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Probing the Relevance of Lectin Clustering for the Reliable Evaluation of Multivalent Carbohydrate Recognition

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Carbohydrate−receptor interactions are important recognition events that regulate a myriad of biological and pathological processes.[1] The clustered arrangement of both carbohydrates and lectins (carbohydrate recognition proteins) on biological surfaces enables their multivalent interaction in global processes that are characterized by high affinities and specificities.[2] This cluster glycoside effect has prompted the development of synthetic multivalent glycoconjugates with the ability to interact with target lectins and, hence, to promote/inhibit natural carbohydrate−receptor interactions.[3,4] Measurement of the binding affinities of novel glycoconjugates toward lectins in solution is routinely realized by e.g. agglutination inhibition assays, ELISA, calorimetry, or surface plasmon resonance (SPR).[2,5] However, these experimental designs frequently represent rough models for mimicking surface-based carbohydrate−lectin interactions since they underestimate the effect derived from the lectin clustering. Indeed, differences in affinity of several orders of magnitude are predicted for surface-based multivalent ligand−receptor interactions relative to those involving disperse soluble species.[6]

With the aim of gaining insight into the fundamental mechanisms of multivalent carbohydrate recognition, here we have compared the outcome of carbohydrate−lectin binding studies in solution (via competitive experiments) and surface-bound direct experiments (with immobilized lectins) by means of SPR (a real time detection technology providing both kinetic and equilibrium data) (Figure 1a,b).[7] This has allowed us to distinguish and quantify the role of the clustered arrangement of lectins in the interaction.[8,9] To that end, we have selected the α-D-mannose binding lectin Concanavalin A (Con A) and three generations of clicked mannosylated GATG (gallic acid-triethylene glycol) dendrimers, containing 3−27 mannose residues ([Gn]-Man, Figure 1c), previously described by our group.[10] The characteristic monodisperse nature of dendrimers, along with the absolute control over their size and branching density, makes them attractive tools for mechanistic studies on multivalency.[4]

The binding ability of [Gn]-Man to Con A in solution was evaluated via a competitive assay. Tetrameric Con A (10 μM) was preincubated for 1 h with increasing concentrations of methyl-α-D-mannopyranoside (Me-Man) or the glycodendrimers, and the residual binding capacity of Con A was evaluated toward a polycarboxylated gold sensor chip coated with α-D-mannose (Figure 1a). IC_{50} concentrations for each competitor were obtained by nonlinear regression, and the macroscopic dissociation constants (K_D) of the complexes were estimated by means of the Cheng−Prusoff equation [Figure 2 and Supporting Information (SI)].[11] [G1]-Man resulted to bind Con A with 8-fold increased affinity compared to Me-Man, while [G2]-Man and [G3]-Man experienced a 112- and 372-fold binding enhancement, respectively. A different outcome resulted, however, when relative affinities were expressed on a per sugar basis. In this case, a significant increase in relative affinity

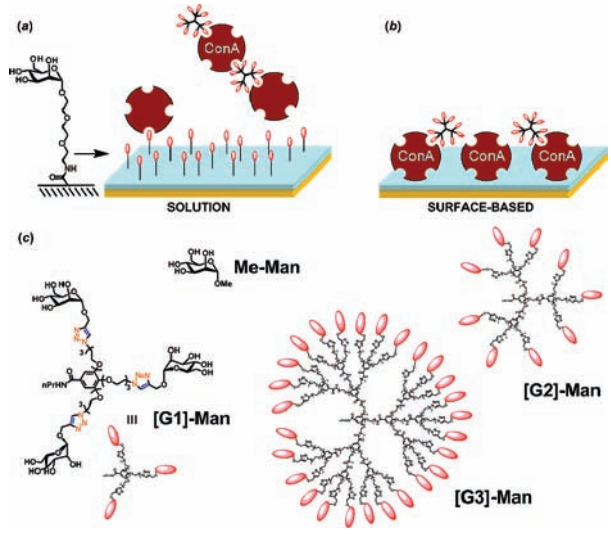

Figure 1. (a) SPR competitive and (b) direct binding assays. (c) Mannosylated GATG dendrimers.

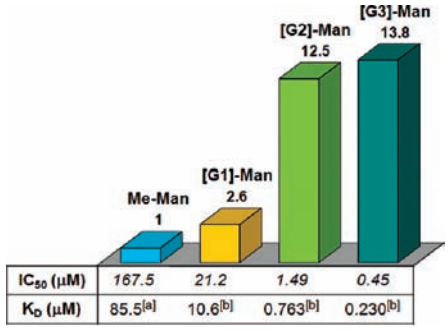

Figure 2. IC_{50}, K_D, and relative affinities per mannose (bars) of the complexes [Gn]-Man-Con A in solution obtained from SPR competitive experiments. (a) Obtained from a SPR direct binding assay. (b) Estimated from the corresponding IC_{50} values.

was observed only up to G2 (12.5-fold), with G3 not improving much further its binding to Con A in solution (13.8-fold). This result is in agreement with previous reports by the groups of Kiessling and Cloninger for multivalent glycoconjugates not spanning multiple lectin binding sites[12,13] and reveals that higher dendrimer generations do not always represent any real advantage for solution-based carbohydrate−lectin interactions.[14]

For the SPR direct binding experiments (Figure 1b), Con A was covalently bound to a polycarboxylated sensor chip to yield high density coverage (Con A-HD, 6900 μRiU, 1 μRiU = 0.73 RU). As a control experiment, the binding of Me-Man to Con A-HD was evaluated by sequentially injecting increasing concentrations of the monosaccharide. The resulting sensorgrams showed on and

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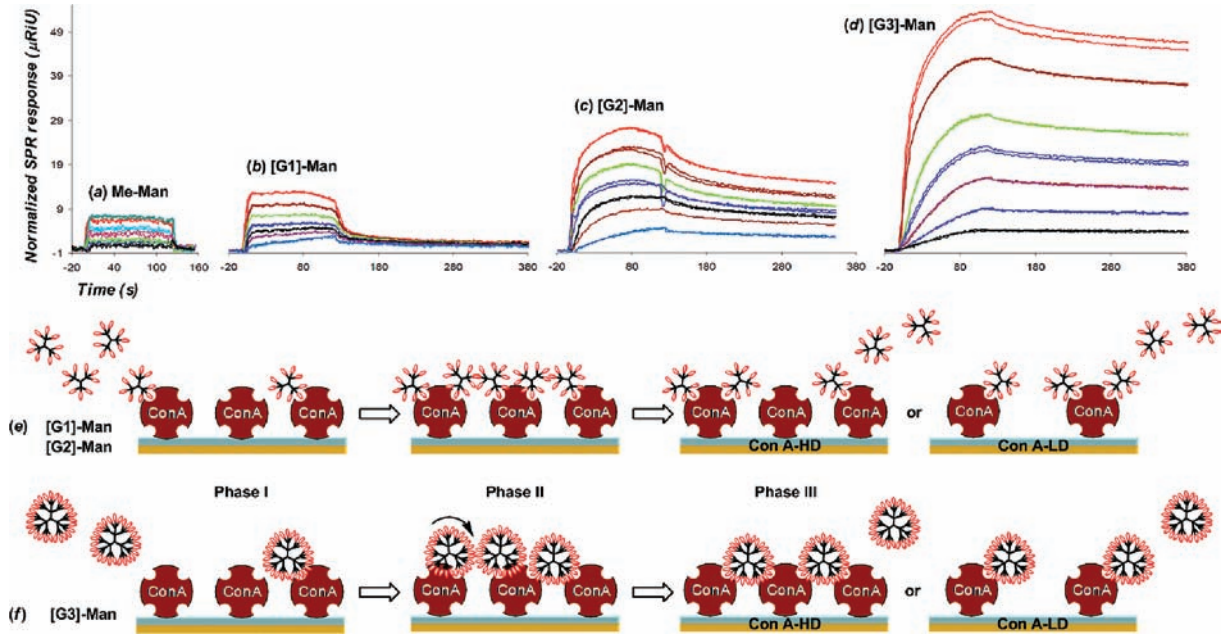

Figure 3. Sensorgrams of the interaction of Con A-HD (6900 μRiU) with (a) Me-Man (400−6.3 μM), (b) [G1]-Man (33.3−0.83 μM), (c) [G2]-Man (11.3−0.095 μM), and (d) [G3]-Man (3.0−0.012 μM). SPR responses have been normalized to represent the number of mannoses in close proximity to surface. (e,f) Schematic representation of direct surface-bound experiments between Con A clusters and mannosylated glycodendrimers.

Table 1. Binding Data from Direct SPR Assays: K_D (μM)

Analyte	Con A-HD (6900 μRiU)		Con A-LD (1100 μRiU)	
	Low affinity	High affinity	Low affinity	High affinity
Me-Man[a]	85.5			
[G1]-Man[b]	50	0.47	88	1.1
[G2]-Man[b]	30	0.40	49	1.4
[G3]-Man	5-30[b]	0.07[b]		0.84[a]
[G3]-Man[c] kinetic analysis	0.078 k_{on} 2.9 10^5 k_{off} 2.3 10^{-2}	0.002 k_{on} 1.2 10^5 k_{off} 2.4 10^{-4}		

[a] Steady state analysis fitting to a 1:1 Langmuir model. [b] Steady state analysis fitting to a heterogeneous two-site model. [c] Kinetic analysis fitting to a heterogeneous two-site model (k_{on} in $M^{-1}s^{-1}$; k_{off} in s^{-1}).

off rates that were too fast to accurately determine kinetic constants (Figure 3a). In the steady state analysis, the binding isotherm fitted well to a 1:1 Langmuir model, yielding a K_D of 85.5 μM (Table 1 and Figure S2). This value agrees well with affinity data determined by other methods,[13,15] and to the best of our knowledge it constitutes the first SPR direct binding assay of a carbohydrate with the molecular weight below 200 Da. Similarly, when the binding affinity of [Gn]-Man was investigated, a higher avidity and a much more complex binding profile were observed, consistent with the multivalent nature of the dendrimers and the clustered arrangement of the lectin (Figure 3b−d). The association phases of [Gn]-Man sensorgrams exhibited pronounced slopes indicative of fast-rate bindings. However, major differences occurred at the dissociation rate that decreased dramatically with generation. Two-step dissociation processes resulted, with an initial fast signal decay followed by a slower one at longer dissociation times (with rates at least 10^2-fold lower than those for the Con A-Me-Man complex).[16]

In the equilibrium analysis, the binding isotherms of [Gn]-Man sensorgrams fitted well to a two-site binding model, indicative of a fraction of the glycodendrimers binding Con A-HD with nanomolar affinity and the remaining dendrimers with micromolar affinity. Noteworthy, affinity increased with generation, with [G3]-Man being the best binder studied (Table 1 and Figure S2).[17]

Attempts to fit [G1]-Man and [G2]-Man sensorgrams to a two-site heterogeneous ligand kinetic model failed, mainly because dendrimers dissociate slower than predicted from the theoretical curves. However, a reasonably good fitting was obtained for [G3]-Man at 12−192 nM, where the slow dissociation profile prevails. This fitting revealed again two binding modes characterized by similar fast on rates but remarkable different off rates (Table 1 and Figure S3). The much lower K_D resulting from this kinetic analysis compared to the steady state analysis suggests that an additional stabilization by rebinding is occurring during the dissociation phase. In addition, the sensorgrams at the lowest concentrations of [G3]-Man (12−24 nM) fitted well to a kinetic 1:1 Langmuir model, yielding a K_D of 1 nM (3100-fold affinity enhancement on a per sugar basis relative to Me-Man), in agreement with the prevalence of the high affinity binding mode at low analyte concentrations.

The normalized SPR sensorgrams of the four analytes injected at approximately the same concentration on a per-sugar basis revealed an increased relative accumulation capacity of glycodendrimers on the surface at higher generations (2.0- and 5.1-fold higher for [G2]-Man and [G3]-Man at the steady state, relative to [G1]-Man) (Figure 3b−d). Interestingly, the differences in accumulation capacity increased during the dissociation phase (% of remaining glycodendrimers on the surface after 230 s of dissociation relative to the steady state: 15% for [G1]-Man, 56% for [G2]-Man, and 87% for [G3]-Man). This much higher affinity of [G3]-Man relative to [G2]-Man contrasts with the results obtained in solution (Figure 2) and reflects the importance of lectin clustering in the binding. Also, relative and absolute affinity data between series of multivalent ligands toward receptors clustered on biological surfaces must be carefully interpreted from experiments in solution and better determined from surface-based experiments.

The binding of [Gn]-Man was also studied on a lower density surface of Con A (Con A-LD, 1100 μRiU) (Table 1 and Figure S4).[17] It was found that the three generations of dendrimers bind to Con A-LD with faster dissociation rates and weaker affinities than to Con A-HD.[9] The drop in affinity for [G1]-Man and [G2]-

Man was moderate (~3.0-fold lower than to Con A-HD in the equilibrium analysis), while a 12.0-fold lower affinity was obtained for [G3]-Man. The same trend resulted when comparing the dendrimer accumulation capacities of [Gn]-Man on both surfaces at the steady state and during dissociation. These affinity data have been interpreted considering that [G1]-Man and [G2]-Man are smaller than the average interlectin distance in both surfaces, while [G3]-Man is smaller for Con A-LD but larger for Con A-HD (Figure 3e,f).[18] So, the size of each dendrimer determines a threshold of interlectin distance (cluster density). For larger distances, statistical or local concentration effects, also referred to as "bind and slide" (associated to the efficient recapture of glycodendrimers on lectin clusters), stabilize the binding more strongly the higher the cluster density is ([G1]-Man, [G2]-Man, and [G3]-Man toward Con A-LD).[2] Shorter distances result in dendrimers benefitting from cross-linking lectins as an additional source of greater stabilization ([G3]-Man toward Con A-HD).[18] Therefore, for the correct evaluation of absolute affinities toward surface-bound receptors, cluster density should be selected to mimic as much as possible the biological environment. In our opinion, this match between glycoconjugate size and lectin density is envisioned as a potential source of selectivity with biomedical implications (drug delivery and polymer therapeutics), as a large number of transmembrane receptors are clustered and many of them are found in lipid rafts forming microdomains on cell surfaces.[19]

In light of these results, the binding of [Gn]-Man to Con A clusters has been interpreted as resulting from an initial fast on-rate binding, comparable to the monosaccharide (Figure 3e,f, Phase I). Subsequent competition between dendrimers for the establishment of stronger interactions results in two limiting binding modes: (i) a low affinity binding with the K_D in the range of the monosaccharide, associated with dendrimers binding the Con A surface monovalently, and (ii) a high affinity nanomolar binding mode, associated with dendrimers binding the surface with higher functional valency (statistical effects and cross-linking) (Phase II). The high affinity binding mode prevails at low concentrations and during the dissociation phase, as dendrimers binding with low affinity are rapidly expelled out of the lectin surface, leaving free receptor sites to be occupied by higher functional valency dendrimers (Phase III).[20] This results in an enhanced stabilization governed by rebinding favored by lower entropic costs. Accordingly, glycodendrimers rebinding a lectin cluster could be visualized as a rack and pinion biosystem of dendritic glycopinions sequentially engaging their carbohydrate teeth on a lectin rack.

To summarize, SPR binding experiments illustrate the relevance of lectin density for the reliable evaluation of binding efficiencies in surface-based multivalent carbohydrate recognition. The difference between affinity data obtained by solution and surface-based experiments is also stressed.

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Supporting Information Available: Materials, experimental procedures, and data analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) The k_{off} of the slow process was estimated by fitting the dissociation phase of the sensorgrams monitored beyond 250 s to a 1:1 binding model. Since the Con A-Me-Man dissociation kinetics is out of the SPR detection limit, a value of $k_{off} \geq 10^{-1}$ s^{-1} was considered.

(17) In the case of [G3]-Man, the data points of the binding isotherm describing the low affinity binding mode were scarce due to solubility problems. Therefore, the $K_{D,low}$ for Con A-HD is best described by a range of values, while, for Con A-LD, the binding isotherm fitted better with a 1:1 Langmuir model.

(18) The $K_{D,high}$ value of 2 nM for [G3]-Man towards Con A-HD supports this scenario. Thus, according to the equation $[K_N^{poly} = (K^{mono})^{\alpha N}]$ proposed by Whitesides (ref 2d), an $\alpha N = 2.14$ results, indicative of a bivalent binding mode. See also: Wolfenden, M. L.; Cloninger, M. J. *J. Am. Chem. Soc.* **2005**, *127*, 12168.

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