Enhanced Inhibition of Human Anti-Gal Antibody Binding to Mammalian Cells by Synthetic α-Gal Epitope Polymers

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Abstract: Neoglycopolymers of polyacrylamide backbone conjugated with varying densities of Galα1−3Galβ1−4Glcβ trisaccharide epitopes (α-Gal epitopes) were designed and synthesized to study the inhibition of the binding of human natural anti-Gal antibodies to either α-Gal-containing glycoproteins or α-Gal antigens on the surface of mammalian cells. An inhibition ELISA using mouse laminin and a flow cytometry assay using pig kidney cells (PK15) were established to determine the binding affinity of the synthesized polymers. In comparison to the α-Gal monomer (Galα1−3Galβ1−4GlcNHAcβ), the α-Gal polymers dramatically enhanced the inhibition of human anti-Gal antibodies (IgG, IgM, and IgA) binding to mouse laminin or mammalian cells. Increases of 7.8×10^3- and 5.0×10^4 -fold in inhibitory potential of polymer **7C** to IgA and IgM (with IC_{50}s of 7.0 and 5.6 nM respectively) were observed over the monomer in inhibition ELISA. The results also indicated that binding enhancement of α-Gal polymers is greater for anti-Gal IgA and IgM than for IgG. Such amplified binding differences among the three anti-Gal isotypes can be utilized to selectively inhibit or remove a particular isotype of anti-Gal antibodies. Moreover, it was demonstrated through the flow cytometry assay that certain α-Gal polymers are effective in inhibition of anti-Gal antibody (in human serum) binding to pig kidney (PK15) cells. Thus, such synthetic carbohydrate polymers may find practical applications in cell xenotransplantations.

Introduction

Xenotransplantation,[1] which was considered as a means of overcoming the shortage of human organs, tissues, and cells for transplantation, has been hampered because of the severe immunological rejection by the human body. Recent studies indicated that the initial step of this rejection is the recognition of human natural anti-Gal antibody to carbohydrate epitopes bearing a Galα1−3Galβ terminus[2] (termed as α-Gal epitope or α-Gal) on the surface of animal cells. Trisaccharides Galα1−3Galβ1−4Glcβ−R (**1**) and Galα1−3Galβ1−4GlcNAcβ-R′ (**2**) and pentasaccharide Galα1−3Galβ1−4GlcNAcβ1−3Galβ1−4Glcβ-R″ (**3**) (Figure 1) have been identified as the major α-Gal epitopes, which are abundantly expressed on the cells of most mammals with the exception of humans, apes, and other Old World primates.[3] Conversely, anti-Gal antibodies (known as anti-Gal) are the most abundant human natural antibodies in blood serum, constituting 1−2% of total serum IgG and 3−8% of total IgM.[4] Interestingly, each individual subclass of anti-Gal plays a quite distinctive role in the human immune rejection of xenografts.[2b,3b] In the initial stage of the rejection (termed hyperacute rejection or HAR), anti-Gal IgG binds to α-Gal epitopes expressed on the surface of xenograft cells triggering the antibody-dependent cell-mediated cytotoxicity by human blood monocytes and macrophages. The IgM isotype of anti-Gal is believed to be responsible for the complement activation that leads to complement-mediated lysis of the xenograft cells. Various approaches have been studied to prevent the hyperacute rejection and to prolong xenograft cell survival in xenotransplanation based on elimination or reduction of the interaction between α-Gal and anti-Gal. These methods include encapsulation of cells for the cellular replacement therapy,[5] depletion of

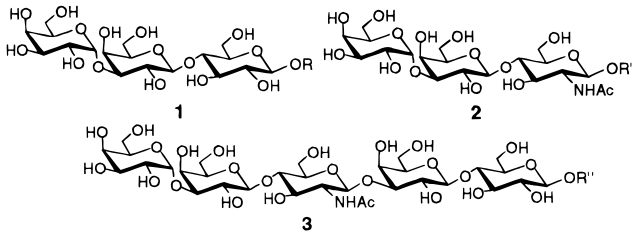

Figure 1. The structures of major α-galactosyl epitopes.

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anti-Gal antibodies from the human recipients using α-Gal epitope affinity column (anti-Gal immunoadsorption approach),[6] and inhibition of anti-Gal antibody binding to xenograft cells by infusing soluble synthetic α-Gal oligosaccharides (anti-Gal neutralization approach),[7] etc. In attempts to prevent the rejection using neutralization approach, a number of α-Gal oligosaccharides have been tested for the inhibition of anti-Gal binding to the porcine endothelial cells. Results indicated[8] that it would require a millimolar level of α-Gal trisaccharide Galα1−3Galβ1−4GlcNAc to achieve 90% inhibition of anti-Gal binding to the porcine endothelial cells, which is not sufficient for practical application. Thus, how to conquer low binding affinity of α-Gal to anti-Gal antibody becomes a focal point in this approach.

In biological systems the interactions between cell surface carbohydrates and sugar-binding proteins are of polyvalent nature. Although low affinity and poor specificity are intrinsic in protein binding, carbohydrates function as very important signaling molecules in a wide variety of biological recognition events. Their high avidity can be attributed, at least in part, to their multivalent presentation pattern on the cell surface. Carbohydrates are typically expressed on the cell surface in clusters; thus their overall binding capacity with protein receptors (commonly with multiple binding sites) is enhanced over the affinity of individual monovalent ligands through cooperative multiple interactions. Therefore, it is of logical choice to use multivalent ligands as potent synthetic inhibitors to effectively block the recognition process. The multivalent inhibitors of carbohydrate-binding proteins, bacteria, and viruses have been well studied primarily by Lee,[9a,b,g] Whitesides,[9c,10a,b] Roy,[9d,e] and others[9f,10d] in recent years. They have demonstrated that the multivalent forms of carbohydrate ligands, either polymers or dendrimers, often have amplified inhibitory effects over their monovalent counterparts, although the levels of enhancement vary.

In this report, we synthesized a series of polymers with varied densities of α-Gal epitopes to investigate their binding affinity to anti-Gal antibodies. The inhibition ELISA and flow cytometry assays were established accordingly to evaluate these polymers. Our results clearly demonstrated that the overall avidity of certain α-Gal-containing polymers toward anti-Gal antibodies was significantly enhanced by their "multivalent" or "polyvalent effects".

Results and Discussion

Synthesis of α-Gal-Containing Polymers (7A−7F). The syntheses of α-Gal-containing polymers were achieved by the reaction of preactivated poly [*N*-(acryloyloxy) succinimide] (pNAS) (**5**) with an α-Gal trisaccharide derivative (**6**), followed by capping the active esters with aqueous ammonia (Scheme 1).[10] All α-Gal polymers (**7A**−**7F**) used in this study were prepared from one single batch of pNAS, which was obtained by polymerization of *N*-(acryloyloxy) succinimide (**4**). The molecular weight of this "parent" polymer was determined by gel filtration chromatography after its complete hydrolysis to poly (acrylic acid) sodium salt. The average molecular weight of the hydrolyzed polymer was $M_w = 252$ kD with a relatively narrow molecular weight distribution ($M_w/M_N = 1.5$). The degree of polymerization is $\sim 1.8 \times 10^3$. By varying the ratio of α-Gal trisaccharide to active esters in pNAS, we obtained a series of polymers with different densities of α-Gal. The ratios of α-Gal unit to acrylamide unit calculated from the integration of trisaccharide signals and acrylamide signals in ^{1}H NMR spectra were 1:1.8 (**7A**), 1:2.5 (**7B**), 1:3.3 (**7C**), 1:6.4 (**7D**), 1:15 (**7E**), and 1:29 (**7F**), respectively. The degrees of functionalization of polymers with α-Gal trisaccharide were 36% (**7A**), 28% (**7B**), 23% (**7C**), 13% (**7D**), 6% (**7E**), and 3% (**7F**), respectively.

The synthesis of α-Gal trisaccharide derivative[11] {*N*-[*O*-α-D-galactopyranosyl)-(1→3)-*O*-(β-D-galactopyranosyl)-(1→4)-1-β-D-glucopyranosyl]-5-aminopentamide} **6** started with a glycosylation reaction between donor **8** and acceptor **9** promoted by NIS/TfOH in dichloromethane at −30 °C with 4 Å MS as water scavenger. Trisaccharide derivative **10** was afforded in 90% yield with 20:1 (α/β) selectivity. A mild hydrogenation condition with PtO_2 in methanol selectively reduced the azido group of **10** to primary amine, which was immediately reacted with 5-chlorovaleryl chloride to give compound **11** in 87% yield. Sequential debenzylation and acetylation reactions of **11** produced peracetylated trisaccharide **13** in 89% yield. Nucleophilic substitution of **13** with sodium azide in DMF gave **14** in 90% yield. The fully deprotected α-Gal trisaccharide **6** was therefore obtained from **14** in 93% yield after deacetylation and hydrogenation.

A shortcut to compound **13** was the chemo-enzymatic synthesis[11] illustrated in Scheme 2. Glycosylation of UDP-glucose (**16**) and acceptor **17** produced trisaccharide **18** in 65% yield using a fusion enzyme (GalE-GalT)[12] as a catalyst. This enzyme contained both uridine-5′-diphospho-galactose 4-epimerase (GalE) and α(1→3) galactosyltransferase (GalT) and was recently cloned in our laboratory by in-frame fusion of the *Escherichia coli* gene *galE* to the 3′-terminus of a truncated bovine *galT* gene within a high-expression plasmid. It has dual

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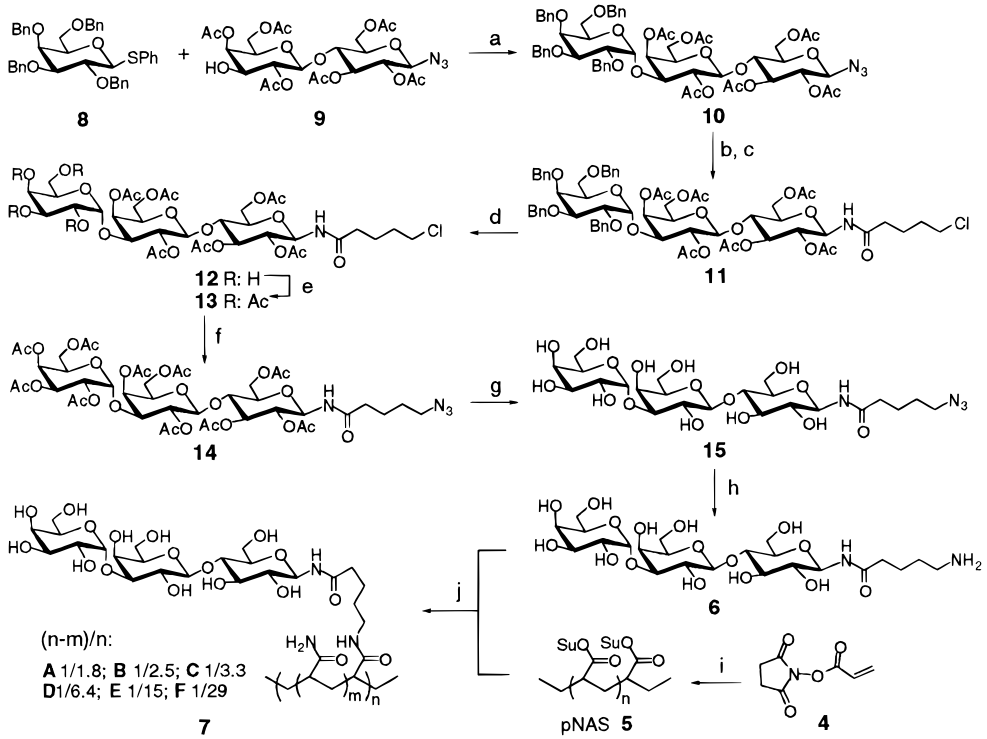
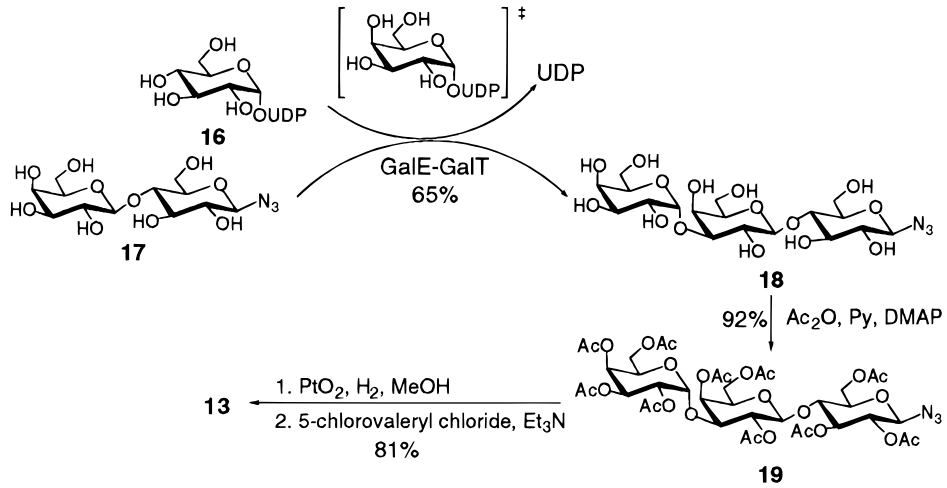
Scheme 1. Chemical Synthesis of α-Gal Polymers (**7A**−**F**)[a]



[a] Reagents: (a) NIS, TfOH, CH_2Cl_2, 4A MS, −30 °C, 90%; (b) PtO_2, H_2, MeOH; (c) 5-chlorovaleryl chloride, Et_3N, CH_2Cl_2, 87% (b and c); (d) Pd/C, H_2, MeOH; (e) Py/Ac_2O, DMAP, 89%; (f) NaN_3, DMF, 70 °C, 90%; (g) NaOMe, MeOH; (h) PtO_2/H_2, H_2O-MeOH, 93% (g and h); (i) AlBN, PhH, reflux; (j) DMF, rt, 24 h; 65 °C, 6 h; rt, 24 h then $NH_3 \cdot H_2O$, rt, 24 h.

Scheme 2. Enzymatic Chemical Synthesis of **13**

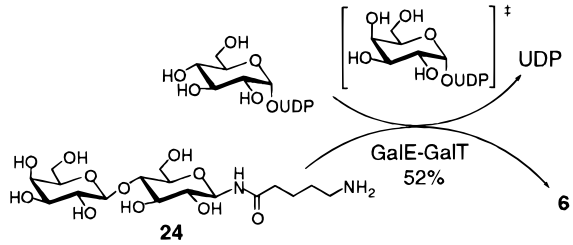


functions both as an epimerase and as a galactosyltransferase that allows the use of relatively inexpensive UDP-glucose instead of the high-cost UDP-galactose as the donor. The compound **13** was then obtained from **18** after peracetylation, reduction, and reaction with 5-chlorovaleryl chloride sequentially.

In addition, a more efficient synthesis of **6** was also achieved using this bifunctional fusion enzyme. Reaction of disaccharide derivative **24** with UDP-glucose gave compound **6** in 52% yield.

Polylactose[13] **25** with lactose/acrylamide ratio of 1:2.5 was synthesized as a negative control for bioassays. The synthetic

Scheme 3. Chemoenzymatic Synthesis of **6**



route was shown in Scheme 4. Compound **21** was obtained in 79% yield using the same transformation from **10** to **11** as illustrated in Scheme 1. Nucleophilic substitution of chloride in **21** with sodium azide gave **22** in 84% yield. The fully deprotected lactose derivative **24** was then obtained from **22** in

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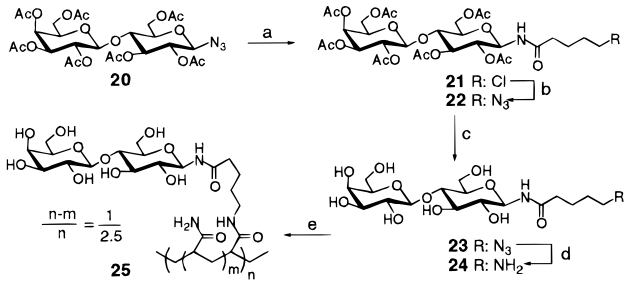
Scheme 4. Synthesis of Polylactose **25**[a]



[a] Reagents: (a) PtO_2, H_2, MeOH, then 5-chlorovaleryl chloride, Et_3N, 79%; (b) NaN_3, DMF, 70 °C, 84%; (c) NaOMe, MeOH; (d) PtO_2/H_2, H_2O-MeOH, 91% (two steps); (e) pNAS, DMF, rt, 24 h; 65 °C, 6 h; rt, 24 h, then $NH_3 \cdot H_2O$, 24 h.

Table 1. Inhibition of α-Gal Polymers and Monomer against the Binding of Purified Human Anti-Gal Antibody (Male, Blood Type: AB) to Mouse Laminin

		IC_{50} [μM][b]		
compound	ratio[a]	IgG	IgA	IgM
monomer **26**		69 ± 5	55 ± 16	277 ± 24
polymer **7A**	1/1.8	0.37 ± 0.08	0.016 ± 0.006	0.0068 ± 0.0016
polymer **7B**	1/2.5	0.28 ± 0.05	0.0053 ± 0.0023	0.0063 ± 0.0015
polymer **7C**	1/3.3	2.8 ± 0.9	0.0070 ± 0.0032	0.0056 ± 0.0014
polymer **7D**	1/6.4	7.2 ± 2.7	0.043 ± 0.017	0.027 ± 0.018
polymer **7E**	1/15	60 ± 5	0.86 ± 0.28	165 ± 13
polymer **7F**	1/29	93 ± 16		
polylactose **25**	1/2.5	N/I	N/I	N/I

[a] Ratio of the unit of α-Gal epitope (or lactose) to that of acrylamide in polymer. [b] The concentration of inhibitor at 50% inhibition of the binding between purified anti-Gal antibody (16 μg/mL) and mouse laminin in inhibition ELISA. Errors are reported as 2σ. N/I: no inhibition.

91% yield after deacetylation and hydrogenation. Polylactose **25** with lactose/acrylamide ratio of 1:2.5 was prepared by reaction of pNAS (**5**) and intermediate **24** using the same procedure as in the preparation of polymer **7B**.

Evaluation of α-Gal Epitope Polymers. The evaluation of the binding affinities of synthesized α-Gal-containing polymers **7** to anti-Gal antibodies was accomplished by inhibition ELISA[14] (enzyme-linked immunosorbent assay) with purified human (male, blood type AB) anti-Gal antibody as the primary antibody and mouse laminin as a natural source of α-Gal. The concentrations of α-Gal polymers at 50% inhibition (IC_{50}) of anti-Gal antibody binding to α-Gal epitopes on mouse laminin were measured and the IC_{50} data were summarized in Table 1. All of the IC_{50} data presented in the text are the net α-Gal trisaccharide concentrations (micromolar) calculated from the degree of functionalization and polymerization of each polymer, to compare with the corresponding α-Gal monomer explicitly. Results indicated that polymers **7A**−**7D** inhibit the antibody/antigen binding better than the α-Gal monomer, Galα1−3Galβ1−4Glcβ−NHAc (**26**), in all cases. The efficacy can be illustrated by the IC_{50} result for polymer **7C** of 5.6 nM, which is 5.0×10^4-fold better than its monomeric analogue **26** in inhibiting anti-Gal IgM. Polymer **7B** has an IC_{50} of 5.3 nM with 10^4-fold enhancement over the α-Gal monomer **26** in inhibiting anti-Gal IgA. The enhancement proved to be greater for anti-Gal IgM and IgA than for anti-Gal IgG. For example, the activity enhancement of polymer **7B** is 246-, 1.0×10^4-, and 4.4×10^4-fold toward anti-Gal IgG, IgA, and IgM, respectively. This observation is consistent with the increasing numbers of binding sites from IgG to IgA to IgM. In human serum, IgM exists as a pentamer with 10 equivalent binding sites in one molecule; 80% of IgA exists as a monomer with two binding sites and 20% as higher oligomers with a higher number of binding sites; IgG exists as a monomer with two binding sites. Therefore, the "multivalent" effect is certainly more pronounced for antibodies (IgM, IgA) with more protein−carbohydrate interaction sites.

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Table 2. Inhibition of α-Gal Polymers and Monomer against the Binding of Human Serum (male, Blood Type: AB) to Mouse Laminin

		IC_{50} [μM][b]		
compound	ratio[a]	IgG	IgA	IgM
monomer **26**		74 ± 11	306 ± 22	268 ± 31
polymer **7A**	1:1.8	12± 3	1.14± 0.26	0.070 ± 0.001
polymer **7B**	1:2.5	9.4± 0.9	0.12 ± 0.04	0.035± 0.002
polymer **7C**	1:3.3	18 ± 5	0.091± 0.003	0.031± 0.009
polymer **7D**	1:6.4	52± 8	3.3± 0.7	0.38± 0.02
polymer **7E**	1:15	>500	>1000	89± 27
polylactose **25**	1:2.5	N/I	N/I	N/I

[a] Ratio of the unit of α-Gal epitope (or lactose) to that of acrylamide in polymer. [b] The concentration of inhibitor at 50% inhibition of the binding between human serum (10 μL) and mouse laminin in inhibition ELISA. Errors are reported as 2σ. N/I: no inhibition.

Table 3. Inhibition of α-Gal Polymers and Monomer against the Binding of Human Serum (Male, Blood Type: AB) to Pig Kidney (PK15) Cells

		IC_{50}[μM][b]		
compound	ratio[a]	IgG	IgA	IgM
monomer **26**		~1000	>1000	>1000
polymer **7A**	1:1.8	595		5.2
polymer **7B**	1:2.5	407	63	2.2
polymer **7C**	1:3.3	774		>1000
polylactose **25**	1:2.5	N/I	N/I	N/I

[a] Ratio of the unit of α-Gal epitope (or lactose) to that of acrylamide in polymer. [b] The concentration of inhibitor at 50% inhibition of the binding between human sera and pig kidney cells in flow cytometry assay. N/I: no inhibition.

The effectiveness of α-Gal polymers varied not only with antibody isotype but also with the density of α-Gal epitopes conjugated to the polymer. There is a steady increase in inhibition efficacy for IgG and IgA from polymer **E** to **B** with increased α-Gal densities in the polymers (Table 1). The same trend can be observed for IgM from polymer **E** to **C**. However, polymer **7A**, with a higher density of α-Gal residues, exhibited a lower inhibition than polymer **7B** for IgA binding and the same level as **7B** for IgG and IgM. This activity drop can be rationalized by the thermodynamics of polyvalent interactions in a biological system recently reviewed by Whitesides, et al.[9c,10b] Negative cooperative interactions and the enthalpically diminished binding can be caused by the unfavorable spatial conformation of polymers at high ligand concentrations. The enhancements of inhibition by introducing more α-Gal epitopes in **7A** were obviously overcome by the simultaneous formation of the noncooperative spatial conformation of the polymer as well as steric hindrance. Similar to the positive cooperative multivalent interactions, this negative effect is more evident for antibodies with more binding sites, such as IgA and IgM. Work is in progress to elucidate the detailed physical mechanism of this enhancement. As a control in the assays, polylactose did not show any inhibitory activities up to 1 mM.

To assess the practical application of α-Gal polymers, we tested intact human serum (male, blood type: AB) instead of purified human anti-Gal antibody using the inhibition ELISA

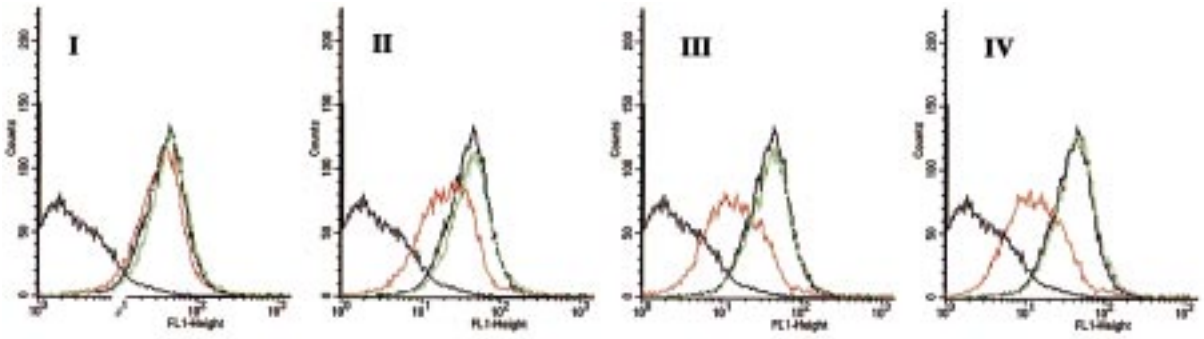

Figure 2. Flow cytometry demonstration of enhanced inhibition of α-Gal polymer B versus monomer against the binding of anti-Gal IgM in human serum to pig kidney (PK15) cells: horizontal axis, fluorescence intensity; vertical axis, cell counts. Peaks: no staining (PK15 cells alone), left black; maximum staining (PK15 cells + human serum + secondary Ab), right black; staining in the presence of polymer **B**, red; staining in the presence of monomer, green. Concentrations of net trisaccharide for both polymer and monomer: 0.1 μM (**I**), 1 μM (**II**), 10 μM (**III**), and 100 μM (**IV**).

(Table 2). The results also indicated the activity enhancement of the α-Gal polymers as compared to the monomer **26**. The same trends were also observed in the interaction of α-Gal polymers with different isotypes of the antibody and with the varied densities of the α-Gal epitope conjugated to the polymer. Interestingly, the IC_{50}s observed with the purified antibodies were consistently lower than the IC_{50}s with human sera. One explanation is that the purified antibodies were obtained from affinity column immobilized with α-Gal trisaccharide similar in structure to the epitope on the polymer. Therefore, subsets of antibodies selected during the purification would bind most tightly to the polymer. Since the purified antibodies from our affinity column contained 89% of IgM, 50% of IgA, and 42% of IgG of corresponding anti-Gal in human sera,[4b,6a] the increased proportion of IgM in the purified anti-Gal would contribute to the greater inhibitory effect of the α-Gal polymers.

To more realistically mimic cell transplantation situations, we carried out a flow cytometry assay[15] in which the inhibition of synthetic polymers against the binding of human natural anti-Gal antibodies in intact serum to α-Gal epitopes on pig kidney cells (PK15, ATCC) was measured (Table 3). As expected, results from flow cytometry analysis demonstrated that α-Gal polymers exhibited enhanced inhibition activities in comparison to the monomer **6**. With IC_{50}s of approximately 5.2 and 2.2 μM, respectively, high levels of inhibition were achieved with polymers **7A** and **7B** against anti-Gal IgM binding to PK15 cells. In sharp contrast, the α-Gal monomer **26** has no significant inhibitory effect up to 1 mM, whereas polylactose did not exhibit any inhibition up to 1 mM. This inhibition enhancement in anti-Gal IgM binding is clearly illustrated by the downshift of the fluorescence intensity peak of polymer **B** (red curve) relative to that of the α-Gal monomer (green curve) shown in cytometry histograms (Figure 2, where low fluorescence represents higher inhibition of the anti-Gal binding). In all concentrations tested, ranging from 0.10 μM to 100 μM, no inhibition was observed with monovalent α-Gal trisaccharide. One can see that the enhancement of inhibitory activity of the polymer is concentration-dependent. At a net trisaccharide concentration of 0.1 μM, the enhancement of the polymer over the monomer was minimal, while at concentration of 100 μM, the inhibition of anti-Gal binding to PK15 cells by the polymer jumped to 68%.

Our results also demonstrated that IC_{50}s of the polymers against antibody binding to cells were much higher (orders of magnitude) than the values for binding to laminin. This may be ascribed to the lower density of epitopes on the cell surface.[16,4,17] Moreover, the concentration of human sera used in the cell assay was 2.5 times more than that in laminin assay, which would bind more α-Gal epitopes on the polymer. Other factors such as non-α-Gal antigen−antibody binding[18] may contribute to the difference at the same time.

Conclusion

In summary, the synthetic α-Gal-conjugated polymers significantly enhanced activities in the inhibition of human anti-Gal antibody binding to mouse laminin glycoproteins and mammalian PK15 cells. Such enhancement is greater for anti-Gal IgA and IgM than for IgG. The amplified binding differences among the three anti-Gal isotypes can be utilized to selectively inhibit and remove particular isotype antibodies. Moreover, it was demonstrated through flow cytometry analysis that certain α-Gal polymers are effective in inhibiting anti-Gal antibodies in human serum binding to pig kidney cells. Thus, such synthetic carbohydrate polymers not only can serve as tools in studying α-Gal/anti-Gal interactions but also may find practical applications in cell xenotransplantations.

Experimental Section

General. ^1H and ^{13}C spectra were recorded on 400 MHz Varian VXR400 NMR and 500 MHz Varian Unity spectrometers. Mass spectra were run at the mass spectrometry facility at the University of California, Riverside. Baker silica gel (40 μm) was used for column chromatography and E. Merck precoated TLC plates for thin-layer chromatography. Size-exclusion chromatography was performed on Biogel P2 resin using distilled water as the eluent. Dialysis was performed using Spectra/Por Molecularporous membrane (16 mm cylinder diameter, molecular weight cutoff 14 kD) against deionized water.

Poly[*N*-(acryloyloxy)succinimide] (5). A mixture of *N*-(acryloyloxy)succinimide (**4**) (6.86 g, 40.5 mmol) and AIBN (40 mg, 0.006 equiv) in benzene (300 mL) was heated at 60 °C for 36 h. After the solution was cooled to room temperature, a white precipitate was formed. This precipitate was filtered and washed with dry tetrahydrofuran (3 × 100 mL). Drying in vacuo afforded poly[*N*-(acryloyloxy)-succinimide] (**5**) (6.21 g, 91%; Lit.[10a,b] 98%) as a white fluffy solid. The polymer was taken up in dry THF (500 mL), vigorously stirred

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for 3 days, filtered, and dried in vacuo. ^{1}H NMR (400 MHz, DMSO) δ 2.09 (br., CH_2), 2.80 (br., CH_2CH_2), 3.13 (br., CH).

Determination of Molecular Weight of Poly[*N*-(acryloyloxy)-succinimide] (pNAS). To a solution of **5** (50 mg) in DMF (2 mL) was added 1 N NaOH (10 mL). The mixture was heated at 65 °C for 24 h. After cooling to room temperature, the solution was exhaustively dialyzed against distilled H_2O. The solution of poly(acrylic acid) sodium salt thus obtained was analyzed by HPLC (Waters Ultrahydrogel Linear) using the following standards: poly(acrylic acid) sodium salt of MW 20, 60, 140, and 225 kD. Found: M_w = 252 kD, M_N = 172 kD, M_w/M_N = 1.5.

***O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl azide (10).** To a solution of *O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl azide (**9**) (0.27 g, 0.43 mmol) and phenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-*β*-D-galactopyranoside (**8**) (0.41 g, 0.65 mmol) in dichloromethane (15 mL) was added 4 Å molecular sieves (0.8 g). The mixture was stirred for 1 h at room temperature and then cooled to −30 °C. *N*-Iodosuccinimide (NIS, 95%; 0.25 g, 0.65 mmol) and trifluoromethanesulfonic acid (TfOH; 0.02 mL) were added. The reaction mixture was stirred for 1 h at −30 °C. Triethylamine was added to adjust the pH to 8.0. The mixture was filtered through a pad of Celite and washed with chloroform. The combined solution was successively washed with aqueous sodium thiosulfate and water. After concentration, the residue was chromatographed on a silica gel column (hexane/ethyl acetate: 2:1, 3:2, sequentially) to give the product **10** (0.42 g, 86%, R_f = 0.50, 1:1 hexane/ethyl acetate) as a white foamy solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.82 (s, 3H), 1.93 (s, 3H), 2.01 (s, 3H), 2.07 (s, 3H), 2.08 (s, 3H), 2.09 (s, 3H), 3.49 (d, J = 6.4 Hz, 2H), 3.64−3.82 (m, 6H), 3.97−4.12 (m, 3H), 4.31 (d, J = 7.8 Hz, 1H), 4.37−4.51 (m, 4H), 4.60−4.73 (m, 4H), 4.80−4.93 (m, 3H), 5.04−5.12 (m, 2H), 5.19 (m, 2H), 5.43 (d, J = 3.0 Hz, 1H), 7.22−7.34 (m, 20H). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 170.4, 170.2, 170.1, 169.5, 169.4, 168.8, 138.6, 138.5 (m), 138.0, 128.3 (m), 128.2 (m), 128.1 (m), 127.9 (m), 127.7 (m), 127.6 (m), 127.5, 127.4, 101.1, 95.0, 87.7, 78.4, 75.7, 75.3, 75.2, 74.9, 74.8, 73.6, 73.3, 73.2, 72.9, 72.6, 71.1, 71.0, 70.5, 69.8, 68.5, 64.8, 61.9, 61.3, 20.7 (m), 20.6 (m), 20.5, 20.4. MS (*m*/*e*) 1164 (M + Na^+). HRMS calcd for $C_{58}H_{67}N_3O_{21}Na^+$ 1164.4165, found 1164.4117.

***N*-[*O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-chloropentamide (11).** A solution of *O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl azide (**10**) (9.08 g, 7.96 mmol) in methanol (200 mL) was hydrogenated for 2 h at room temperature and 4 atm pressure in the presence of platinum(IV) oxide hydrate (380 mg). The solid was filtered off, and the filtrate was concentrated under reduced pressure to give a white solid. Then anhydrous dichloromethane (300 mL) was added to dissolve the solid and the mixture was cooled to −78 °C. Triethylamine (3.32 mL, 23.88 mmol) and 5-chlorovaleryl chloride (1.27 mL, 11.94 mmol) were added sequentially to the reaction mixture. The mixture was then warmed to room temperature and stirred for 3 h. After concentration, the residue was dissolved in ethyl acetate (300 mL) and then washed successively with HCl (0.5 N aqueous, 50 mL), water (100 mL), and saturated aqueous sodium bicarbonate solution. After solvent removal, the residue was chromatographed on a silica gel column (hexane/ethyl acetate: 1:1, 2:3, sequentially) to afford product **11** (8.54 g, 87%, R_f = 0.45, 1:1 hexane/ethyl acetate) as a white foamy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.78 (m, 4H), 1.85 (s, 3H), 1.96 (s, 3H), 2.05 (s, 3H), 2.08 (s, 3H), 2.10 (s, 3H), 2.11 (s, 3H), 2.22 (m, 2H), 3.53 (m, 5H), 3.70 (m, 1H), 3.76 (m, 1H), 3.81 (m, 4H), 4.00−4.17 (m, 4 H), 4.33 (m, 1H), 4.42 (m, 2H), 4.53 (m, 2H), 4.66 (m, 1H), 4.73 (m, 2H), 4.84 (m, 2H), 4.94 (m, 1H), 5.10 (m, 2H), 5.29 (m, 2H), 5.46 (d, J = 3.2 Hz, 1H), 6.29 (d, J = 9.2 Hz, 1H), 7.26−7.40 (m, 20H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 173.7, 172.5, 171.6, 171.5, 171.4, 170.5, 169.9, 139.7 (m), 139.1, 129.5 (m), 129.4 (m), 129.3 (m), 129.2 (m), 128.8 (m), 128.7 (m), 128.6 (m), 128.5 (m), 101.9, 96.1, 79.5, 79.1, 76.7, 76.5, 76.3, 75.9, 75.7, 74.8, 74.4, 74.3, 74.0, 73.4, 72.2, 72.1, 71.5, 70.9, 69.5, 65.9, 63.2, 62.5, 45.5, 36.7, 32.9, 23.5, 21.9, 21.8 (m), 21.5. HRMS calcd for $C_{63}H_{76}NO_{22}ClNa^+$ 1256.4445, found 1256.4476.

***N*-[*O*-(α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-chloropentamide (12).** A solution of *N*-[*O* -(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-chloropentamide (**11**) (8.39 g, 6.80 mmol) in methanol (200 mL) was hydrogenated at room temperature and 4 atm pressure in the presence of palladium on activated carbon (10%) (2 g) overnight. The reaction mixture was diluted with methanol (100 mL), filtered through a pad of Celite (caution: Pd/C can easily cause fire), washed with methanol (200 mL), and concentrated to give debenzylated product **12** (5.96 g, 100%, R_f = 0.25, 8:1 chloroform/methanol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.75 (m, 4H), 2.04(1) (s, 3H), 2.04(4) (s, 3H), 2.08 (s, 3H), 2.09 (s, 3H), 2.11 (s, 3H), 2.16 (s, 3H), 2.20 (m, 2H), 3.52 (t, J = 6.5 Hz, 2H), 3.55 (m, 1H), 3.64−3.92 (m, 9H), 4.09 (m, 2H), 4.21 (m, 1H), 4.41 (m, 2H), 4.82 (dd, $J_1 = J_2$ = 9.0 Hz, 1H), 4.98 (m, 2H), 5.20 (dd, $J_1 = J_2$ = 9.0 Hz, 1H), 5.27 (m, 1H), 5.45 (m, 1H), 6.39 (d, J = 9.0 Hz, 1H). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 173.4, 172.1, 171.9, 171.4, 171.3, 170.7, 170.4, 101.4, 97.9, 78.6, 76.5, 75.2 (m), 73.1, 71.6 (m), 71.3, 70.7, 70.5, 69.3, 66.5, 62.8, 62.6, 62.1, 45.1, 36.2, 32.4, 23.1. HRMS calcd for $C_{35}H_{52}NO_{22}ClNa^+$ 896.2567, found 896.2530.

***N*-[*O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-chloropentamide (13) (from 12).** *N*-[*O* -(α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-chloropentamide (**12**) (6.04 g, 6.91 mmol) was dissolved in a mixture of pyridine (100 mL) and acetic anhydride (70 mL). 4-(Dimethylamino)pyridine (100 mg) was added, and the solution was stirred at room temperature for 3 h. After removal of pyridine and acetic anhydride, the residue was dissolved in ethyl acetate (300 mL) and the solution was successively washed with HCl (0.5 N aqueous, 50 mL), water (100 mL), and saturated aqueous sodium bicarbonate solution. After concentration, the residue was chromatographed on a silica gel column (hexane/ethyl acetate: 1:1, 1:2, sequentially) to give the product **13** (6.39 g, 89%, R_f = 0.5, 1:3 hexane/ethyl acetate) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.73 (m, 4H), 1.91 (s, 3H), 2.01 (m, 6H), 2.02 (s, 3H), 2.03 (s, 3H), 2.04 (s, 3H), 2.06 (s, 3H), 2.10 (s, 6H), 2.12 (s, 3H), 2.18 (m, 2H), 3.49 (t, J = 6.0 Hz, 2H), 3.77 (m, 4H), 4.14 (m, 6H), 4.35 (m, 2H), 4.78 (dd, $J_1 = J_2$ = 9.9 Hz, 1H), 5.03−5.29 (m, 7H), 5.41 (d, J = 3.0 Hz, 1H), 6.22 (d, J = 9.0 Hz, 1H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 172.5, 171.2, 170.3, 170.2 (m), 170.1 (m), 169.8, 169.7, 169.2, 168.6, 100.7, 93.5, 77.9, 75.6, 74.5, 72.9, 72.2, 70.9, 70.8, 69.6, 67.6, 67.1, 66.8, 66.5, 64.6, 61.9, 61.1, 61.0, 44.3, 35.5, 31.7, 22.4, 20.6−20.4 (m). HRMS calcd for $C_{43}H_{60}NO_{26}ClNa^+$ 1064.2990, found 1064.3017.

***N*-[*O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-azidopentamide (14).** A mixture of *N*-[*O* -(2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-chloropentamide (**13**) (6.31 g, 6.06 mmol) and sodium azide (0.79 g, 12.12 mmol) in DMF (20 mL) was heated at 70 °C for 12 h. After removal of DMF, the residue was dissolved in ethyl acetate (300 mL) and the solution was filtered through a pad of Celite and washed with ethyl acetate (100 mL). The combined filtrate was concentrated, and the residue was chromatographed on a silica gel column (hexane/ethyl acetate: 1:1, 1:2, sequentially) to give the product **14** (5.71 g, 90%, R_f = 0.45, 1:3 hexane/ethyl acetate) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.58 (m, 2H), 1.65 (m, 2H), 1.94 (s, 3H), 2.04(2) (s, 3H), 2.04(5) (s, 3H), 2.05(3) (s, 3H), 2.05(8) (s, 3H), 2.07 (s, 3H), 2.09 (s, 3H), 2.13 (s, br., 6H), 2.16 (s, 3H), 3.28 (t, J = 6.4 Hz, 2H), 3.80 (m, 4H), 4.16 (m, 6H), 4.38 (m, 2H), 4.81 (dd, $J_1 = J_2$ = 9.6 Hz, 1H), 5.06−5.44 (m, 6H), 5.44 (d, J = 3.0 Hz, 1H), 6.24 (d, J = 9.6 Hz, 1H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 173.6, 172.4, 171.5, 171.4 (m), 171.3 (m), 170.9, 170.8, 170.3, 169.7, 101.9, 94.5, 79.0, 76.8, 75.6, 74.0, 73.3, 72.0, 71.9, 70.7, 68.7, 68.2, 67.9, 67.6, 65.7, 63.0, 62.3, 62.1, 52.1, 36.8, 29.3, 23.3, 21.9, 21.8 (m), 21.7 (m), 21.6 (m), 21.5. HRMS calcd for $C_{43}H_{60}N_4O_{26}ClNa^+$ 1071.3393, found 1071.3362.

***N*-[*O*-α-D-Galactopyranosyl)-(1→3)-*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-glucopyranosyl]-5-azidopentamide (15).** To a solution of *N*-[*O* -(2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-azidopentamide (**14**) (1.01 g, 0.96 mmol) in absolute methanol (500 mL) was added sodium methoxide to adjust the pH value of the solution to 9. The mixture was stirred for 5 h and then neutralized with Dowex 50WX2−100 (H^+) resin. The resin was filtered and washed with methanol. The combined methanol solution was concentrated to give product **15** (0.57 g, 93%, R_f = 0.75, 8:3:3 2-propanol/water/ethyl acetate) as a white solid. ^{1}H NMR (300 MHz, D_2O) δ 1.48 (m, 2H), 1.56 (m, 2H), 2.21 (t, J = 6.5 Hz, 2H), 3.20 (t, J = 6.5 Hz, 2H), 3.30 (m, 1H), 3.51−3.73 (m, 12H), 3.80 (m, 2H), 3.87 (m, 2H), 4.37 (d, J = 8.0 Hz, 1H), 4.83 (d, J = 9.5 Hz, 1H), 4.99 (d, J = 4.0 Hz, 1H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 178.4, 103.5, 96.1, 79.8, 78.8, 77.8, 77.0, 75.8, 75.7, 72.1, 71.5, 70.2, 69.9, 69.8, 68.8, 65.5, 61.7, 61.6, 60.7, 51.4, 35.8, 28.1, 22.9. HRMS calcd for $C_{23}H_{40}N_4O_{16}H^+$ 629.2517, found 629.2504.

***N*-[*O*-α-D-Galactopyranosyl)-(1→3)-*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-glucopyranosyl]-5-aminopentamide (6) (from 15).** A solution of *N*-[*O*-α-D-Galactopyranosyl)-(1→3)-*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-glucopyranosyl]-5-azidopentamide (**15**) (0.54 g, 0.86 mmol) in methanol (35 mL) was hydrogenated at room temperature and 4 atm pressure in the presence of platinum dioxide (60 mg). The reaction mixture was diluted with methanol (50 mL) and filtered through a pad of Celite. The Celite pad was washed with methanol (50 mL). The combined methanol solution was concentrated to gave **6** [0.52 g, 100%, R_f = 0.15, 7:3:2 2-propanol/NH_4OH (27% aqueous solution)/water] as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 1.43 (m, 4H), 2.17 (t, J = 7.5 Hz, 2H), 2.62 (m, 2H), 3.27 (m, 2H), 3.46−3.68 (m, 12H), 3.75 (m, 1H), 4.00 (m, 2H), 4.33 (d, J = 8.0 Hz, 1H), 4.79 (d, J = 8.5 Hz, 1H), 4.95 (d, J = 3.5 Hz, 1H). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 179.0, 103.4, 96.0, 79.7, 78.6, 77.7, 76.9, 75.8, 75.6, 72.0, 71.4, 70.1, 69.9, 69.7, 68.8, 65.4, 61.6, 61.5, 60.5, 40.7, 36.3, 29.6, 23.2. HRMS calcd for $C_{23}H_{42}N_2O_{16}H^+$ 603.2613, found 603.2629.

Chemoenzymatic Synthesis of Trisaccharide Derivative 13. (A) *O*-α-D-Galactopyranosyl)-(1→3)-*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-Glucopyranosyl azide (18). To a mixture of 1-azido-*β*-D-lactoside (**17**) (0.20 g, 0.54 mmol), UDP-glucose (0.33 g, 0.54 mmol), bovine serum albumin (BSA) (0.1%), $MnCl_2$ (10 mM) in Tris-HCl (100 mM, pH = 7.0, 27 mL) was added fusion enzyme GalE-GalT (~5 units). The reaction mixture was shaken gently for 2 days at room temperature and then passed through chloride anion exchange column (Dowex-Cl 1 × 8−200). The elute was concentrated and then purified with gel permeation chromatography (Bio-Gel P2) to give trisaccharide **18** (0.19 g, 65%, R_f = 0.25, 7:1:2 2-propanol/NH_4OH (27% aqueous solution)/water) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 3.13 (t, J = 9.0 Hz, 1H), 3.46−3.84 (m, 15H), 4.00 (d, J = 2.5 Hz, 1H), 4.01 (t, J = 6.5 Hz, 1H), 4.34 (d, J = 8.0 Hz, 1H), 4.59 (d, J = 8.5 Hz, 1H), 4.96 (d, J = 3.5 Hz, 1H). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 102.7, 95.3, 89.8, 77.8, 77.0, 76.6, 74.9, 74.3, 72.4, 70.7, 69.5, 69.2, 69.0, 68.1, 64.7, 60.9, 60.8, 59.8. HRMS calcd for $C_{18}H_{31}N_3O_{15}Na^+$ 552.1652, found 552.1639.

(B) *O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl azide (19). Azid **19** (0.26 g, 92%, R_f = 0.70, 1:3 hexane/ethyl acetate, white solid) was prepared from *O*-α-D-Galactopyranosyl)-(1→3)-*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-Glucopyranosyl azide (**18**) (0.16 g, 0.36 mmol) using the same procedure described in the transformation from **12** to **13**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.92 (s, 3H), 2.01 (s, 3H), 2.03 (s, 6H), 2.04 (s, 6H), 2.09 (s, 3H), 2.10 (s, 6H), 2.12 (s, 3H), 3.70 (m, 1H), 3.79 (m, 3H), 3.99−4.17 (m, 6H), 4.43 (m, 2H), 4.62 (d, J = 8.5 Hz, 1H), 4.82 (t, J = 8.5 Hz, 1H), 5.06 (m, 1H), 5.14 (t, J = 11.5 Hz, 1H), 5.20 (m, 3H), 5.30 (m, 1H), 5.41 (m, 1H). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.1, 170.9 (2C), 170.8 (2C), 170.6, 170.4, 170.2 (2C), 169.4, 101.8, 94.1, 88.3, 76.2, 75.5, 73.5, 73.2, 71.6, 71.5, 70.3, 68.3, 67.8, 67.5, 67.1, 65.3, 62.4, 61.9, 61.7, 21.4 (m), 21.3 (m), 21.2 (m), 21.1. MS (FAB) $C_{38}H_{51}N_3O_{25}$ 988 (M + K^+).

(C) *N*-[*O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-chloropentamide (13) (from 19). Compound **13** (0.19 g, 81%) was prepared from azide (**19**) (0.22 g, 0.23 mmol) using the same procedure described in the preparation of **11**. Spectra data was shown in the preparation of **13** from **12**.

***N*-[*O*-α-D-Galactopyranosyl)-(1→3)-*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-glucopyranosyl]-5-aminopentamide (6) (from 24).** Compound **6** (73 mg, 52%) was prepared from **24** (103 mg, 0.23 mmol) using the same procedure described in the preparation of **18**. Spectra data was shown in the preparation of **6** from **15**.

***N*-[*O*-(2,3,4,6-tera-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-gluco pyranosyl]-5-chloropentamide (21).** Compound **21** (1.56 g, 79%, R_f = 0.45, 1:2 hexane/ethyl acetate, white foamy solid) was prepared from **20** (1.74 g, 2.73 mmol) using the same procedure described in the preparation of **11**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.72 (m, 4H), 1.93 (s, 3H), 2.01 (s, 3H), 2.02 (s, br., 6H), 2.04 (s, 3H), 2.08 (s, 3H), 2.13 (s, 3H), 2.18 (m, 2H), 3.49 (t, J = 6.4 Hz, 2H), 3.72 (m, 2H), 3.86 (t, J = 6.8 Hz, 1H), 4.05 (m, 3H), 4.41 (d, J = 11.6 Hz, 1H), 4.45 (d, J = 8.0 Hz 1H), 5.07 (dd, J_1 = 8.0 Hz, J_2 = 10.0 Hz, 1H), 5.18 (t, J = 9.5 Hz, 1H), 5.26 (t, J = 9.0 Hz, 1H), 5.32 (d, J = 3.5 Hz, 1H), 6.31 (d, J = 8.8 Hz, 1H). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 173.2, 171.9, 171.0 (2C), 170.8, 170.7, 169.9, 169.6, 101.5, 78.7, 76.6, 75.2, 73.1, 71.7 (2C), 71.4, 69.7, 67.3, 62.6, 61.6, 45.0, 36.2, 32.4, 23.1, 21.5, 21.4, 21.3, 21.2 (m), 21.1. HRMS calcd for $C_{31}H_{44}NO_{18}ClNa^+$ 776.2145, found 776.2135.

***N*-[*O*-(2,3,4,6-tera-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-*β*-D-glucopyranosyl]-5-azidopentamide (22).** Compound **22** (1.04 g, 84%, R_f = 0.40, 1:2 hexane/ethyl acetate, white solid) was prepared from **21** (1.23 g, 1.63 mmol) using the same procedure described in the preparation of **14**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.58 (m, 2H), 1.66 (m, 2H), 1.96 (s, 3H), 2.04 (s, br., 6H), 2.05 (s, 3H), 2.06 (s, 3H), 2.11 (s, 3H), 2.15 (s, 3H), 2.21 (m, 2H), 3.28 (t, J = 6.4 Hz, 2H), 3.75 (m, 2H), 3.87 (t, J = 6.8 Hz, 1H), 4.12 (m, 3H), 4.42 (d, J = 11.6 Hz, 1H), 4.46 (d, J = 8.0 Hz 1H), 4.82 (t, J = 9.2 Hz, 1H), 4.94 (dd, J_1 = 3.2 Hz, J_2= 10.4 Hz, 1H), 5.10 (dd, J_1 = 7.6 Hz, J_2= 10.4 Hz, 1H), 5.21 (t, J = 9.2 Hz, 1H), 5.29 (dd, J_1 = 8.8 Hz, J_2= 10.0 Hz, 1H), 5.34 (dd, J_1 = 3.6 Hz, J_2= 1.0 Hz, 1H), 6.31 (d, J = 8.8 Hz, 1H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 173.7, 172.4, 171.5, 171.4, 171.3, 171.2, 170.5, 170.1, 102.0, 79.0, 77.1, 75.5, 73.4, 72.1, 72.0, 71.7, 70.0, 67.7, 63.0, 61.9, 52.1, 36.8, 29.3, 23.3, 22.0, 21.9, 21.8 (2C), 21.7, 21.6. HRMS calcd for $C_{31}H_{44}N_4O_{18}Na^+$ 783.2548, found 783.2547.

***N*-[*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-glucopyranosyl]-5-azidopentamide (23).** Compound **23** (0.56 g, 91%, R_f = 0.75, 5:1:1 2-propanol/H_2O/EtOAc, white solid) was prepared from **22** (1.01 g, 1.33 mmol) using the same procedure described in the preparation of **15**. ^{1}H NMR (500 MHz, D_2O) δ 1.49 (m, 2H), 1.54 (m, 2H), 2.22 (t, J = 7.0 Hz, 2H), 3.20 (t, J = 6.0 Hz, 2H), 3.30 (m, 1H), 3.50−3.67 (m, 8H), 3.77 (m, 2H), 4.31 (d, J = 7.5 Hz, 1H), 4.84 (d, J = 9.0 Hz 1H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 178.5, 103.5, 79.7, 78.4, 77.0, 76.0, 75.8, 73.1, 72.1, 71.6, 69.2, 61.7, 60.5, 51.4, 35.8, 28.1, 22.9.

***N*-[*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-glucopyranosyl]-5-aminopentamide (24).** Compound **24** (0.51 g, 100%, R_f = 0.20, 7:3:2 2-propanol/NH_3H_2O/H_2O, white solid) was prepared from **23** (0.54 g, 1.16 mmol) using the same procedure described in the preparation of **6** from **15**. ^{1}H NMR (500 MHz, D_2O) δ 1.35 (m, 2H), 1.48 (m, 2H), 2.18 (t, J = 6.5 Hz, 2H), 2.56 (t, J = 7.0 Hz, 2H), 3.26 (m, 1H), 3.37 (dd, J_1= 8.0 Hz, J_2 = 10.0 Hz), 3.47−3.65 (m, 8H), 3.75 (m, 2H), 4.28 (d, J = 7.5 Hz, 1H), 4.80 (d, J = 9.0 Hz 1H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 179.2, 104.0, 80.3, 78.9, 77.6, 76.6, 76.3, 73.7, 72.6, 72.1, 69.7, 62.3, 61.0, 40.9, 36.5, 30.6, 23.4. HRMS calcd for $C_{17}H_{32}N_2O_{11}Na^+$ 463.1904, found 463.1913.

A Typical Procedure for Preparation of α-Gal Polymers (Polymers 7A−7F) and Polylactose (25). A solution of *N*-[*O* -α-D-Galactopyranosyl)-(1→3)-*O*-(*β*-D-galactopyranosyl)-(1→4)-1-*β*-D-gluco- pyranosyl]-5-aminopentamide (**6**) (72 mg, 119.6 μmol) in DMSO/DMF (1:1, 2 mL) containing diiospropyl ethylamine (30 μL) was added to a stirred solution of pNAS **5** (100 mg, 590 μmol) in DMF (12 mL). The mixture was stirred at room temperature for 24 h, heated at 65 °C for 6 h, and then stirred continuously at room temperature for 24 h. $NH_3 \cdot H_2O$ (concentrated aqueous, 1.5 mL) was added dropwise to the reaction mixture followed by stirring at room temperature for 24 h.

The resulting mixture was then dialyzed exhaustively against distilled H_2O to yield a polymer containing α-Gal epitopes as a white powder. The density of α-Gal in this polymer is 1:6.4 (polymer **7D**) calculated from the integration of trisaccharide signal and acrylamide signal in ^{1}H NMR spectra. Using different ratios of **6** to **5**, polymers **7A**−**7F** were obtained in the ratio of 1:1.8, 1:2.5, 1:3.3, 1:6.4, 1:15, and 1:29, respectively. Polylactose **25** (1:2.5) was prepared by reaction of **24** with pNAS **5** using the same procedure as above.

Bioassays for the Inhibition of Anti-Gal Antibody Binding to Mouse Laminin and Pig Kidney (PK15) Cells by Synthetic α-Gal Polymers. (A) Materials. Pig kidney (PK15) cell line was purchased from the American Type Culture Collection (ATCC). Bovine serum albumin (BSA), human serum (male, type AB), mouse laminin, peroxidase-conjugated goat anti-human IgG, IgM, or IgA and FITC-conjugated goat anti-human IgG, IgM, or IgA antibodies were purchased from Sigma. NHS-activated sepharose was purchased from Pharmacia Biotech. Other reagents were all from commercially available sources.

(B) Isolation of Polyclonal anti-Gal Antibody from Human Serum. Polyclonal anti-Gal antibody was isolated from human sera using an α-Gal [immobilized trisaccharide (α-Gal1−3Galβ1−4Glc-NHCO$(CH_2)_4NH_2$ **6**) on sepharose beads] affinity chromatography column.[6a,6b,19] Human serum was heated in a 56 °C water bath for 30 min to inactivate the complement and then passed through the column. After an extensive washing with phosphate buffered saline (PBS buffer, pH7.4), the bound anti-Gal antibody was eluted with a glycine-HCl buffer (pH 2.8). Monitored with UV detector (280 nm), the elute containing antibodies was immediately adjusted to pH 7.2 using 0.1 M NaOH. The resulting antibody solution was stored as frozen aliquots in PBS buffer.

(C) ELISA Inhibition Assay with Mouse Laminin. An ELISA was conducted using mouse laminin, a basement membrane glycoprotein containing 50−70 α-Gal epitopes per molecule, as the solid-phase antigen. The purified human polyclonal anti-Gal antibodies (32 ng/mL) or human sera (2.5-fold dilute) was first incubated with varying concentrations of α-Gal compounds for 1.5 h at room temperature with gentle shaking. An aliquot (50 μL) of the mixture was then added to each microtiter plate well precoated with mouse laminin (50 μL/well of 10 μg/mL in 0.1 N Na_2CO_3−$NaHCO_3$ buffer, pH = 9.5). After incubation for 1.5 h at room temperature, unbound antibodies were washed out with PBS-tween (pH = 7.4, 0.5% tween, 5 × 200 μL). A secondary antibody (1:1000 peroxidase-conjugated goat anti-human IgG or IgM or IgA; 50 μL/well) was introduced, and the incubation was allowed to proceed for 1 h at room temperature. After being washed with PBS-tween buffer (5 × 200 μL), standard substrate (3,3′,5,5′-tetramethylbenzidine: H_2O_2, 9:1; 100 μL/well) was added. The enzymatic oxidation reaction produced a blue stain in each well. The staining reaction was stopped by adding 1 N H_2SO_4 (100 μL/well). Readings of optical absorption were taken at 450 nm (BioRad Microplate Reader, model 3550-UV). PBS with the secondary antibody was used as a background control. Purified anti-Gal antibody or human sera with secondary antibody as maximum staining (0% inhibition) was used. The percent inhibition was calculated with eq 1:

$$(M - S)/(M - B) = \%\ \text{inhibition} \quad (1)$$

S was the OD_{450} reading of the sample with different concentrations of α-Gal inhibitors (mouse laminin + α-Gal polymer + purified anti-Gal Ab or human sera + 2nd Ab). *B* was the OD_{450} value of the background staining (mouse laminin + 2nd Ab), while *M* was the OD_{450} value of the maximum staining (mouse laminin + purified anti-Gal Ab or human sera + 2nd Ab). The percent inhibition versus the concentration of inhibitors was plotted for each α-Gal compound. IC_{50} was calculated from the curve obtained.

(D) Flow Cytometry Assay with Pig Kidney (PK15) Cells. Polymers **7A**, **7B,** and **7C** were analyzed with a flow cytometry assay to determine their inhibitory potential against the binding of human anti-Gal antibodies to PK15 cells. PK15 cells were detached by versene solution (PBS containing 0.68 mM of EDTA and 1.1 mM of D-glucose) and then resuspended and blocked with RPMI 1640 serum-free medium (SFM) containing 1% BSA for 30 min at 0 °C. Cells (6×10^5) were added into polystyrene tubes (Falcon, 6 mL) and incubated with a series of cocktails (100 μL) for 60 min on ice. The cocktails were made by incubating 50 μL of heat-inactivated human sera with 50 μL of varying concentrations of α-Gal monomer or polymers for 60 min at 4−7 °C. The cells were separated and washed with RPMI extensively (3 × 1 mL). FITC-conjugated goat anti-human IgG, IgM, or IgA antibody (secondary antibody, 80 μg/mL, 200 μL) was then added, and the resulting mixture was incubated for 30 min on ice. After washing with RPMI (3 × 1 mL), the cells were suspended in PBS (400 μL, pH 7.4) and subjected to fluorescence analysis performed on a FACScan apparatus (Becton-Dickinson) with 448 nm argon laser equipment. Data were processed using PC-LYSYS Software, version 1.1 (1994), and the means of fluorescence intensity were calculated. PK15 cells with the secondary antibody were used as a background control. PK15 cells with human sera and secondary antibody were used as maximum staining (0% inhibition). The percent inhibition was calculated with eq 2:

$$(M - S)/(M - B) = \%\ \text{inhibition} \quad (2)$$

S was the mean of fluorescence intensity with different concentrations of α-Gal compounds (PK15 cells + α-Gal polymer + human sera + 2nd Ab). *B* was the mean of fluorescence intensity for background staining (PK15 cells + 2nd Ab), while *M* was the mean of fluorescence intensity for maximum staining (PK15 cells + human sera + 2nd Ab). The percent inhibition versus the concentration of inhibitor was plotted for each α-Gal compound. IC_{50} was calculated from the curve obtained.

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Supporting Information Available: Spectral data for compounds **5**−**7**, **10**−**15**, **18**−**19**, and **21**−**26**. This material is available free of charge via the Internet at http://pubs.acs.org.

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