P2}_{50}$ , signals belonging to the end group could be discerned (see spectra in Supporting Information). Specific rotations measured in water ranged from  $+162^\circ$  for  $\text{P2}_{25}$  to  $+170^\circ$  for  $\text{P2}_{100}$  (Scheme 3, box). The  $M_n$ -values based on aqueous GPC were larger than expected, as observed with the  $\text{P1}$ -series. The dispersity of  $\text{P2}_{100}$  (2.3) was larger than that of the protected polymer,  $\text{P2}'_{100}$  (1.6), but the  $\text{DP}_{\text{GPC}}$  did not decrease, as observed with  $\text{P1}_{100}$ .

In comparison to the glc-derived polymers previously reported,<sup>10</sup> the gal-derived polymers of all molecular weights showed high water solubility. All polymers were found to be soluble at 100 mg/mL; higher concentrations were not investigated. We did not observe the formation of precipitates, as was the case with glc-derived polymers. This trend was most evident when comparing  $\text{P2}_{25}$  to a glc-derived polymer of approximately the same molecular weight with the same end group. After 2 days of incubation at  $37^\circ\text{C}$ , aqueous solutions of  $\text{P2}_{25}$  remained clear, whereas solutions of the glc-derived polymer became cloudy (Figure S2, Supporting Information). Polymer solubility is a complex phenomenon, and therefore it is challenging to give a precise rationale for why changing the orientation of the  $\text{C}_4$  hydroxyl from equatorial to axial affects solubility dramatically. However, we speculate that the  $\text{C}_4$ -hydroxyl group of the gal-derived repeat unit, which points into the plane above the pyranose ring, may prevent stacking between PAS chains.

The cytotoxicity of the gal-derived PAS polymers was evaluated using an MTS cell viability assay in three cell lines: human cervix adenocarcinoma (HeLa), human liver carcinoma (HepG2), and Chinese hamster ovary (CHO). As shown in Figure 1,  $\text{P2}_{25}$  and  $\text{P2}_{100}$  at a concentration of 2.0 mg/mL in media did not decrease cell viability after 48 h of incubation in comparison to the media control, indicating no cytotoxicity.

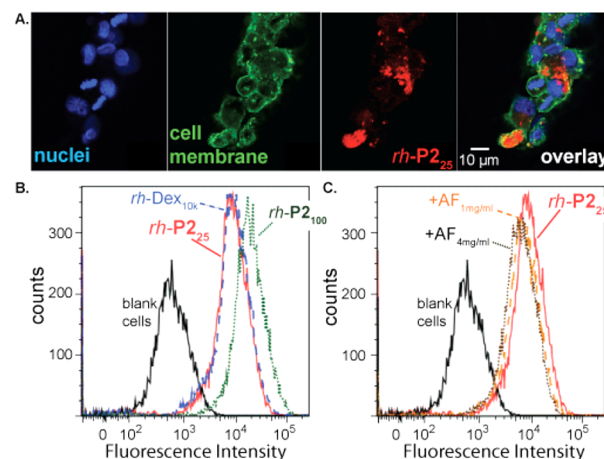
Neutral, hydrophilic polysaccharides, such as dextran, are known to be taken into cells via endocytosis that is not receptor mediated, and therefore fluorophore-labeled dextrans have been used to image cellular fluid flow in endosomes and lysosomes.<sup>14</sup> However, macromolecules containing pendant galactose groups can also be taken into liver cells by an active mechanism. They are recognized and transported into the cell by the asialoglycoprotein (ASGP) receptor, a hepatic lectin that binds some galactose derivatives with free hydroxyls at the  $\text{C}_3$  and  $\text{C}_4$  positions.<sup>15</sup> Therefore, we investigated the uptake of



**Figure 1.** Cell viability for HeLa, HepG2, and CHO cell lines measured after incubating cells with  $\text{P2}_{25}$  and  $\text{P2}_{100}$  (2.0 mg/mL in media) for 48 h using an MTS assay. Polymers  $\text{P2}_{25}$  and  $\text{P2}_{100}$  do not cause a statistically significant decrease in survivability in any of the cell lines. All experiments were done at  $n = 3$ , and error bars represent standard deviation of the mean.

PASs in a human hepatocyte line expressing the ASGP receptor to determine whether this receptor would actively transport the polymers into the cell. Understanding the nature of the interaction between gal-derived PASs and gal-specific receptors is essential in defining applications for these polymers. Hydrophilic polymers, such as PEG, are often used to shield materials from specific biological interactions. If gal-derived PASs are not recognized by gal receptors, their neutrality and high water solubility suggest their potential as shielding polymers. Conversely, a strong interaction with gal receptors would suggest their use in applications where this specific targeting is desired.

To monitor cell uptake,  $\text{P2}_{25}$  and  $\text{P2}_{100}$  were labeled with a fluorescent rhodamine derivative via conjugation with the free amine of the end group and named  $rh\text{-P2}_{25}$  and  $rh\text{-P2}_{100}$ , respectively. To confirm that the polymers entered hepatocytes, the cells were incubated with  $rh\text{-P2}_{25}$  for 24 h and then imaged with a confocal microscope (Figure 2A). The cells were fixed



**Figure 2.** (A) Confocal microscopy of fixed HepG2 cells after a 24 h incubation with  $rh\text{-P2}_{25}$  showing endocytosis. Cells were visualized with Hoechst stain (nucleus) and con A 488 conjugate (cell membrane). (B) Uptake of rhodamine-labeled PAS polymers ( $rh\text{-P2}_{25}$  and  $rh\text{-P2}_{100}$ ) was monitored using flow cytometry and compared to rhodamine-labeled dextran ( $rh\text{-Dex}_{10k}$ ) after 24 h. (C) To probe the mechanism of PAS uptake, asialofetuin (AF), a competitive binder of the ASGP receptor, was added at 1 and 4 mg/mL. Significant inhibition of  $rh\text{-P2}_{25}$  uptake was not observed, confirming that uptake is not strongly dependent on the gal-specific ASGP receptor. The concentration of rhodamine-labeled PAS was 25  $\mu\text{g}/\text{mL}$  for all experiments.



and stained to visualize the nucleus (Hoescht stain, blue) and membrane (concanavalin A 488 conjugate, green). The fluorescence signal of *rh-P2<sub>25</sub>* (red) localized within the cytosol, confirming uptake. To quantify this uptake, flow cytometry was performed with *rh-P2<sub>25</sub>* and *rh-P2<sub>100</sub>* and, for comparison, a rhodamine-labeled dextran with a molecular weight of 10 kDa (*rh-Dex<sub>10k</sub>*). After a 24 h incubation, *rh-P2<sub>25</sub>* and *rh-Dex<sub>10k</sub>* showed similar amounts of uptake, and *rh-P2<sub>100</sub>* displayed slightly higher uptake. At 4 °C, where metabolic activity was decreased, uptake of *rh-P2<sub>25</sub>* and *rh-Dex<sub>10k</sub>* was strongly inhibited, confirming that uptake was a result of endocytosis rather than passive diffusion into the cell (See Figure S3; Supporting Information).

To further probe the mechanism of uptake, we investigated *rh-P2<sub>25</sub>* uptake in the presence of asialofetuin (AF), a competitive binder for the ASGP receptor.<sup>16</sup> If *rh-P2<sub>25</sub>* was actively endocytosed by the ASGP receptor, we would expect AF to inhibit uptake through competition. As shown in Figure 2C, addition of 1 and 4 mg/mL of AF did not significantly decrease *rh-P2<sub>25</sub>* uptake. This result suggested that uptake was not strongly dependent on the gal-specific receptor. The fact that *rh-P2<sub>25</sub>* and *rh-P2<sub>100</sub>* displayed similar amounts of uptake relative to *rh-Dex<sub>10k</sub>*, a polymer that does not contain galactose and, thus, was not actively taken up, also agreed with the conclusion that the gal-derived PAS polymers were not being recognized by the ASGP receptor. Finally, under the same conditions with the same rhodamine label, glucose-derived PASs of similar molecular weights showed comparable uptake into hepatocytes, further strengthening the argument that uptake was not mediated by the ASGP receptor (data not shown).

In summary, the synthesis and characterization of gal-derived PASs are reported. Polymerization using either an acyl chloride or pentafluorophenol ester initiator results in complete consumption of monomer when 4 or 2 mol % initiator is employed. When 1 mol % initiator is used, some (<20%) unreacted monomer remains with both initiators. However, unreacted monomer is removed by dialysis to obtain pure polymers in good to high yields. The resulting polymers are characterized using NMR, optical rotation, IR, and GPC. The polymers of all molecular weights are highly water soluble, in contrast to the previously reported glc-derived polymers,<sup>10</sup> and are nontoxic to HeLa, HepG2, and CHO cell lines. On the basis of microscopy and flow cytometry studies, the polymers are taken into cells via endocytosis that is not dependent on the gal-specific receptor on hepatocytes. These results suggest that  $\alpha$ -N-1,2-D-gal PASs are of interest for applications requiring hydrophilic polymers that do not strongly interact with biological receptors. Future work will focus on identifying biomedical applications where the unique architecture and properties of these water-soluble, neutral, structurally defined carbohydrate polymers can offer advantages over existing systems.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Experimental details, supplemental figures, and <sup>1</sup>H and <sup>13</sup>C NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## Notes

The authors declare no competing financial interest.

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