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Probing the Lactose·GM3 Carbohydrate−Carbohydrate Interaction with Glycodendrimers

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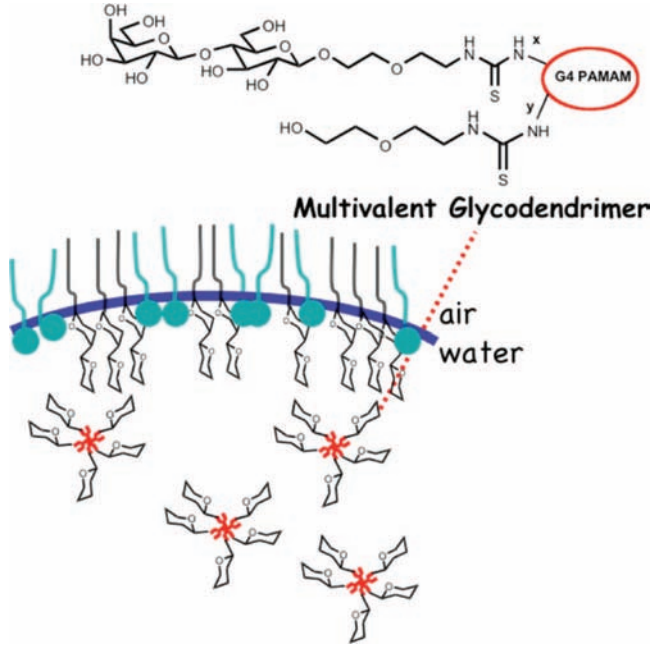
ABSTRACT



Multivalent glycoconjugates were prepared using generation-4 PAMAM dendrimers, and their interaction with Langmuir monolayers containing GM3 was investigated. Excessive carbohydrate valency adversely affects the carbohydrate−carbohydrate interaction. The GM3 monolayer selectively interacts with lactose-functionalized dendrimers in the presence of calcium ions.

Carbohydrate−carbohydrate interactions (CCIs) between cell surface glycans mediate cellular recognition and adhesion.[1] For example, the adhesion of mouse B16 melanoma cells to the endothelial cells is mediated by a CCI between the melanoma cell glycolipid GM3 (**1**) and the glycolipid lactosylceramide (LacCer, **2**) on the endothelial cell.[2] The metastatic potential and LacCer adhesion of several B16 cell line variants is correlated with levels of GM3 present on the cell surface.[3] Binding of carbohydrates to GM3 initiates signal transduction pathways within B16 melanoma cells,

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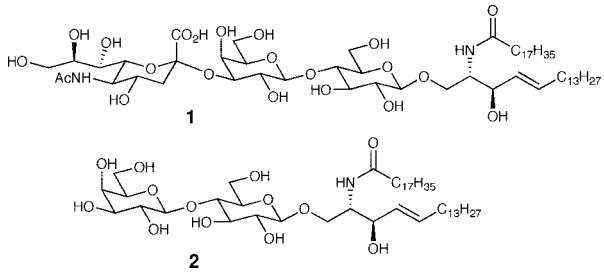
suggesting that this CCI may have functions beyond simply mediating preliminary cell adhesion.[4]



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Biophysical studies of the lactose (Lac)•GM3 CCI have been carried out using model systems to probe the interaction between these two oligosaccharides. We and others have reported on the use of Langmuir monolayers to study the interaction between GM3-containing monolayers and multivalent glycoconjugates.[5,6] These interactions are manifested as changes in surface pressure when the glycoconjugates are injected into the aqueous subphase beneath the monolayer. We have shown that monolayers containing GM3 selectively interact with glycolipid micelles containing lactose but do not interact with structurally similar disaccharides.[6d] A limitation of using micelles to examine interactions with monolayers is that most glycolipids are also surface-active, and therefore the effects of non-specific insertion into the monolayer need to be taken into account. The CCI of GM3 has also been examined with surface plasmon resonance (SPR) experiments.[7]

To circumvent non-specific insertion into the monolayer, we explored the use of glycodendrimers, which have been extensively employed in studies of carbohydrate−protein interactions.[8] We report here that glycodendrimers can also be used to examine the CCI of lactose with GM3 monolayers. We have used polyamidoamine (PAMAM) dendrimers, a well-studied multivalent platform that is commercially available. Although it is possible to vary carbohydrate multivalency by increasing or decreasing the dendrimer generation number, we elected to vary carbohydrate density for a single generation of PAMAM generation-4 (P-G4) (vide infra).

We describe here the synthesis of P-G4 glycodendrimers with varying carbohydrate valency and the results of their interaction with mixed monolayers of GM3 and dipalmitoyl phosphatidylcholine (DPPC). We have also investigated the influence of subphase and monolayer composition on the CCI.

Table 1. Activity of G4 PAMAM Glycodendrimers

entry	P-G4-Lac_xCap_y	% lac	$\Delta\pi^a$ (mN/m)	[lactose]^b (μM)
1	P-G4-Lac_0Gly_{56}	0	0.9 ± 0.4	0.0
2	P-G4-Lac_7Gly_{48}	13	1.2 ± 0.8	7.3
3	P-G4-$Lac_{17}Gly_{27}$	31	5.3 ± 0.7	18.5
4	P-G4-$Lac_{25}Gly_{30}$	45	8.1 ± 2.0	26
5	P-G4-$Lac_{35}Gly_9$	64	6.2 ± 1.0	38
6	P-G4-$Lac_{42}Gly_2$	75	3.2 ± 0.3	44
7	P-G4-$Lac_{25}Cell_{22}Gly_5$	45	1.1 ± 1.7	26
8	P-G4-$Lac_{25}Malt_{21}Gly_2$	45	1.7 ± 1.0	26
9	P-G4-$Cell_{26}Gly_{21}$	48^c	2.5 ± 0.2	29^c
10	P-G4-$Malt_{23}Gly_{30}$	42^d	3.2 ± 0.9	25^d

^{a} Binding to 1:1 GM3-DPPC monolayer in 1 mM $CaCl_2$ was measured. See Supporting Information for details. ^{b} All dendrimer concentrations in the subphase were kept at 1.0 μM. ^{c} Refers to [cellobiose]. ^{d} Refers to [maltose].

We have followed well-established protocols to functionalize amine-terminated PAMAM dendrimers using glycosyl isothiocyanates.[9] Carbohydrates that are attached to a diethyleneglycol isothiocyanate linker have been extensively used in glycodendrimer synthesis.[9c,10] The use of an isothiocyanate linker has the advantage that no additional coupling reagents are required for the reaction, facilitating purification. Glycodendrimers with different amounts of lactose (expressed as % lac in Table 1) were prepared by reaction of P-G4 with the appropriate amount of lactosyl isothiocyanate **3** (Scheme 1). The degree of lactose functionalization was determined using ^{1}H NMR and MALDI-TOF MS. The remaining amines on the dendrimer were capped with the glycol isothiocyanate **4** or glycosyl isothiocyanates **5** and **6**. The carbohydrate ligands are functionalized randomly on the dendrimer scaffold, giving rise to a homogeneous distribution of carbohydrate epitopes on the dendrimer surface.[10a] Acetyl groups on the sugars were removed using sodium methoxide, and the final products were purified by dialysis with water followed by lyophilization. The molecular weights of the final glycoconjugates were determined by MALDI-TOF MS. Glycodendrimers functionalized with the disaccharides cellobiose or maltose in place of lactose were prepared as controls.[11]

Langmuir monolayer binding studies were carried out by injecting glycodendrimers into a 1 mM $CaCl_2$ subphase underneath a monolayer of GM3 and DPPC (1:1) that was

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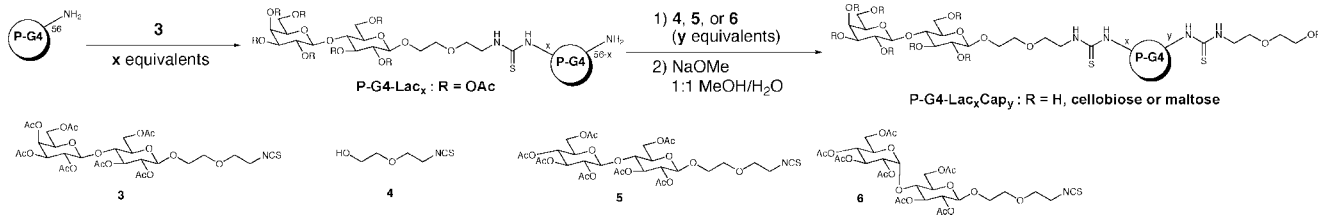
(11) A complete list of all glycodendrimers prepared, along with characterization information, is found in Supporting Information.

Scheme 1. Synthesis of G4 PAMAM Glycodendrimers



held at a pressure of 30 mN/m. Upon injection, the subsequent change in surface pressure ($\Delta\pi$) was monitored using a Wilhelmy probe. In all cases, the dendrimer concentration was held constant at 1 μM, resulting in different subphase carbohydrate concentrations due to variations in the extent of sugar functionalization on the dendrimer. None of the dendrimers used in these studies had intrinsic surface activities higher than 1 mN/m at 1 μM concentrations.

In preliminary studies we found that leaving the unreacted terminal amines uncapped resulted in large $\Delta\pi$ values at low carbohydrate loadings.[12] However, unfunctionalized P-G4 gave rise to an even higher $\Delta\pi$ value, indicating that binding was mediated by electrostatic interactions between the positively charged dendrimers and the anionic monolayer. This non-specific interaction could be significantly reduced by capping any free amines with the glycol **4** (Table 1, entry 1).

The first set of experiments examined the role of valency in the Lac•GM3 CCI. Our preliminary studies with noncapped dendrimers had also indicated that no appreciable changes in surface pressure were observed for generation-1, -2, or -3 PAMAM dendrimers, so all of our subsequent experiments were carried out with generation-4 dendrimers. As the degree of carbohydrate functionalization of P-G4 increased, increasing $\Delta\pi$ values were observed for the interaction of the dendrimers with the GM3 monolayer (Table 1, entries 1−4). Glycodendrimer P-G4-Lac$_{25}$Gly$_{30}$, which has approximately half of its terminal amines functionalized with lactose residues, had the strongest interaction with the monolayer.[13] However, the $\Delta\pi$ values started to decrease as more than half of the surface was functionalized with lactose (Table 1, entries 5 and 6). Reduced activities at high carbohydrate valencies have previously been reported in studies of carbohydrate−protein interactions and are typically attributed to overcrowding of the recognition interface.[9c,14]

To further probe the role of steric effects on the CCI, we examined the effect of using the diastereomeric disaccharides cellobiose or maltose as the capping agents instead of diethylene glycol. The glycodendrimers P-G4-Lac$_{25}$Cell$_{22}$Gly$_5$ and P-G4-Lac$_{25}$Malt$_{21}$Gly$_2$ were prepared by reaction of the lactosyl dendrimer P-G4-Lac$_{25}$ with **5** and **6**, respectively. We and others[10d] have observed that it is generally difficult to functionalize more than 90% of the amines on a G4 PAMAM dendrimer with carbohydrate residues, so attempts were made to cap as many of the remaining free amines with **4**. Both of these glycodendrimers interacted very weakly with the monolayer, with $\Delta\pi$ values that were less than half of the value recorded for the glycol-capped lactodendrimer P-G4-Lac$_{25}$Gly$_{30}$ (Table 1, entries 4, 7, 8). These results indicate that the use of large capping ligands disrupts the Lac•GM3 CCI.

We previously reported that GM3-DPPC monolayers interact strongly with micelles of lactosyl lipids but interact weakly with micelles that present the stereoisomeric disaccharides cellobiose or maltose.[6d] We find that this carbohydrate selectivity is retained when using glycodendrimers in place of micelles (entries 4, 9 and 10).

Further insight into the role of calcium and electrostatic effects was provided by carrying out binding experiments using different subphase and monolayer compositions (Table 2). The magnitude of $\Delta\pi$ is sensitive to the amount of GM3 in the monolayer.[15] The $\Delta\pi$ value decreased from 8.1 to 3.8 mN/m when the amount of GM3 in the monolayer was lowered from 50% to 20%. However, P-G4-Lac$_{25}$Gly$_{30}$ continued to interact preferentially with the monolayer relative to the cellobiose analog (3.8 vs 1.4 mN/m). Both dendrimers interacted very weakly (∼1 mN/m) with a monolayer consisting of only DPPC.

However, this lactose/cellobiose selectivity was lost when the binding was carried out over a subphase of pure water. In this event, the 1:1 GM3-DPPC monolayer no longer preferentially interacted with a lactodendrimer. Instead, the monolayer exhibited an inexplicably strong interaction with the cellobiose functionalized dendrimer, while its interaction with the lactose and maltose functionalized dendrimers were of comparable magnitude.[16] When the interactions of P-G4-Lac$_{25}$Gly$_{30}$ or P-G4-Cell$_{26}$Gly$_{21}$ with monolayers containing 20% or 0% GM3 were examined, both dendrimers gave rise

(12) P-Lac$_7$NH$_{49}$, 5.71 ± 0.62; P-Lac$_{24}$NH$_{32}$, 7.81 ± 0.81; P-G4, 8.07 ± 0.31

(13) The magnitudes of the $\Delta\pi$ values obtained with the dendrimers are lower than than those observed with glycolipid micelles.[6c,d] These lower $\Delta\pi$ values arise as a consequence of the lack of intrinsic surface activity of the glycodendrimers, which do not non-specifically insert into the monolayer.

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(15) While a 50% GM3 monolayer has a higher GM3 content than would be found on the cell surface, it should be noted that the percentage of glycolipid in a lipid raft can be significantly higher: Grauby-Heywang, C.; Turlet, J.-M. *Chem. Phys. Lipids* **2006**, *139*, 68–76.

(16) The $\Delta\pi$ value for P-G4-Malt$_{23}$Gly$_{30}$ over pure water for a 50% GM3 monolayer is 8.5 mN/m.

Table 2. Activity of Glycodendrimers with and without Ca^{2+} at Various GM3 Loadings in the Monolayer

	1 mM $CaCl_2$ subphase		water subphase	
% GM3 in monolayer	P-G4-$Lac_{25}Gly_{30}$	P-G4-$Cell_{26}Gly_{21}$	P-G4-$Lac_{25}Gly_{30}$	P-G4-$Cell_{26}Gly_{21}$
0	1.1 ± 1.4	0.6 ± 0.2	4.2 ± 0.4	4.3 ± 0.6
20	3.8 ± 0.9	1.4 ± 0.9	7.6 ± 1.0	7.2 ± 0.2
50	8.1 ± 2.0	2.5 ± 0.2	9.9 ± 0.8	16.9 ± 2.5

to similar $\Delta\pi$ values. Notably, in each of these cases the magnitude of the pressure changes were larger in the absence of calcium chloride than in its presence. It is possible that both calcium and chloride ions inhibit non-specific electrostatic interactions, the former by screening the anionic GM3 in the monolayer and the latter by screening the positively charged dendrimers. The lack of carbohydrate specificity and the larger $\Delta\pi$ values observed over a water subphase suggest that the dendrimer interacts with the monolayer primarily through non-specific electrostatic interactions in these cases. Consistent with this hypothesis, binding studies to a 50% GM3 monolayer carried out over a 1 mM NaCl subphase also do not show any selectivity.[17] Thus, the results in Table 2, taken in toto, indicate that calcium is required for the CCI between lactose-functionalized dendrimers and GM3-containing monolayers.

Prior experiments employing glycolipid micelles showed that high concentrations of sodium chloride provided a stabilizing effect to Lac•GM3 CCIs.[6c] However, no measurable activity with 50% GM3 monolayers was observed with any of the glycodendrimers in subphases containing 100 mM NaCl, even in the presence of 1 mM $CaCl_2$. The latter result suggests that there is an electrostatic component to the interaction of the lactosyl dendrimer with the GM3 monolayer.

(17) $\Delta\pi$ values for binding to a 50% GM3 monolayer over a 1 mM NaCl subphase are P-G4-$Lac_{25}Gly_{30}$, $\Delta\pi = 7.4 \pm 1.4$ mN/m; P-G4-$Cell_{26}Gly_{21}$, $\Delta\pi = 9.6 \pm 1.2$; P-G4-$Malt_{21}Gly_{30}$, $\Delta\pi = 8.6 \pm 0.4$.

The calcium ion requirement for the Lac•GM3 CCI has also been observed in experiments using both liposomes and micelles.[6c,18] In contrast, while the interaction of a lactosylated polystyrene with a GM3 monolayer is not sensitive to the presence of calcium, it should be noted that these experiments were carried out using monolayers containing solely GM3.[6a,b] Calcium condenses gangliosides in monolayers by cross-linking their anionic headgroups.[19] It is possible that the influence of calcium on ganglioside conformation and aggregation influences the interaction of the monolayer with the lactose containing conjugates.

In summary, we have demonstrated that lactosyl dendrimers engage in a CCI with GM3 in a Langmuir monolayer. This CCI is dependent on both the carbohydrate density on the dendrimer as well as the density of glycolipid within the monolayer. A specific CCI is observed only in the presence of calcium ions and when at least one-fifth of the monolayer is composed of GM3. These results provide the first example of the use of glycodendrimers as model systems for studying CCI. Glycodendrimers may serve as useful agents for probing CCI in vivo and may also find application as targeted diagnostic and antimetastatic agents.[3,20,21]

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Supporting Information Available: Synthetic procedures and characterization data for isothiocyanates and glycodendrimers as well as protocols for Langmuir binding studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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