Evaluation of α-D-mannopyranoside glycolipid micelles–lectin interactions by surface plasmon resonance method

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It is established that achieving higher binding affinities in carbohydrate–protein interactions requires multivalent presentations of the sugar ligands at the receptor binding site. Several inhibition, calorimetric, mass balance, and other studies have reiterated the beneficial effects of molecular level clustering of the sugar ligands for tight binding to the receptors. We have undertaken an effort to study the multivalent effects involving larger assemblies, represented by micelles, and their lectin interactions. The micelles were constituted with monomer bearing one- or two-sugar moieties at the monomolecular level and with varying the distances between the sugar moieties. Micellar aggregation studies and dynamic light scattering (DLS) studies afforded details of the aggregation numbers and the hydrodynamic diameters of various glycolipid (GL) micelles. The GL micelles were used as analytes of surface plasmon resonance (SPR) experiments on a lectin concanavalin A (Con A)-immobilized surface. SPR studies of the micelle–lectin interactions demonstrate that the ligand–receptor binding can be fit into the bivalent analyte model of interaction. Furthermore, micelles formed from two-sugar containing GLs are able to elicit favorable kinetic association rate constants in comparison to the micelles constituted with one-sugar containing GLs. The kinetic rate constants across the micelles and the effect of the sugar valencies in the GLs are discussed.

Key words: carbohydrates/glycolipids/kinetics/lectins/micelles/surface plasmon resonance

Introduction

Molecular level clustering of the sugar ligands has been observed consistently to enhance an otherwise weak binding affinity of monomeric sugar ligands with lectins. Logarithmic enhancements in binding efficiencies by clustered sugar ligands are referred often as “glycoside cluster effect” (Lee and Lee, 1995). Although such enhancements in binding affinities are not very common, unless satisfying the exact geometric requirements and complementarities between the ligands and the receptors, a nominal enhancement in binding affinities is observed often, resulting primarily from incremental advantages because of kinetic and thermodynamic factors (Mammen et al., 1998; Dam and Brewer, 2002; Lundquist and Toone, 2002; Srinivas and Jayaraman, forthcoming). Multisite binding, emanating from intermolecular associations, leads largely to aggregation or cross-linking, and the role of clustering of the ligand resides in increasing the strength of ligand–receptor interactions in a kinetically controlled manner. On the contrary, intramolecular multisite binding, that is chelation, occurring with the aid of exact complementarities by binding site requirements of the oligomeric receptor and the clustered sugar ligand, often gains thermodynamic advantages in addition to kinetic ones (Kitov et al., 2000; Fan et al., 2000; Kitov et al., 2003; Thoma et al., 2005). In either case, binding affinity enhancements result and allow the ligand–receptor interactions that are physiologically relevant. In the absence of information of the exact structural and geometric requirements of the receptor sites for a complementary ligand design, clustering of sugar ligands is thought to exploit kinetic advantages, with an overall incremental increase in binding affinities. Intermolecular interactions, leading to cross-linking and aggregation, require there to be sufficient spatial separations between the interacting ligand (sub) units, so as to envelop appropriate intermolecular receptor sites (Kisseling et al., 1998; Pierce et al., 2002). Spatial disposition of the ligand being one of the major requirements, the role of intermittent, non-receptor-bound ligands in a sugar cluster is not understood at present. The intermittent ligands may be functionally inactive (Jayaraman et al., 2005), yet such ligands may be expected to play a role when present at the binding site environments. To identify the role of spatially and topologically restricted sugar ligands and their effects at the binding site environments, studies of the glycolipids (GLs) bearing either one- or two-sugar ligands were undertaken in this study. The GLs are prone to form micellar aggregates. The aggregate state not only elicits a clustered format of the ligands (Kingery-Wood et al., 1992; Charych et al., 1993; Spevak et al., 1993; Song et al., 1998) but also facilitates intermolecular interactions, as a result of the nanometric sizes of the micelles. In this respect, the micellar systems are considered as multivalent ligands. In the absence of knowledge on how a neighboring sugar ligand would affect the binding of a given sugar ligand, a study was aimed to assess the effect of two-sugar containing monomers in comparison to monomer bearing a single sugar ligand, given both types of monomers form micelles. Importantly, the distance between the sugars in the two-sugar-containing monomer is not sufficient to envelop two...
independent lectin-binding sites, thereby allowing the observation of effects attributable directly to the presence or absence of the neighboring second sugar ligand within the monomer. In this report, this comparison is extended to a series of GLs with α-D-mannopyranoside units, so as to obtain relative rate constants and/or binding constants with concanavalin A (Con A) lectin. We present the synthesis of the various GLs, assessment of their micellar state, and the kinetic constants of the binding processes of these GL micelles with Con A. The interaction profiles are determined with the aid of surface plasmon resonance (SPR), which is known to provide a real time binding profile (Garland, 1996; Schuck, 1997; Monsigny et al., 2003; Surolia et al., 2003).

Results and discussion

The aim of the study was to assess the interaction of GL micelles with a high-affinity lectin. The GL micelles were incorporated with two important features: (1) the GL monomers were constituted with either one- or two-sugar moieties and (2) there were variable distances between the sugar units at the monomolecular level. The distance between the sugar units was anticipated to allow control over the micelle diameter. As a case study, α-D-mannopyranosides were utilized as the sugar ligands and Con A as the lectin. Target GL monomers featuring variable length ethylene glycol spacer arms are presented in Figure 1.

Synthesis of GLs

Syntheses of the above GLs were performed by utilizing standard glycosylation methods, wherein the hydroxyl group-protected glycosyl donors and hydroxyl group-bearing aglycon acceptors were subjected to glycosylation. Removal of the protecting groups after glycosylation led to the isolation of the GLs GL-2 to GL-6. The GLs were synthesized by Koenig–Knorr glycosylation of primary hydroxyl groups of 2-O-alkylated glycerol moieties 10, 11, and 12 with required molar equivalents of the glycosyl donor 14. Thus, the glycosylation with 1 and 2 molar equivalents of mannosyl bromide 14 resulted in the formation of one- (GL 2 and GL 3) and two-sugar containing GLs (GL 4, GL 5, and GL 6), respectively (Scheme 1). The purities and structural homogeneities were ascertained by routine physical methods. The GLs and non-sugar 12 (NS1) were partially soluble in water at room temperature.

Critical micellar concentration measurements

The critical micellar concentrations (CMC) of the GLs were determined by fluorescence measurements at 25°C, using anilinonaphthalene sulfonic acid (ANSA) as the probe. In a hydrophobic environment, fluorescence emission intensity of ANSA increases dramatically, and therefore, a break in the plot of emission intensity versus concentration provides the CMC of the lipid under study (Goddard et al., 1985; Roe and Griffiths, 2000). The fluorescence emission spectra of ANSA obtained in the presence of various concentrations of GL-3 are presented in Figure 2. The break-point in the plot of the emission intensity at 490 nm versus the GL concentration (Figure 2b) afforded a CMC of GL-3 at 1.42 ± 0.3 μM. The CMCs measured in a similar manner for other GLs are summarized in Table I. CMCs for these non-ionic GLs were in the micromolar concentrations, as expected (Engberts et al., 2003). Upon estimating the CMCs of various GLs, an assessment of the aggregation number was undertaken. The aggregation numbers of the GLs were determined

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Fig. 1. Molecular structures of various glycolipids (GL-2 to GL-6), and non-sugar lipid (NS-1).
by fluorescence quenching using cetyl pyridinium chloride (CPCl), a cationic surfactant, as the quencher (Q) (Bhattacharya et al., 2003). The surfactant at very low concentration resides inside the GL micelles and acts as a Q of probe ANSA emission. A ratio of the fluorescence intensity of the probe in the presence of Q (I) to the intensity in the absence of Q (I₀) is related to CMC and the aggregation number (n) as

\[
\ln\left(\frac{I}{I_0}\right) = n \frac{[Q]}{[S] - \text{MC}}
\]

For the aggregation number determination, a concentration of GL [S] of 100 times the value of the respective CMC was chosen. Because [S] is much higher than the CMC, \(\ln\left(\frac{I}{I_0}\right) = n\frac{[Q]}{[S]}\).

The slope of a plot of \(\ln(I/I₀)\) versus [Q] hence provides n (Prieto et al., 1995). The data for the thus determined aggregation numbers are summarized in Table I.

From the CMC values and the aggregation numbers of GL-2 and GL-3, it is observed that the ethylene glycol spacer length alone does not have a considerable influence on either the CMC or the aggregation numbers. A lack of the effect of added ethylene glycol units on the CMC and aggregation numbers in gemini surfactants have been observed previously (Engberts et al., 2003). On the contrary, the additional sugar unit in the two-sugar-containing GLs moderates the CMC values and the aggregation numbers. For GL-5, we obtained

\[
\text{Table I. The critical micellar concentration (CMC), aggregation number (n), and hydrodynamic diameter (dₘ) of glycolipid micelles made from GL-2 to GL-6 at 25°C}
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<th>Glycolipid</th>
<th>Fluorescence methods</th>
<th>Dynamic light scattering</th>
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<tr>
<td></td>
<td>CMC (μM)</td>
<td>Aggregation number (n)</td>
</tr>
<tr>
<td>GL-2</td>
<td>1.68 ± 0.4</td>
<td>16</td>
</tr>
<tr>
<td>GL-3</td>
<td>1.42 ± 0.3</td>
<td>14</td>
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<tr>
<td>GL-4</td>
<td>5.0 ± 0.6</td>
<td>36</td>
</tr>
<tr>
<td>GL-5</td>
<td>3.85 ± 0.3</td>
<td>28</td>
</tr>
<tr>
<td>GL-6</td>
<td>2.02 ± 0.8</td>
<td>15</td>
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Fig. 2. Fluorescence intensities of ANSA as a function of GL-3 concentration, at 25°C.

Scheme 1. Synthesis of glycolipids. Reagents and conditions: 8: C₁₆H₃₃Br; 13: TsO(CH₂CH₂O)ₙCH₂CH₂OBn; 14: 2,3,4,6-tetra-O-benzoyl-α-D-mannopyranosyl bromide; (i) NaH, THF, 65°C, 36 h; (ii) H₂, 10% Pd-C, EtOAc-MeOH (1:1), rt, 12 h; (iii) Hg(CN)₂, HgBr₂, CH₂Cl₂, 2 Å Mol. sieves, rt, 24 h; (iv) 0.5 N NaOMe/MeOH, rt, 12 h.
CMC and aggregation number values about twice as high as for the mono-sugar counterpart GL-2. However, additional ethylene glycol units in the former series of GLs provide enhanced hydrophobicity responsible for micellization, countering the hydrophilicity of the sugar head groups. Thus, across the series GL-4 to GL-6, the CMC values and the aggregation numbers decrease periodically from GL-4 to GL-6.

**Dynamic light scattering experiments to determine the sizes of the GL micelle**

Having determined the CMCs and the associated aggregation numbers of the GL micelles, the equilibrium aggregate sizes were assessed through dynamic light scattering (DLS). The micelle diameters thus obtained are summarized in Table I. The increasing number of ethylene glycol units led to a small but measurable micelle sizes. On the contrary, a comparison of the hydrodynamic diameter of GL-2 and GL-5, GL-3, and GL-6 revealed that the additional sugar unit in these pairs led to considerable increase in the micelle diameter. The observed differences can be explained both by changes in packing arrangement of the hydrophobic chains within the micelle and by the differences in aggregation numbers. For GL-2 and GL-5, the hydrodynamic diameter increases with increasing aggregation number. For GL-3 and GL-6 on the contrary, the aggregation numbers are similar and the 3-nm increase in diameter for the two-sugar-containing GL micelle hence might be the result of less-coiled conformations of the hydrophilic lipid heads.

**Studies of the interaction of GL micelles with lectins by SPR**

An assessment of the interaction of various GLs with lectin was conducted by means of the SPR technique. This technique has been utilized systematically to study various biochemical interactions (Falt and Karlsson, 1997; Kahne et al., 1999; Nakajima et al., 2001; Myszka et al., 2002; Penades et al., 2002; Tsoi and Yang, 2004), including carbohydrate–protein interactions (Kuziemko et al., 1996; Young et al., 1996; Mackenzie et al., 1997; Matsumoto and Satoh, 1999; Thomas and Suriol, 2000; Kataoka et al., 2003; Sota et al., 2003). Alpha-D-Mannopyranoside-bearing GLs involved studies with a high-affinity lectin, namely, Con A (Goldstein et al., 1974; Becker et al., 1976). In the lectin-GL recognition studies, the lectin was immobilized on the sensor surface and the GLs served as the analytes. As a control, wheat germ agglutinin (WGA) was utilized, as α-D-mannopyranosides do not recognize this lectin.

**Lectin immobilization studies**

The lectin immobilization was accomplished on a carboxymethylated dextran-coated (CM5) sensor chip. To minimize the nonspecific interactions and mass transport limitations, controlled immobilization of the lectin on the sensor chip was required (Haseley et al., 1999; Biacore Application Handbook, 1998). For an efficient and a controlled immobilization, pre-concentration of the lectin on the sensor surface was accomplished by electrostatic interaction initially, between the negative and positive charges on the surface and the lectin, respectively. This process allowed efficient immobilization from relatively dilute lectin solutions (typically 20–100 µg/mL). An immobilization level of 6000 response units (RU) was targeted on the sensor surface. This immobilization level has been chosen because dextran is known to bind lectin Con A (Halkes et al., 2005), and such a binding should not interfere with the GL–lectin binding profile. A Con A immobilization level of 6000 RU is equivalent to about 6000 pg/mm² (1 RU = 1 pg/mm²). This RU value can be translated to an area of 14–15 nm², being occupied by each dimer molecule. The approximate area of Con A tetramer theoretically is 32 nm² (Becker et al., 1976). Thus, the molecular area of the lectin dimer upon immobilization and the theoretical value support that Con A nearly occupies an optimum surface area on the CM5 sensor chip. After the pre-concentration step, the sensor surface was washed with aq. NaOH (10 mM) solution, to remove the electrostatically bound lectin from the CM5 surface. The sensor surface was activated with N-hydroxysuccinimide (NHS) in H2O (0.1 M) and N-ethyl-N′-(dimethyl-aminoethyl) carbodiimide in H2O (0.1 M). The lectin was then injected at a concentration of 100 µg/mL in NaOAc buffer (pH 4.3) (10 mM). After coupling of the lectin, remaining NHS esters were blocked by the addition of ethanolamine hydrochloride (1.0 M). The following procedure was adopted to ensure a homogeneous Con A surface necessary for the kinetic studies. Con A was immobilized as dimer at pH 4.3. Upon immobilization, the stability of Con A surface was confirmed after three to five washes with the regeneration solution (10 mM glycine–HCl [pH 2.5]). The first wash with regeneration solution resulted in a significant decrease in response. The amount of loss in surface reduced for subsequent regeneration steps, and the surface response stabilized after four washes. After confirming the stability of Con A surface in the buffer wash and the regeneration solution wash, the kinetic experiments was performed. The lectin WGA was immobilized to the same extent as Con A on another parallel flow cell. WGA acted as a reference, so as to determine the level of nonspecific binding and sample bulk refractive contributions.

**GL micelle-Con A interaction studies**

Sensograms obtained for the interaction of the various GLs (injected at concentrations well above CMC), with Con A and WGA-immobilized surfaces are presented in Figure 3. Binding of the GL micelles with Con A resulted in a significant increase (>800) in RU for the association phase. Upon attaining an equilibrium phase, the complex was dissociated with HEPES buffer for 8 min, and then the Con A surface was regenerated by using a 1-min pulse of glycine hydrochloride (pH 2.55). The corresponding RU increase for the WGA-immobilized surface is insignificant, confirming that WGA is not recognized by α-D-mannopyranoside.

A NS lipid, NS-1, was also tested for its interaction with Con A, and again only a small RU change was noticed. The sensogram obtained for buffer injection on Con A functionalyzed surface, along with the corresponding sensograms for GL and NS injection, are presented in Figure 3a. From Figure 3, it is conspicuous that GL micelle–Con A interaction is specific, and such an interaction is absent for NS, WGA surface, and buffer alone. The following trends emerged in relation to the ethylene glycol spacer length and the one- or two-sugar constitutions of the GLs.
Effect of ethylene glycol spacer length. Sensograms given in Figure 4a and b show that the micelles constituted of ethylene glycol spacer containing GL-3, GL-5, and GL-6 reach faster equilibrium response than the micelles from GL-2 and GL-4. Flexibilities to the sugar residues in the former series, originating from the presence of the spacer, allow faster association and dissociation for the binding of the sugar ligands to the lectin. GL-4, without the spacer, showed the largest equilibrium SPR response amongst the range of GLs studied (Figure 3b).

Effect of one- and two-sugar-bearing nature of the GLs. The two-sugar-bearing GL micelles exhibited relatively faster association and dissociations (Figure 4), resulting in reduced equilibrium binding response. We further observed that these GL micelles (GL-4, GL-5, and GL-6) exhibited nearly overlapping dissociation phases (Figure 3b). These observations indicated that the presence of additional sugar ligand in these two-sugar-bearing GL micelles influenced the ligand–receptor interactions.

Concentration-dependent studies of the GL micelle–Con A interactions. The SPR studies were continued further to assess the GL interactions with lectin Con A in a concentration-dependent manner. Sensograms obtained at various concentrations of GL-2 and GL-4 are presented in Figure 5. Below the CMC, GL-4–lectin interaction elicited a plateau response of <100 RU (Figure 5b), descriptive of the

Fig. 3. Sensogram obtained for the binding of various glycolipids to Con A surface at a particular lipid concentration (250 μM): (a) binding of one-sugar-containing glycolipid micelles, non-sugar and buffer to Con A surface; (b) binding of two-sugar-containing glycolipid micelles to Con A and reference WGA surface.

Fig. 4. Sensogram obtained for the binding interactions of Con A with glycolipid micelles (glycolipid concentration: 250 μM), having (a) mono-ethylene glycol and (b) di-ethylene glycol spacers.
response level for the monomolecular GL–lectin interaction. As the concentration of the GL was increased above the CMC leading to the formation of a supramolecular ligand, a significant increase in the plateau values was attained.

**Kinetic studies of the GL micelle–Con A interactions**

Kinetic studies of GLs, GL-2 to GL-6, were performed at five different concentrations, from 16 to 250 μM, over the sensor chip containing immobilized Con A. Figure 6 shows the relative responses thus obtained for GL-2–Con A and GL-4–Con A interactions. To check mass transport limitations, analyte at one particular concentration was injected at different flow rates (5, 15, and 75 μL/min), and the observed binding rates were found to be independent of the flow rates, thus precluding mass transfer effects during the ligand–receptor interactions. The mass transfer limitations were checked for all the GLs, and mass transfer effects were found to be negligible. For all kinetic studies, a flow rate of 20 μL/min was used because at this flow rate artifacts from analyte rebinding and mass transfer effects could be ruled out.

**Analysis of the sensogram**

Sensogram analysis of each GL micelle–lectin interactions was performed with the available Biacore software tools (Biacore Evaluation Handbook, 1998). Primarily, the kinetic data were fitted to Langmuir 1:1 binding, equilibrium analysis, and the bivalent binding model. An equilibrium state of the GL micelle–Con A interactions was attained in all experiments. From the equilibrium response values ($R_{eq}$) and the analyte concentrations, the corresponding association constants ($K_A$) were derived. A plot of the $R_{eq}/C$ versus $R_{eq}$ (Scatchard plot) provided the $K_A$. However, it was found that the $\chi^2$ values, that measure the closeness of the fit and the reliability of the values derived thereupon, were consistently >10, indicative of a non-optimal curve fit and hence low confidence in
the derived $K_A$ values. The bivalent binding model assumes that the analyte having two independent binding sites interact with two receptor sites with identical binding properties. The kinetic analysis showed that the bivalent analyte model provided $\chi^2$ values of <10, therefore considered to more accurately describe the sensograms. A visual inspection of the plot of theoretical model versus experimental data provided that the $\chi^2$ fitting agreed the bivalent analyte model. Thus, based on the $\chi^2$ fitting, the bivalent analysis appeared to reflect the mode of binding better than the other two models, showing that the GL–lectin interactions are not true one-to-one binding. The association and dissociation rate constants, representing the first and second stage of the binding process, can thus be obtained from the bivalent analyte model. Detailed analysis of the bivalent analyte model of the interaction of various GL micelles with lectin Con A is summarized in Table II. By comparison of the first association and dissociation rate constants, it was established that in the one-sugar-bearing GL micelle-Con A interactions had thus emerged in a manner that faster $k_{a1}$ coincided with slower $k_{d1}$ and vice versa.

True association constant calculations are difficult to obtain in a bivalent analyte model. However, for the purpose of comparing various GL micelle-lectin interactions, ratios of the association–dissociation rate constants are useful accessible parameters. Table II summarizes that the $k_{a1}/k_{d1}$ remained largely uniform for all the GLs, whereas the $k_{a2}/k_{d2}$ were about 1000 times larger for the two-sugar-containing GL micelle-lectin interactions than for the one-sugar-containing series. This difference had evolved largely during the second association rate constants ($k_{a2}$). Putting together the above observations, it emerges that the additional sugar unit in the two-sugar-containing GL micelles provides a favorable complexation between the sugar ligand and the lectin for a bivalent interaction to occur. The favorable interaction relates to the relative first and second association rate constants, $k_{a1}$ and $k_{a2}$, respectively, across the series of GL micelles studied herein. Specifically, the second association rate constants were found to be orders of magnitude faster for the two-sugar-containing GL micelles when compared to the one-sugar-containing GL micelles. Although this observation of favorable bivalent interaction is consistent for the GL micelles GL-4, GL-5, and GL-6, it remains unclear as to how the additional sugar unit in these ligands at the monomolecular level assists the bivalent binding process. The distances between the sugar units at the monomolecular level do not permit interaction of the individual sugar units with separate receptor sites. The

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<th>Glycolipid</th>
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<tr>
<td></td>
<td>$k_{a1}$ (M$^{-1}$s$^{-1}$)</td>
</tr>
<tr>
<td>GL-4</td>
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</tr>
<tr>
<td>GL-2</td>
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<tr>
<td>GL-6</td>
<td>$2.25 \times 10^3$</td>
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additional sugar unit in the two-sugar-containing GL micelles can however be juxtaposed to the ligand–receptor site, which might influence the ligand–receptor interactions favorable for the two-sugar-containing GL micelles.

Conclusion

A series of one- and two-α-D-mannopyranoside-containing GLs were synthesized and their CMCs, aggregation properties, and micellar diameters were assessed. The nanometric GL micelles were used as analytes in SPR studies, involving lectin Con A-immobilized surfaces. Control studies with lectin WGA, to which α-D-mannopyranoside do not bind, were conducted, apart from studies to eliminate possibilities of mass transport phenomena in the SPR studies. GL micelles–Con A interactions were found to fit in a bivalent analyte model, in which the micelle would bind to two receptor sites independently. The identification of the bivalent interaction model allowed us to assess the association–dissociation rate constants. The kinetic analysis revealed that the two-sugar-containing GL micelles elicited favorable association rate constants, $k_{11}$ and $k_{22}$, when compared to the one-sugar-containing GL micelles. These results demonstrate the beneficial effects of additional sugar unit at the binding site vicinity, so as to alter the kinetics of the ligand–receptor interactions.

Materials and methods

General methods

Solvents were dried and distilled according to literature procedures. All chemicals were purchased from commercial sources and were used without further purifications. Analytical thin layer chromatography (TLC) was performed on commercial plates coated with silica gel GF254 (0.25 mm). Silica gel (100–200 mesh) was used for column chromatography. Microanalyses were performed on an automated C, H, and N analyzer. High-resolution mass spectra were obtained from a Q-TOF instrument by electrospray ionization (ESI). $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectral analyses were performed on a spectrometer operating at 300 and 75 MHz, respectively, and residual solvent signal was used as the internal standard. The SPR studies were performed on a Biacore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden) operated using the software version 1.3. The research grade carboxymethylated dextran matrix (CMS) was used in the analysis, and the chip contained four flow cells of dimensions $(l \times w \times h) = 2.4 \times 0.5 \times 0.05$ mm. Lectin Con A (salt free, lyophilized powder) was purchased from Sigma (St. Louis, MO). All experiments were performed at 25°C. Aqueous solutions were prepared from double-distilled water purified through a Milli Q to 18.2 MΩ resistance. Biacore experiments were performed in 10 mM HEPES buffer containing 150 mM NaCl, 1 mM CaCl$_2$, and 1 mM MnCl$_2$. All buffer solutions were filtered (0.2 μm) and thoroughly degassed.

General procedure for synthesis of GL-2 to GL-6

To a suspension of appropriate 2-O-hexadecyl glycerol derivative (10 or 11 or 12), Hg(CN)$_2$, HgBr$_2$ and molecular sieves (4Å) in CH$_2$Cl$_2$, a solution of 2,3,4,6-tetra-O-benzoyl-α-D-mannopyranosyl bromide 14 (Ness et al., 1950) in CH$_2$Cl$_2$ was added under stirring at room temperature and under argon atmosphere. The mixture was stirred for 36 h, then it was filtered through celite, washed with CH$_2$Cl$_2$, and the organic layer was washed with aq. Na$_2$SO$_4$ (10%), aq. NaHCO$_3$ (5%), and H$_2$O. The CH$_2$Cl$_2$ layer was dried (Na$_2$SO$_4$), filtered, concentrated in $\text{v} \times \text{c}$uo, and the residue was purified (SiO$_2$, petroleum ether/EtOAc) to afford the O-benzoyl-protected GLs. A suspension of protected GL in MeOH was admixed with NaOMe/MeOH (0.5 M, 0.5 mL) and left stirring for 12 h, then neutralized with Amberlite IR-120 resin (H$^+$ form), filtered, and the filtrate concentrated in $\text{v} \times \text{c}$uo. The resulting gummy syrup was triturated with Et$_2$O and lyophilized to afford the GLs as white foamy powder.

The one-sugar-containing analog GL-1 was also synthesized. However, because of the insolubility of GL-1 in aqueous solutions, it was not considered for studies in this report.

GL-2. Yield: 37%, $R_f = 0.5$ (MeOH/CHCl$_3$ = 1 : 4). [α]$_D^{25} = +24^\circ$ (c = 1.0, MeOH). $^1$H NMR [CDCl$_3$ + dimethylsulfoxide (DMSO)–d$_6$, 300 MHz]: δ 4.82 (br s, 1 H), 3.8–3.3 (band, 21 H), 1.52 (m, 2 H), 1.26 (br, 26 H), 0.87 (t, 3 H, $J = 7.2$ Hz). $^{13}$C NMR (CDCl$_3$ + DMSO–d$_6$, 75 MHz): δ 100.03, 77.58, 72.79, 72.44, 71.41, 70.65, 70.58, 70.52, 70.27, 67.64, 66.32, 61.82, 61.11, 31.73, 29.90, 29.46, 29.44, 29.33, 29.3, 25.89, 22.5, 14.1. HR-MS $m/z$: calculated for C$_{26}$H$_{58}$O$_{10}$Na: 589.3928, found: 589.3946 (M + Na). Elemental analysis: calculated for C$_{26}$H$_{58}$O$_{10}$C: 61.46, H 10.31, found: C 61.24, H 10.28.

GL-3. Yield: 40%, $R_f = 0.45$ (MeOH/CHCl$_3$ = 1 : 4). [α]$_D^{25} = +26^\circ$ (c = 1.0, MeOH). $^1$H NMR (DMSO–d$_6$, 400 MHz): δ 4.71 (t, 1 H, $J = 4.8$ Hz), 4.6 (s, 1 H), 4.57 (t, 1 H, $J = 5.4$ Hz). 4.53 (dd, 1 H), 4.42 (t, 1 H, $J = 5.6$), 3.64 (m, 2 H), 3.52–3.21 (band, 23 H). 1.43 (m, 2 H), 1.22 (br, 26 H), 0.84 (t, 3 H, $J = 5.4$). $^{13}$C NMR (DMSO–d$_6$, 100 MHz): δ 100.39, 79.57, 77.72, 74.3, 72.75, 71.36, 70.97, 70.92, 70.69, 70.59, 70.56, 70.13, 69.92, 69.7, 67.37, 66.14, 61.66, 60.64, 31.69, -22.49, 14.35. HR-MS $m/z$: calculated for C$_{35}$H$_{68}$O$_{15}$Na: 677.4452, found: 677.4449 (M + Na). Elemental analysis: calculated for C$_{35}$H$_{68}$O$_{15}$C: 61.66, H 10.16, found: C 61.24, H 10.28.

GL-4. Yield: 58%. [α]$_D^{25} = +35^\circ$ (c = 1.0, MeOH). $^1$H NMR (300 MHz, DMSO–d$_6$): δ 4.75 (m, 4 H), 4.58 (br s, 4 H), 4.48 (m, 2 H), 3.62–3.42 (band, 11 H), 1.42 (m, 2 H), 1.22 (br, 26 H), 0.83 (t, 3 H, $J = 6.9$ Hz). $^{13}$C NMR (75 MHz, DMSO–d$_6$): δ 100.3, 79.2, 73.9, 71.0, 70.3, 69.5, 66.9, 66.4, 61.2, 31.4, 29.7, 29.1, 29.0, 28.7, 25.6, 22.1, 14.1. HR-MS $m/z$: calculated for C$_{35}$H$_{68}$O$_{15}$Na: 663.3932, found: 663.3915 (M + Na). Elemental analysis: calculated for C$_{35}$H$_{68}$O$_{15}$C: 60.52, H 10.16, found: C 60.23, H 9.85.

GL-5. Yield: 61%. [α]$_D^{25} = +31^\circ$ (c = 1.0, MeOH). $^1$H NMR (300 MHz, DMSO–d$_6$): δ 4.74 (m, 4 H), 4.61 (br s, 4 H), 4.42 (m, 2 H), 3.67–3.34 (band, 19 H), 1.43 (m, 2 H), 1.26 (br, 26 H), 0.89 (t, 3 H, $J = 6.9$ Hz). $^{13}$C NMR (75 MHz, DMSO–d$_6$): δ 99.2, 73.86, 70.94, 70.52, 70.29, 69.78, 68.23, 67.12, 66.92, 66.61, 61.25, 31.29–22.10, 13.97. HR-MS $m/z$: calculated for C$_{35}$H$_{68}$O$_{15}$Na: 751.4456.
found: 751.4489 (M+Na). Elemental analysis: calculated for C_{35}H_{68}O_{14} + 2H_{2}O: C 54.97, H 9.32; found: C 54.19, H 9.20.

GL-6. Yield: 69%. [d]_{25}^{20} = +32\(^\circ\) (c = 1.0, MeOH). \(^1\)H NMR (300 MHz, DMSO-d\(_6\)): \(\delta\) 4.74 (m, 4 H), 4.6 (br s, 4 H), 4.43 (m, 2 H), 3.57–3.31 (band, 27 H), 1.42 (m, 2 H), 1.21 (br s, 26 H), 0.83 (t, J = 7.2 Hz). \(^13\)C NMR (75 MHz, DMSO-d\(_6\)): \(\delta\) 100.27, 79.46, 77.74, 75.19, 71.23, 70.82, 70.57, 70.01, 69.82, 69.59, 67.23, 66.02, 61.53, 30.60–22.41. HR-MS \(m/z\): calculated for C_{35}H_{76}O_{17}Na+: 839.4980, observed: 839.5049 (M + Na). Elemental analysis: calculated for C_{35}H_{68}O_{15} + 2H_{2}O: C 56.69, H 9.32; found: C 56.69, H 8.98.

2-O-Hexadecyl-1,3-di-O-benzyl glycerol (\(^9\)). 1,3-Di-O-benzyl glycerol (2.5 g, 9.0 mmol) (Cassel et al., 2001) was added to a suspension of NaH (60% in mineral oil) (0.43 g, 14.1 mmol) was added slowly. The mixture was stirred at 60\(^\circ\)C for 36 h, cooled, and concentrated in vacuo. The crude residue was dissolved in CH\(_2\)Cl\(_2\), washed with water, dried (Na\(_2\)SO\(_4\)), and the solvent was evaporated in vacuo. The residue was purified (SiO\(_2\)) (EtOAc/hexane) to afford \(^9\) (2.6 g, 58%). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.4–7.2 (m, 10 H), 4.5 (s, 4 H), 3.6–3.5 (m, 7 H), 1.6 (m, 2 H), 1.3 (s, 26 H), 0.87 (t, J = 6.6 Hz, 3 H). \(^13\)C NMR (75.5 MHz, CDCl\(_3\)): \(\delta\) 138.3, 128.3, 127.6, 127.5, 77.8, 73.3, 70.6, 70.1, 31.9–22.6. 14.1. HR-MS \(m/z\): calculated for C_{33}H_{52}O_{3}Na+: 519.3814, observed: 519.3826 (M + Na).

2-O-Hexadecyl-1,3-di-O-ethylglycerol (\(^10\)). A solution of 2-O-hexadecyl-1,3-di-O-ethylglycerol (2.5 g, 4.0 mmol) in EtOAc and MeOH (1:1) was treated with Pd-C (10%) (0.25 g), stirred followed by purification (SiO\(_2\), EtOAc) afforded \(^10\) (1.32 g, 82%) as a white powder. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 3.7 (m, 2 H), 3.6–3.5 (m, 7 H), 1.5–1.4 (m, 2 H), 1.23 (br s, 26 H), 0.86 (t, J = 7.4 Hz, 3 H). \(^13\)C NMR (75.5 MHz, CDCl\(_3\)): \(\delta\) 79.5, 79.0, 62.1, 31.87–26.03, 22.7, 14.13. HR-MS \(m/z\): calculated for C_{19}H_{40}O_{3}Na+: 339.2875, observed: 339.2889 (M + Na).

Mono- and diethylene glycol spacer containing derivatives \(^11\) and \(^12\) were synthesized by treating 2-O-hexadecylglycerol (\(^10\)) with ethylene glycol derivative \(^13\), following a similar protocol as described above.

2-O-(Hexadecyl-1,3-di-O-(2-hydroxyethyl)glycerol (\(^11\)). Yield: 49%. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 3.72–3.53 (band, 23 H), 1.42 (m, 2 H), 1.29 (br s, 26 H), 0.88 (t, J = 6.6 Hz, 3 H). \(^13\)C NMR (75.5 MHz, CDCl\(_3\)): \(\delta\) 78.7, 73.5, 71.9, 71.5, 71.1, 70.8, 61.8, 32.6–23.3, 14.1. HR-MS \(m/z\): calculated for C_{27}H_{50}O_{7}Na+: 515.3924, observed: 515.3937 (M + Na).

Preparation of sensor surface

After equilibration of the sensor surface with HEPES buffer (pH 7.4), the sensor surface (CM5) was activated at a flow rate of 10 \(\mu\)L/min with a 5-min pulse of 1:1 mixture of freshly prepared 0.1 M NHS and 0.1 M N-ethyl-N-(dimethylamino)propylcarbodiimide. Con A, at a concentration of 100 \(\mu\)g/mL in 10 mM NaOAc buffer (pH 4.3), was injected into channel 2. The remaining NHS esters were blocked by the addition of 1.0 M ethanolamine hydrochloride at pH 8.5 for 7 min. Similarly, WGA was immobilized on CM5 channel 1 surface, and this surface was used as a reference to the Con A functionalized surface.

Biocore experiments

All binding experiments with GLs were performed at a flow rate of 20 \(\mu\)L/min using HEPES buffer as eluant (BIAcore Manual, 1998). Injection times of GLs were 8 min, followed by 8 min dissociation. Regeneration was performed using a 1-min pulse of glycine–HCl, pH 2.55 for removal of GLs from the Con A surface. For the calculations of kinetic constant, each GL was diluted in HEPES buffer concentrations of 250, 125, 62.5, 31.25 to 15.6 \(\mu\)M. The GLs were diluted in the running buffer (HEPES, pH 7.4) to minimize the bulk effects attributable to the change in the refractive index of the solution. Each analyte GL solutions were injected into channel 2 (Con A) and channel 1 (WGA). The specific response of the Con A surface toward GLs was obtained by subtracting the channel 2 response from channel 1 response. In the bivalent analyte analysis, the unit of k_{a2} is RU\(^{-1}\) s\(^{-1}\) and this is modified to M\(^{-1}\) s\(^{-1}\) by the following expression: k_{a2} (M\(^{-1}\) s\(^{-1}\)) = k_{a2} (RU\(^{-1}\) s\(^{-1}\)) \times 100 \times molecular weight of analyte. Monomer molecular weight was considered in all calculations.

Fluorometry

The CMC and aggregation number for each GL in water were measured by using fluorometry. ANSA emission spectra were obtained by exciting the samples at 410 nm, and emission was recorded in the range from 450 to 550 nm. The GL solutions with known concentration were added progressively, and the emission intensities at 490 nm were measured. The CMCs and aggregation number of each GL were determined by using fluorometry.

DLS measurements

The particle size (hydrodynamic diameter) of the GL-micelles was determined by using a high performance particle size (HPPS) analyzer (Malvern, Worcestershire, UK). Essentially, DLS measures Brownian motion of the particles suspended within a liquid and relates this to the size of the particles (Berne and Pecora, 1976). The velocity of the Brownian motion is defined by a property known as the translational diffusion coefficient (D). The size of a particle (\(d_p\)) is calculated from the Stokes–Einstein equation: \(d_p = kT/3\pi\eta D\).

Where \(\eta\), D, \(k\), and \(T\) are the viscosity coefficient of the medium, the translational diffusion coefficient, the Boltzmann constant, and the absolute temperature, respectively. The
GL micelle sizes were measured, at the concentrations of 250 μM, at 25°C.

Supplementary material
Supplementary data are available at Glycobiochemistry online (http://glycob.oxfordjournals.org/).

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Conflict of interest statement
None declared.

Abbreviations
ANSA, anilinonaphthalene sulfonic acid; CMC, critical micellar concentration; Con A, concanavalin A; DLS, dynamic light scattering; DMSO, dimethylsulphoxide; GL, glycolipid; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; NS, non-sugar lipid; RU, response unit; Q, quencher; SPR, surface plasmon resonance; WGA, wheat germ agglutinin.

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