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Exploring the effect of sialic acid orientation on ligand–receptor interactions†

Rohan Yadav and Raghavendra Kikkeri*

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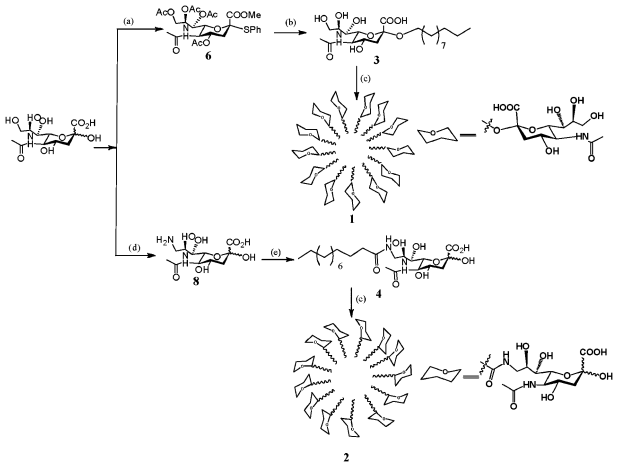
Here, we present the synthesis of two sialo-micelles to validate the significance of sialic acid orientation during specific carbohydrate–protein and carbohydrate–carbohydrate interactions. Our data clearly suggest that orientation of carboxylic acid and glycerol side chains of sialic acid moieties exert fine tuning of ligand–receptor interactions.

Sialic acids (sias) occur as the terminal part of glycan chains on glycoproteins/glycolipids expressed by the deuterostome lineage of animals and on certain bacterial species.[1] Sias appear to dictate a wide variety of biological functions such as the binding of hormones, toxins and viruses, maintenance of surface negative charge, and contribution to the viscosity of mucins, *etc*.[2] Further, a significant difference exists between sialylation patterns in normal cells and their malignant counterparts.[3] Therefore, sia-analogs are crucial in order to fine-tune their biological behaviour. Over fifty different naturally occurring sia species have so far been identified. In mammals, the most abundant sias are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (NeuGc) which exist either as *O*-acetate, lactate, sulfate or phosphate ester, or methyl ether forms at 4, 7, 8, and 9 positions giving rise to a great variety of compounds and isomers.[1a,4] These substitutions alter the biological properties of sias compared to their parental Neu5Ac ligand. For example, C-9 and C-5 *O*-acetylated Neu5Ac are expressed by human tissues such as brain, colon, salivary and gastric mucins and peripheral blood cells to offer resistance against bacterial or viral neuramidases.[5] Similarly, sialic acid–CD22 interaction was modulated by introducing an aromatic substituent at the C-9 position of sias.[6] Furthermore, it has been shown that lactamized-sialyl 6-sulfo Lewis^X, but not conventional sialyl Lewis^X, serves as the major ligand for L-selectin.[7] Overall, a recent survey along with experimental evidence clearly indicates that modified sias are more widely distributed than the parental Neu5Ac ligand.[8]

In this communication, we describe that sialic acid locked in two opposite orientations on micelles effectively tunes sia dependent ligand–receptor interactions in their native context. To obtain sialo-micelles that could serve as multivalent probes,[9] we conjugated amphiphilic groups at C-2 and C-9 positions of sia respectively. Upon dissolution of amphiphiles in water, self-assembled highly regular micelles were obtained. The bioavailability of resultant sialo-micelles with plant and human sialic acid binding protein (SBP) was evaluated by surface plasma resonance (SPR) and *in vitro* assay. *Sambucus nigra* agglutinin (SNA), *Limax flavus* agglutinin (LFA),[10] P, E-selectin and CD22 (Siglec-2) were selected as sialic acid binding proteins (SBP), where SNA and LFA bind to all common sialic acid residues,[10,11] human CD22-Fc recognizes siaα(2-6)-linked sias,[12] and E and P-selectin, which belong to the subgroup of the C-type lectins that mediate leukocyte trafficking, are specific to sialyl Lewis^X and sialyl Lewis^a glycans respectively.[13]

The syntheses of compounds **3** and **4** are depicted in Scheme 1. The synthesis of *O*-sialoside **3** was carried out with β-thioglycoside donor **6**[14] which was glycosylated with *n*-undecanol, followed by de-acetylation. 9-Amidosialic acid **4** was synthesized starting from mono-tosylation of sialic acid **7**, followed by azidation, hydrogenation of **8**[6b] and coupling with dodecanoic acid (Scheme 1). The structures of **1** and **2** could be unambiguously confirmed by mass spectrometry and



Scheme 1 Synthesis of sialic acid based amphiphiles (**1** and **2**); (a) amberlite-H$^+$/MeOH; Ac_2O/pyridine; (b) $PhSH/BF_3{\cdot}Et_2O$; (c) undecanoic acid/NIS/MS 4 Å/TfOH, NaOMe/MeOH; (d) H_2O; (e) TsCl/pyridine; (f) NaN_3/acetone/water, Pd–C/H_2; (g) dodecanoic acid/DIC/NHS/dioxane/water.

Indian Institute of Science Education and Research, Sai Trinity Building, Pashan, Pune 411021, India. E-mail: rkikkeri@iiserpune.ac.in; Fax: +91-20-25899790; Tel: +91-20-25908207

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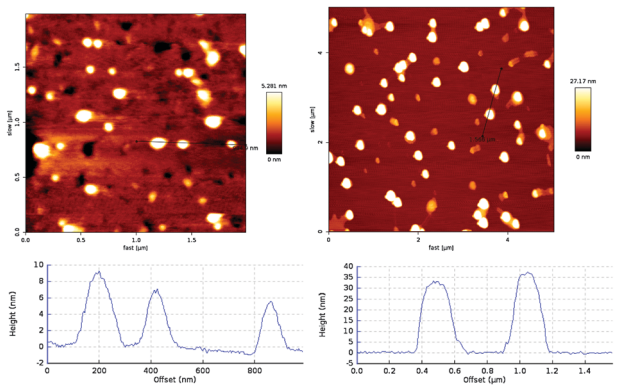

Fig. 1 AFM topographical images of (a) compound **1**, (b) compound **2** and its corresponding topology. The image scale is in micrometers.

NMR spectrometry in MeOD. Both ^{1}H and ^{13}C NMR spectra displayed characteristic signals of aliphatic chains and sialic acid units.

Upon dissolution of 10 mg ml^{-1} of **1** and **2** in water, self-assembled behaviour of sia based micelles was characterized by means of dynamic light scattering (DLS) and atomic force microscopy (AFM). DLS patterns of **1** and **2** were almost identical and the corresponding hydrodynamic radius (R_H) was between 100 and 150 nm (Fig. S4, ESI†). Evidence for the formation of circular aggregates was provided by AFM experiments (Fig. 1).

After synthesizing sia micelles, the kinetics and mechanism of SBP binding were investigated by using SPR. Kinetic analysis was performed on a 1 : 1 interaction model.[15] SBP of 25 μg concentration was covalently bound to a polycarboxylated CM5 sensor chip. Of the five SBP, four bind to micelle **2** (Fig. 2 and Table 1) to a varying extent and no binding has been observed with CD22-Fc. **1** served as a positive control.

For SNA lectin interaction at 25 °C, there is a marginal decrease in the binding of **2** compared to **1** (0.88-fold decrease in affinity *versus* compound **1**). With the LFA lectin module, there is 0.9-fold decrease in affinity to compound **2**, which also disassociates from LFA much slower than compound **1**. Overall, the degree of plant lectin binding induced by micelle **2** was more or less similar to that of **1**. This may be due to the non-specific recognition of sias including Neu5Ac, Neu5Gc and their respective glycan residues by plant lectins.[11]

For E and P-selectin–micelle interactions, K_D for **2** was almost similar to that for **1**. This may be due to the α-hydroxycarboxylic acid[16] residue of **2** which coordinates with calcium ions in a manner akin to sialyl $Lewis^X$. To confirm this hypothesis, we soaked compound **2** with Ca^{2+} ions. Interestingly, DLS measurements showed aggregates of size ~1000–1500 nm (Fig. S6, ESI†) which was further supported by AFM imaging (Fig. S5, ESI†). Taken together, lectin binding assays indicate that sialic acid orientation has little impact and **2** may be a small potential mimic of the sialyl $Lewis^X$ ligand. In addition, the terminal carboxylic acid group facilitates Ca^{2+} mediated carbohydrate–carbohydrate interactions.

With the CD22-Fc (Siglec-2) module, there was a significant difference between **1** and **2**. Data derived from SPR assay demonstrate no binding activity with **2**, whereas binding of **1** displays a K_D value of 0.43 μM. The binding curves obtained

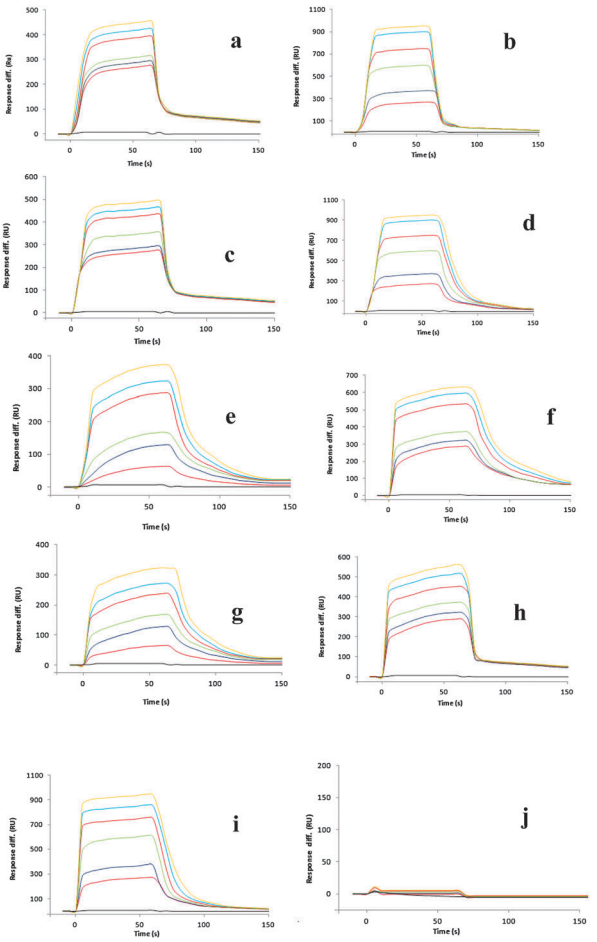

Fig. 2 SPR sensorgrams of different sialic acid binding protein (SBP) binding to captured micelles containing two opposite orientations of sialic acid. Sensorgrams a and b show different concentrations of **1** and **2** binding to the CM5 surface expressing SNA lectin; sensorgrams c and d show interaction of **1** and **2** with LFA lectin; sensorgrams e and f show interaction of **1** and **2** binding with E-selectin lectin; sensorgrams g and h show interaction of **1** and **2** binding with P-selectin lectin; sensorgrams i and j show interaction of **1** and **2** with CD22-Fc; concentrations of **1** and **2**: 0 μM (black line), 5 μM (red line), 10 μM (dark blue line), 20 μM (green line), 30 μM (dark red line) 40 μM (blue line) and 50 μM (orange line) respectively.

Table 1 Equilibrium constant, K_D, of **1** and **2**

	K_D (μM)	
Lectins	Comp **1**	Comp **2**
Sambucus nigra agglutinin (SNA)	0.146	0.129
Limax flavus agglutinin (LFA)	0.149	0.134
E-Selectin	0.32	0.31
P-Selectin	0.35	0.36
CD22-Fc	0.43	No binding

for interaction between **2** and CD22-Fc was comparable to results obtained using only buffer as a negative control. These observations were consistent with those of Kelm and Oetke *et al.*, who reported the requirement of hydroxyl groups at C-9 for sialic acid recognition by CD22.[17]

To further support our assessment by SPR, a CD22 transfected CHO cell line was used to study sialic acid orientation

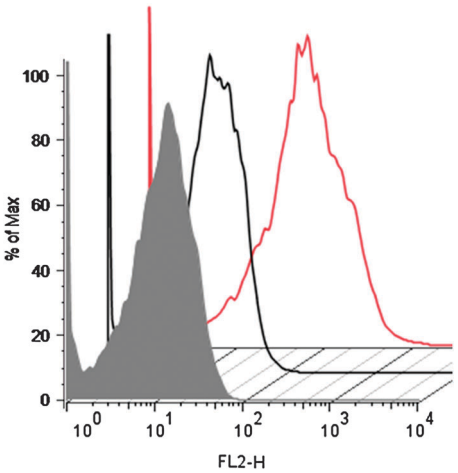

Fig. 3 Flow cytometry graph of CHO-CD22 with **1** and **2**: CHO-CD22 cells were left untreated (tinted area), incubated with 50 μM of compound **1** (red line area, positive control), compound **2** (black line area).

dependent lectin recognition and binding. Fluorescently labeled sialic acid micelles were prepared by mixing 5 mg of fluorescein dye with equal amounts of ligands **3** and **4** in water, followed by 10 K cutoff microcon filtration to obtain fluorescein hosted sialic acid micelles. The sialic acid–CD22 interactions on the cell surfaces were then measured by flow cytometry. As shown in Fig. 3, compound **2** did not bind effectively to CD22-transfected CHO-cells, indicating the requirement of native sialic acid orientation for the recognition. On the other hand, the CHO-K1 cell line served as a negative control and showed no major uptake of either **1** or **2** (data not shown). These findings indicate that compound **2** is a potential sialic acid moiety for fine-tuning sialic acid based ligand–receptor interaction in mammalian SBP.

We have shown that structural rearrangement of sialic acid moieties around a multivalent system could modulate the ligand–receptor interactions. Surface plasmon resonance (SPR) and *in vitro* binding assays show that out of five potential sialic acid binding plant and human lectins (SBP), only CD22 (Siglec-2) abolishes binding with an amphiphile at the C-9 position of sialic acid under biophysical conditions, while *O*-sialoside was used as a positive control. These differences in the relative binding affinities of two human lectins provide a novel lead in the development of inhibitors and biomarkers for specific human SBP. Such studies are currently being considered. Furthermore, stereo-specificity of the carboxylic acid residue of sialic acids also influences Ca^{2+} mediated carbohydrate–carbohydrate interactions. These observations provide a novel lead in the development of inhibitors and biomarkers for specific sialic acid binding proteins. Such studies are currently being considered.

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