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Surface plasmon resonance imaging for real-time, label-free analysis of protein interactions with carbohydrate microarrays

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Abstract Plant lectin recognition of glycans was evaluated by SPR imaging using a model array of *N*-biotinylated aminoethyl glycosides of β-D-glucose (negative control), α-D-mannose (conA-responsive), β-D-galactose (RCA_{120}-responsive) and *N*-acetyl-β-D-glucosamine (WGA-responsive) printed onto neutravidin-coated gold chips. Selective recognition of the cognate ligand was observed when RCA_{120} was passed over the array surface. Limited or no binding was observed for the non-cognate ligands. SPR imaging of an array of 40 sialylated and unsialylated glycans established the binding preference of hSiglec7 for α2-8-linked disialic acid structures over α2-6-sialyl-LacNAcs, which in turn were recognized and bound with greater affinity than α2-3-sialyl-LacNAcs. Affinity binding data could be obtained with as little as 10–20 μg of lectin per experiment. The SPR imaging technique was also able to establish selective binding to the preferred glycan ligand when analyzing crude culture supernatant containing 10–20 μg of recombinant hSiglec7-Fc. Our results show that SPR imaging provides results that are in agreement with those obtained from fluorescence based carbohydrate arrays but with the added advantage of label-free analysis.

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Abbreviations

ConA	concanavalin A
RCA_{120}	the 120 kDa component of *Ricinus communis* agglutinin
WGA	wheat germ agglutinin

Introduction

Increasing awareness of the importance of glycosylation to biological systems [1] has led to recognition of the need to develop better tools for analysis of protein–carbohydrate

interactions. In contrast to template-encoded nucleic acid and protein sequences which aid function assignments, the need for a more empirical, high throughput analysis of potential carbohydrate binding partners has resulted in a variety of approaches for the generation of carbohydrate microarrays [2–6]. For reasons of sensitivity, throughput and compatibility with existing nucleic acid array hardware, fluorescence based measurements is, to date, the prevalent detection principle for such microarrays. Surface plasmon resonance (SPR) has been used for some time for the real-time, label-free analysis of protein–carbohydrate interactions [for instance, 7–10]. Where the stoichiometry of binding is known, SPR provides direct access to association and dissociation kinetics, and indirectly to equilibrium binding affinity. In this latter context, SPR offers advantages over microcalorimetry, for instance, since it consumes substantially less protein [11]—a key issue when analyzing interactions of moderate affinity, such as those typical of lectin–carbohydrate interactions. Although SPR imaging has been used previously to analyze protein carbohydrate interactions [12, 13], it is only with the recent development of commercial SPR imaging instruments [14] that the opportunity for carbohydrate array analysis by SPR presents itself. Indeed, SPR imaging is already becoming established in the analysis of protein and nucleic acid array fields [15, 16].

The Biacore Flexchip SPR imaging instrument was evaluated in the study of carbohydrate arrays described herein [for a survey of the various formats of commercial SPR imaging array instruments, see ref 16]. This instrument, which is capable of handling a 20×20 array defined in a 1 cm^2 window on the sensor chip surface (typical spot size: 150 μm diameter × 500 μm spacing), is designed to allow independent SPR analyses of an entire array of up to 400 spots in a single flow cell, so minimizing sample size and analysis time. Comprehensive evaluation of the instrument for studying antibody-protein interactions has been reported previously [17]. Here we report preliminary studies with plant lectins and our observations on the suitability of SPR imaging to analyze the interactions of a recombinant Fc fusion protein of human Siglec 7 (h-Siglec7-Fc) with a representative selection of sialylated and non-sialylated glycans.

Materials and methods

Biotin-LC-LC-sulfoNHS ester was purchased from Pierce. ConA, RCA_{120}, WGA and goat anti-human-Fc antibody were purchased from Sigma-Aldrich. SPR studies were conducted with a Biacore Flexchip SPR imaging instrument.

Aminoethyl glycosides were prepared using well-established procedures [18–20]. Briefly, this entailed glycosylation of bromo- or chloro-ethanol and subsequent azide displacement of the resulting haloethyl glycoside. Deprotection and reduction gave the desired glycosides in a form suitable for direct derivatization with commercial biotin-LC-LC-sulfoNHS ester. The biotin derivatives were subsequently purified by mixed-bed ion-exchange chromatography or gel filtration to remove residual biotin-LC-LC-CO_2H prior to use. A full list of compounds from the Consortium for Functional Glycomics collection (http://www.functionalglycomics.org/static/index.shtml) that were used in this study can be found in Table 1.

Biotinylated sugars were prepared in phosphate-buffered saline (PBS; 10 mM phosphate buffer pH 7.4, containing 137 mM NaCl and 2.7 mM KCl) at concentrations of 100, 500 and 1000 μg/ml. Samples were spotted onto neutravidin-coated gold chips (Biacore) using a Genomic Solutions Omnigrid contact microarrayer. Split-pin (CMP-5) printing with a pin dwell time of 25 ms gave a delivery volume of *ca.* 1.3 nl, which gave a typical spot size of 150–160 μm diameter. Printed arrays were incubated in a humid atmosphere for 1 h and stored dry at 4°C prior to use.

CHO cell expression of hSiglec7-Fc in serum-free X-VIVO 10 medium (Lonza) was performed as described previously [21]. Multivalent presentation of this protein is required in order to produce interpretable glycan binding data: this was achieved, by cross-linking the Fc fusion protein with anti-Fc antibody prior to use [22–24]. Specifically, a 50 μl mixture of hSiglec7-Fc (2 mg/ml) and goat anti-human-Fc Ab (2 mg/ml) was incubated for 1 h at room temperature. The resulting complex was diluted in PBS (1.6 ml) prior to use.

Regions of interest (ROI) on the printed chip were assigned while the chip was dry, as described by the manufacturer. Each ROI has associated reference spots that surround the ROI and were used to correct for bulk refractive index changes and instrumental drift. Experiments were performed at a flow rate of 500 μl/min at 25°C. The chip was blocked with Flexchip blocking buffer prior to use. The array was subsequently washed for 10 min with PBS running buffer prior to the injection of lectin (in PBS) across the chip for 10 min to observe glycan–lectin association. A further 10 min wash with PBS was used to observe the dissociation phase of the glycan–lectin interaction. Data analysis was performed using the package provided with the Flexchip instrument. Relative ranking is represented as the average value of duplicates from a 'binding late' analysis (as defined by the manufacturer). A full kinetic analysis was not attempted for the hSiglec7-Fc studies due to the mixed valence state of the cross-linked protein-antibody complex.

Results and discussion

Initial studies were performed using the aminoethyl glycosides of β-D-glucose, α-D-mannose, β-D-galactose, and *N*-

Table 1 Biotinylated glycans employed in this study [28]

CFG No	Name	Common name
B80	Lacβ-SpNH-LC-LC-Bt	Lac
B81	LacNAcβ-SpNH-LC-LC-Bt	LN
B82	Galβ1-3GlcNAcβ-SpNH-LC-LC-Bt	LeC
B83	Neu5Acα2-3Galβ1-4Glcβ-SpNH-LC-LC-Bt	3′SLac
B84	Neu5Acα2-3Galβ1-4GlcNAcβ-SpNH-LC-LC-Bt	3′SLN
B85	Neu5Acα2-3Galβ1-3GlcNAcβ-SpNH-LC-LC-Bt	3′SLeC
B86	Neu5Acα2-6Galβ1-4Glcβ-SpNH-LC-LC-Bt	6′SLac
B87	Neu5Acα2-6Galβ1-4GlcNAcβ-SpNH-LC-LC-Bt	6′SLN
B89	Neu5Gcα2-3Galβ1-4Glcβ-SpNH-LC-LC-Bt	3′S(Gc)Lac
B90	Neu5Gcα2-3Galβ1-4GlcNAcβ-SpNH-LC-LC-Bt	3′S(Gc)LN
B93	Neu5Gcα2-6Galβ1-4GlcNAcβ-SpNH-LC-LC-Bt	6′S(Gc)LN
B107	Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ-SpNH-LC-LC-Bt	GD3
B108	Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ-SpNH-LC-LC-Bt	GT3
B111	(Galβ1-4GlcNAcβ)2-SpNH-LC-LC-Bt	Di-LN
B114	GlcNAcβ1-3Galβ1-4Glcβ-SpNH-LC-LC-Bt	LNT2
B115	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-SpNH-LC-LC-Bt	LNnT
B121	GlcNAcβ-SpNH-LC-LC-Bt	GN
B156	Galβ1-3[Fucα1-4]GlcNAcβ-SpNH-LC-LC-Bt	LeA
B157	Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ-SpNH-LC-LC-Bt	SLeX
B174	Neu5Acα2-3Galβ1-3[Fucα1-3]GlcNAcβ-SpNH-LC-LC-Bt	SLeA
B177	Neu5Acα2-3[GalNAcβ1-4]Galβ1-4Glcβ-SpNH-LC-LC-Bt	GM2
B178	Neu5Acα2-3[Galβ1-4GlcNAcβ]2-SpNH-LC-LC-Bt	3′-SLN-LN
B179	Neu5Acα2-6[Galβ1-4GlcNAcβ]2-SpNH-LC-LC-Bt	6′-SLN-LN
B180	GalNAcβ1-4GlcNAcβ-SpNH-LC-LC-Bt	LDN
B181	GlcNAcβ1-3Galβ1-4GlcNAcβ-SpNH-LC-LC-Bt	GN-LN
B184	Neu5Acα2-8Neu5Acα2-3[GalNAcβ1-4]Galβ1-4Glcβ-SpNH-LC-LC-Bt	GD2
B185	(6OSO3)Galβ1-4Glcβ-SpNH-LC-LC-Bt	6′SuLac
B186	Galβ1-4(6OSO3)Glcβ-SpNH-LC-LC-Bt	6SuLac
B187	[3OSO3]Galβ1-4(6OSO3)Glcβ-SpNH-LC-LC-Bt	6, 3′-di-SuLac
B194	Neu5Acα2-3[Galβ1-4GlcNAcβ1-3]3-SpNH-LC-LC-Bt	3′SLN-LN-LN
B197	[3OSO3]Galβ1-4Glcβ-SpNH-LC-LC-Bt	3′SuLac
B202	Neu5Acα2-3[Galβ1-3GlcNAcβ1-4]Galβ1-4Glcβ-SpNH-LC-LC-Bt	GM1
B204	Neu5Acα2-3[Galβ1-3GalNAcβ1-4]Galβ1-4GlcNAcβ-SpNH-LC-LC-Bt	GM2(NAc)
B266	Galβ1-4[Fucα1-3]GlcNAcβ-SpNH-LC-LC-Bt	LeX
B273	Neu5Acα2-3LacDiNAcβ-SpNH-LC-LC-Bt	3′SLDN
B274	Neu5Acα2-6LacDiNAcβ-SpNH-LC-LC-Bt	6′SLDN
B277	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-SpNH-LC-LC-Bt	LeC-LN
B298	Neu5Acα2-3[Neu5Acα2-3Galβ1-3GalNAcβ1-4]Galβ1-4Glcβ-SpNH-LC-LC-Bt	GD4
B299	GlcNAcβ1-3Galβ1-3GlcNAcβ-SpNH-LC-LC-Bt	GN-LeC
B301	Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAcβ-SpNH-LC-LC-Bt	LN-LeC

acetyl-β-D-glucosamine, which were derivatized with commercial biotin-LC-LC-sulfoNHS ester. The resulting biotin adducts were printed onto neutravidin-coated gold sensor chips. These sensor chips are designed for work with nucleic acids; hence they were selected for our work, which includes a range of charged sialic acid- and sulfate-containing glycans. Printing solutions of 100, 500 and 1000 μg/ml biotinylated glycan were employed; in this concentration range, lectin responses (as judged by SPR signal intensity) were essentially independent of sugar loading. Only data for 100 μg/ml glycan are shown for clarity. SPR data for a single chip printed with ten spots each of the biotinylated derivatives of glucose (negative control), mannose (ConA-responsive), galactose (RCA_{120}-responsive) and *N*-acetylglucosamine (WGA-responsive) and interrogated with RCA_{120} are shown in Fig. 1a. The data shows very clearly that RCA_{120} selectively binds to its cognate galactose ligand, while not binding to the other sugars on the chip (Fig. 1b) in a manner consistent with the established specificity of this lectin [for a recent survey of plant lectin specificity see 25]. This experiment was performed with 250 μg of RCA_{120}, which highlights the specificity of the glycan–lectin interactions. The error in the signal intensity of the ten galactose replicate spots is +/− 7% around the mean value. Experience suggests

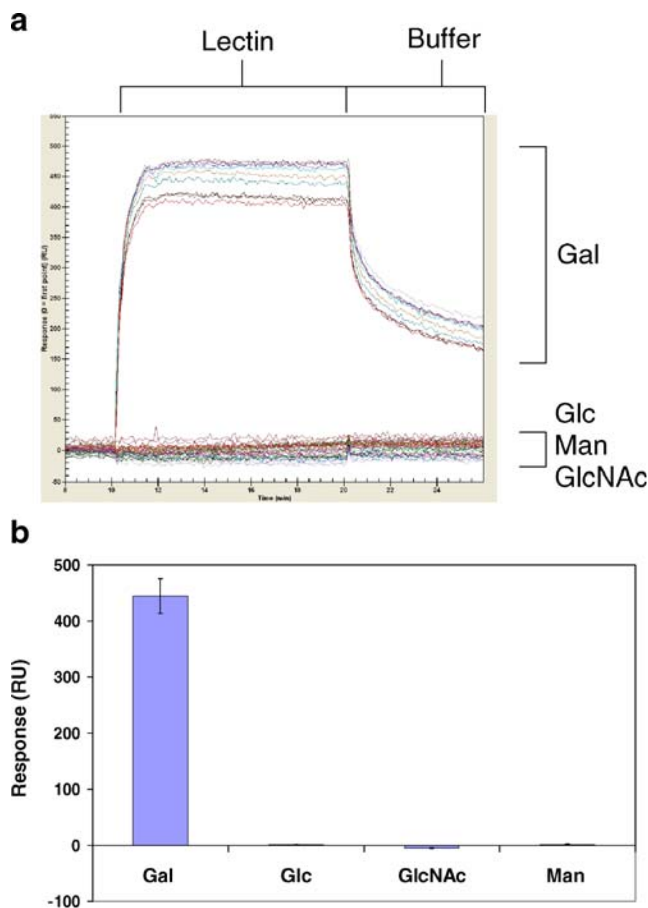

Fig. 1 SPR analysis of a model glycan array: ten spots each of biotinylated Gal, Glc, GlcNAc and Man derivatives were printed from 100 μg/ml glycan stock solution and interrogated with RCA_{120} (250 μg in 1.6 ml buffer). **a** SPR traces (following background subtraction); **b** corresponding bar chart (average of data from 10 spots), showing the specificity and reproducibility of RCA_{120} binding to its cognate Gal ligand

that this error is probably largely due to the printing of the spots for the array.

Subsequently, analysis of glycan binding by recombinant human Siglec 7 [26], a member of the sialic acid-binding immunoglobulin superfamily [27], was achieved by SPR imaging. In this instance, a selection of 40 biotinylated glycans (Table 1) from the Consortium for Functional Glycomics [28] was employed. The need for cross-linking to emulate the cell surface presentation of hSiglec7 *in vivo* has been noted before [22–24]. In keeping with this observation, Fig. 2a shows that without cross-linking, the binding of hSiglec7 gives only rather weak intensity SPR signals (compare Fig. 2a and b; note the difference in *y*-axis range) that are likely attributable to low-affinity, non-specific binding. No preference for binding sialylated over non-sialylated glycans is evident. In stark contrast, when cross-linked (anti-Fc Ab to aggregate the Fc fusion component of this lectin construct), the SPR imaging data for hSiglec7 shows strong intensity signals with low background, indicative a high binding affinity, and selectivity, for the three α2-8-linked disialic acid structures (B107, B108 and B184) amongst the selection of 40 glycans analyzed (Fig. 2b). At much lower sensitivity, cross-linked hSiglec7 also recognized some (B86 and B87) but not all (B179, B274) α2-6-sialylated structures (B93, the *N*-glycolyl version of B87, was not recognized). Further, only 2 of 13 α2-3-sialylated structures (B273 and B298) were recognized by the cross-linked hSiglec7, and then only with very weak affinity.

Our data contrasts with data from ELISA assays employing polyacrylic acid-based glycoconjugates. Using such ELISA assays the hSiglec7 exhibited a high binding affinity for α2-8-diasialic acid; showed good binding to α2-3-sialyl-lactose and very weak binding to α2-6-sialyl-lactose [29]. However, our data are consistent with calorimetric data (α2-8-diasialyl-lactose, K_D 98 μM; α2-6-sialyl-lactose, K_D 242 μM; α2-3-sialyl-lactose, K_D 680 μM), albeit for monovalent ligands binding to non-crosslinked protein in solution [30]. More importantly, a strong affinity of for binding α2-8-diasialylated structures is consistent with data produced by fluorescence-detected array experiments with the same hSiglec7 construct [28]. With the aid of protein crystallography, the α2-8-disialic acid specificity of hSiglec7 has been rationalized [31]. Our studies also show a clear preference for binding 6′sialyl-lactose over 6′sulfo-lactose, which is consistent with the recent literature, which shows that sulfate cannot replace sialic acid in siglec recognition [23]. Data presented in Fig. 2a/b were generated with 100 μg of hSiglec7 per experiment (note the difference in range in the y-axis for Fig. 2a/b). However, similar data were obtainable with 20 μg of protein (not shown). Indeed, high quality binding affinity data were also obtained when analyzing expression culture supernatant containing 16 μg of recombinant hSiglec7-Fc (cross-linked with antibody for analysis; Fig. 2c; For the use of SPR imaging to analyze proteins in crude cell lysates with antibody arrays, see [32]).

In summary, we have demonstrated the ease and utility of SPR imaging for label-free analysis of protein interactions with carbohydrate microarrays. Good data quality with *ca* 15–20 μg for h-Siglec7-Fc on an array of representative *N*-linked glycans was readily achievable. In addition, the ability to assess the carbohydrate selectivity of proteins without prior purification to homogeneity highlights the potential of the SPR-array approach for screening lectin preparations, whether of recombinant or natural origin.

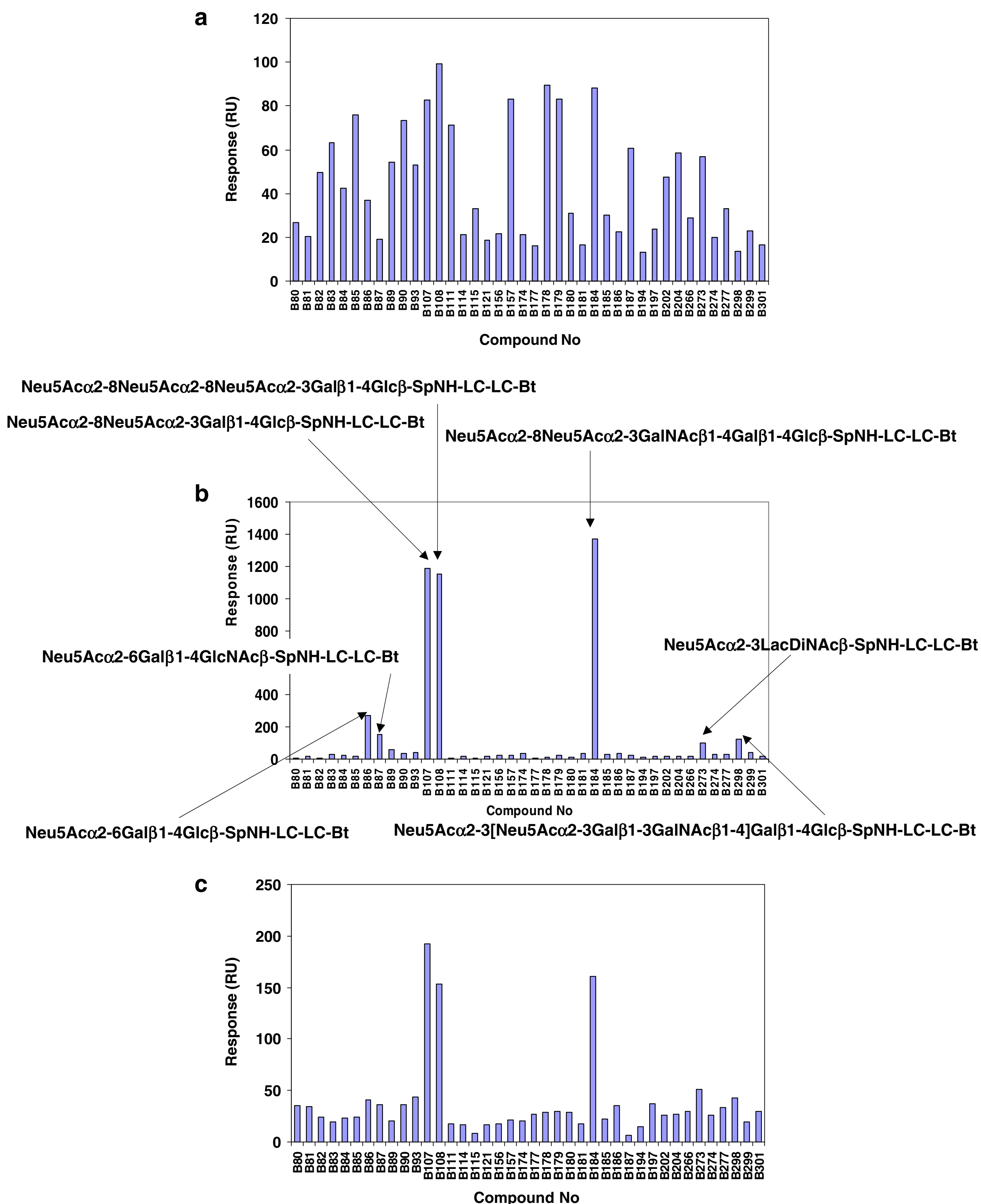

Fig. 2 SPR analysis of glycan array printed with 100 μg/ml glycan and interrogated with: Purified hSiglec7-Fc (100 μg total; not cross-linked) **a**; Purified hSiglec7-Fc (100 μg total) cross-linked with anti-Fc Ab **b**; Unpurified hSiglec7-Fc (16 μg of hSiglec7-Fc) from cell culture supernatant, cross-linked with anti-Fc Ab **c**. Note the difference in *y*-axis in range in each part of the Figure

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