Synthesis and Biophysical Study of Disassembling Nanohybrid Bioconjugates with a Cubic Octasilsesquioxane Core

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Polyhedral oligosilsesquioxanes (POSS) have recently attracted attention as scaffolds for the synthesis of multivalent bioconjugates. The synthesis of glycosyl-octasilsesquioxanes (glyco-POSS) using a copper(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition approach is reported. The problems associated with the use of bases or aqueous media in their preparation are investigated and a comprehensive study of the multivalent interaction between the mannosyl-octasilsesquioxanes and a model lectin, concanavalin A (Con A), using an array of complementary biophysical techniques is presented. The possibility to modulate the half-life of POSS conjugates in aqueous solution and the low toxicity of their constituent monomeric organosilanes offers an advantage over other scaffolds in vivo, preventing bioaccumulation and saturation of complementary receptors (lectins). Despite the hydrolysis in water, the octamannosyl-POSS studied shows a 50-fold higher binding affinity to Con A than methyl α-D-mannopyranoside. These experiments suggest that the novel glyco-POSS are attractive compounds for in vivo applications that require multivalent display of glycans.

1. Introduction

A paradigm of multivalent interactions[1] is the reversible binding of carbohydrates to their protein receptors (lectins), which is involved in many important biological pathways such as immune regulation, cell-cell and cell-pathogen recognition, or cell-growth regulation.[2] Structurally well-defined multivalent glycoconjugates are valuable tools in glycoscience[3] for the study of carbohydrate-protein recognition processes and have potential therapeutic applications in the prevention of early adhesion of pathogens to host epithelial cells,[4] the neutralization of viruses and toxins,[5] the preparation of vaccines,[6] and in carbohydrate-guided targeted drug delivery.[7] Synthetic multivalent glycoconjugates are commonly constructed by attaching a number of glycotopes to a suitable supporting scaffold.[8] A variety of scaffolds have been used for this purpose, including small organic molecules such as pentaerythritol,[9] cyclodextrins,[10] calixarenes[11] or cucurbiturils,[12] dendrimers,[13] polymers,[14] peptides,[15] nucleic acids,[16] and different types of nanoparticles such as carbon nanotubes,[17] fullerenes,[18] quantum dots,[19] gold nanoparticles,[20] magnetic nanoparticles,[21] polymer nanoparticles,[22] and silica nanoparticles.[23] Nanoparticle-based glycoconjugates are particularly appealing because they are generally easy to assemble and a high degree of multivalency (number of glycotopes per particle) can be readily obtained. However, glyconanoparticles have a number of drawbacks in biomedical applications due to their generally ill-defined structures, potential cytotoxicity, low biodegradability and ensuing bioaccumulation.[24]

Polyhedral oligosilsesquioxanes (POSS) have recently joined the arsenal of scaffolds used in the synthesis of multivalent glycoconjugates. POSS are a unique group of hybrid organic-inorganic compounds with the general formula $[RSiO_{1.5}]_n$ (R = organic substituent).[25] The chemistry of POSS has undergone rapid development in the past decade due to their potential applications in areas as diverse as polymers, nanocomposites, optoelectronic materials, liquid crystals, drug carriers, metal catalysts, and cosmetics. POSS present several advantageous features over other inorganic or organic nanobuilding blocks due

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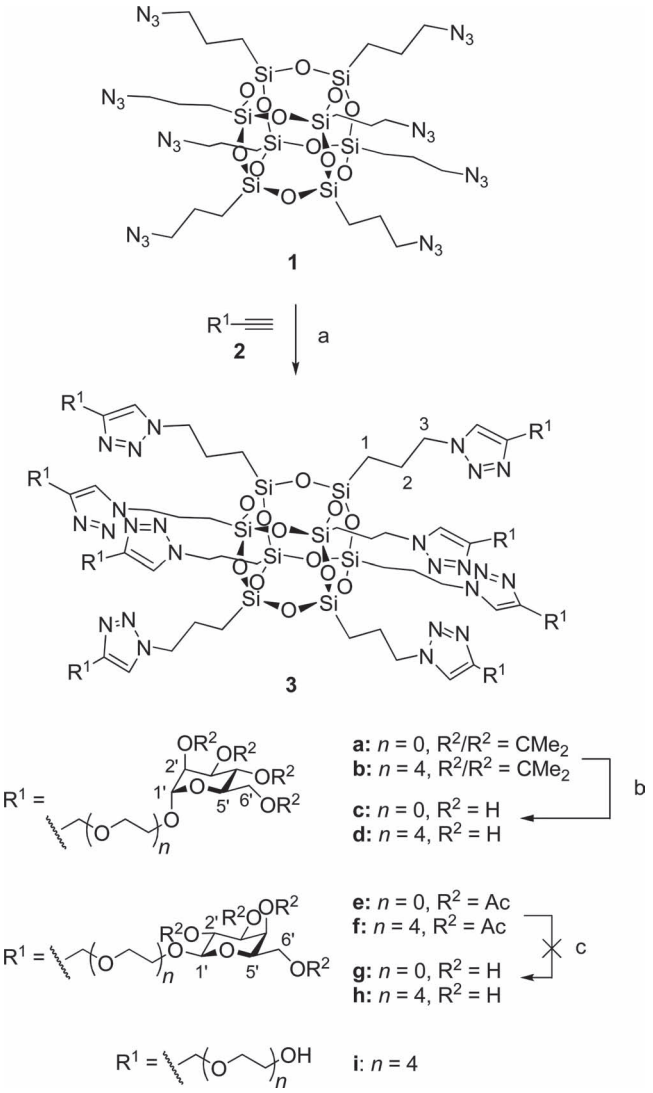
to their facile synthesis and functionalization, high symmetry, nanometer size, and high thermal stability. The most promising POSS nanobuilding blocks are the highly symmetrical and topologically ideal cube-octameric frameworks (T_8), of general formula type $(RSiO_{1.5})_8$.[26] The cubic symmetry of their rigid inorganic framework situates an organic substituent in each octant of Cartesian space, exposing it to interaction with other molecules.[27] This allows the synthesis of clusters and dendrimers with a pseudo-spherical symmetry with smaller generation numbers than conventional cores. In addition, POSS-core dendrimers show an enhanced entrapping ability compared to other dendrimers,[28] a desirable property in drug delivery applications. Due to their low toxicity and high biocompatibility, POSS derivatives and their nanocomposites are being actively developed as high-performance materials for biomedical applications.[29]

Since POSS compounds are formed via hydrolysis-condensation processes, they potentially suffer hydrolysis in aqueous media. This is a unique feature of these compounds compared to other known nanotechnology scaffolds, which could be advantageous for potential therapeutic applications, in that the toxicity and bioaccumulation problems presented by other scaffolds could be avoided. However, available information on the stability toward hydrolysis of water-soluble POSS derivatives is very scarce[27c,30] because most known POSS compounds are insoluble in water. The observed instability of some POSS compounds under physiological conditions and the known low toxicity of their breakdown products, monomeric organosilanes, implicate the suitability of using these compounds for in vivo applications. The degradation of the silsesquioxane cage into smaller POSS fragments may allow a more rapid clearance from the body.[30b]

Cube-octameric POSS have already been used as central platforms for the construction of glycoclusters (glyco-POSS) through three different functionalization reactions: 1) amide bond formation of carbohydrate-derived lactones with octakis(3-aminopropyl)octasilsesquioxane as starting POSS;[27c,30a] 2) photoaddition of carbohydrate-functionalized thiols to octavinyloctasilsesquioxane;[31] and 3) copper(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) of carbohydrate-funtionalized alkynes with octakis(3-azidopropyl)octasilsesquioxane.[32] While some of these glyco-POSS exhibit selective and reversible complexation to carbohydrate-binding proteins,[30a,31,32c] detailed binding studies have not been reported.

Herein we report the synthesis of a series of novel glyco-POSS compounds using a CuAAC approach, as well as a detailed study of their specific binding interactions with a model plant lectin using an array of complementary biophysical techniques. We assessed the stability of these glyco-POSS compounds in aqueous solution under various pH and buffer conditions, as well as the resulting impact on lectin binding.



Scheme 1. Reagents and conditions: a) cat. $CuSO_4{\cdot}5H_2O$, sodium ascorbate, CH_2Cl_2/H_2O (1:1), rt, 2-3.5 h (**3a**, 85%; **3b**, 80%; **3e**, 77%; **3f**, 81%; **3i**, 75%). b) TFA, THF-H_2O (4:1), rt, 12 h; or 80% AcOH in H_2O, rt, 12 h (**3c**, 78%; **3d**, 80%). c) cat. K_2CO_3, MeOH, rt, 3 h (90-98% fully deacetylated product); or NH_3 in MeOH, rt, 3 h (92-99% fully deacetylated product); or cat. KCN, MeOH, rt, 12 h (96% fully deacetylated product); or Mg/MeOH, rt, 48 h (77% fully deacetylated product).

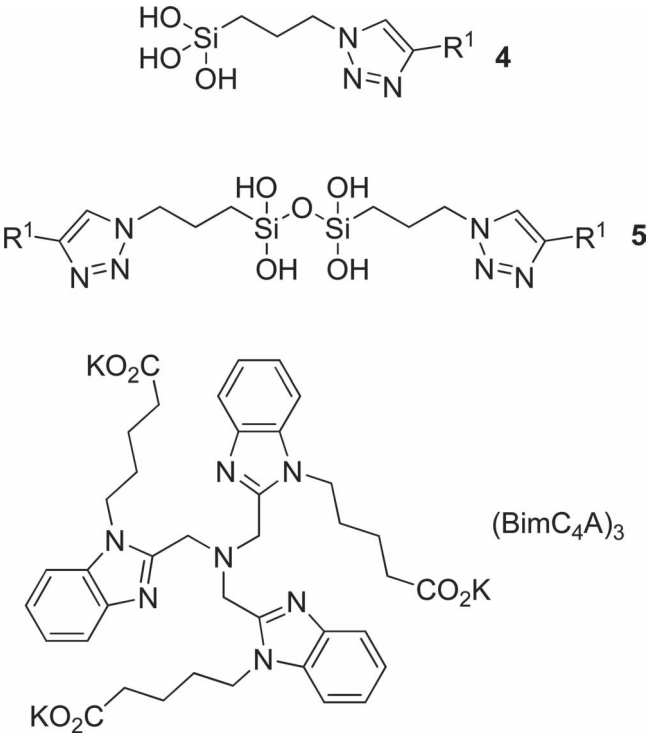
2. Results

2.1. Synthesis of Glyco-POSS Compounds

We have recently described[32a] a mild synthesis of octakis(3-azidopropyl)octasilsesquioxane (**1**) from commercially available octakis(3-aminopropyl)octasilsesquioxane via an efficient diazo-transfer reaction with nonafluorobutanesulfonyl azide.[33] Compound **1** proved to be an excellent nanobuilding block for the efficient synthesis of new functional cubic POSS through CuAAC reaction[32] including acetal-protected α-D-mannosyl-POSS **3a**,[32a] which could be readily deprotected under mild acidic conditions to afford glyco-POSS **3c (Scheme 1)**.[32a] We have now used the same strategy to synthesize the new compound **3d**. The glyco-POSS containing peracetylated β-D-galactose substituents (**3e**,[32c] **3f**) were synthesized under the same optimized CuAAC conditions (cat. $CuSO_4 \cdot H_2O$, sodium ascorbate in CH_2Cl_2/H_2O 1:1 at room temperature) using the appropriate alkyne-functionalized carbohydrate derivatives (**2**) with linkers of different chain length between the anomeric carbon and the

terminal alkyne group (Scheme 1). The ^{1}H, ^{13}C and ^{29}Si NMR spectra of the new hybrid glycoclusters, which showed a single set of signals in each case, together with the HRMS and/or MALDI-TOF data (see Supporting Information) fully confirmed the expected T_8 cubic structure of the new glyco-POSS.

Attemps to deprotect the peracetylated β-D-galactosyl-POSS derivatives **3e** and **3f** using a variety of basic reaction conditions (see Scheme 1) afforded mixtures of fully deacetylated products in all cases as observed from the ^{1}H NMR spectra of the crudes. This result confirms that POSS are more stable to acid than they are to bases and nucleophiles, as previously described.[34] To avoid the post-click deprotection step and obtain the β-D-galactopyranosyl-POSS derivatives, we attemped the synthesis using the CuAAC reaction directly with the unprotected sugar alkynes **2c**, **2d**, **2g**, and **2h**. No reaction took place with the mannose derivative **2c** under the optimized conditions in a CH_2Cl_2/H_2O biphasic solvent mixture due to the insolubility of the starting polyhydroxylated alkyne in the organic phase. However, the starting octa-azide-POSS **1** was fully consumed after 3 hours with the same catalytic system when we used a homogeneous THF-H_2O (2:1) solvent mixture instead, in the presence or absence of $(BimC_4A)_3$[35] as Cu(I) ligand (**Scheme 2**). After gel filtration chromatography through Sephadex G-25 using water as eluent, a product was isolated in high yield. Surprinsingly the ^{1}H NMR spectrum of this product in D_2O (**Figure 1**A) showed two different sets of resonances: a set of well-resolved peaks superimposed on a different set of broad signals slightly shifted to higher field from the first one. The ^{29}Si NMR spectra did not show any discernible signal using similar concentration and acquisition times to those employed for **3c** and **3d** obtained by deprotection of **3a** and **3b** as explained above. However, the more sensitive ^{29}Si–^{1}H HMBC technique revealed the presence of a cross-correlation peak at –39.3 ppm in the ^{29}Si resonance scale (Figure 1B) that correlated exclusively with the well-resolved set of ^{1}H signals at 0.45 (CH_2Si) and 1.88 (CH_2CH_2Si) ppm (Figure 1). This ^{29}Si chemical shift is characteristic for organosilanetriols (T_0)[36] and, accordingly, it was assigned to compound **4c** (Scheme 2). The hydrolysis process was greatly accelerated by subjecting the solution to ultrasonic irradiation in a bath at 40 °C. After one hour of treatment, the set of



Scheme 2 Substituents R^1 are defined as in Scheme 1.

broad signals completely disappeared and only the signals of **4c** could be observed in the ^{1}H NMR spectrum in D_2O (Figure 1A). The ESI-HRMS analysis of this aqueous solution showed only the molecular ion corresponding to **4c** at m/z = 382.1299 $[M+H]^+$, confirming the above assignment. A similar result was obtained when alkynes **2d**, **2g** and **2h** were reacted with **1** under the same conditions. Compounds **4** should have formed through hydrolytic cleavage of the POSS cage, which may have taken place during the reaction, work-up and chromatographic purification stages. Hydrolysis of the cage should generate a mixture of silsesquioxanes of lower symmetry than the starting POSS.[37] As a result,

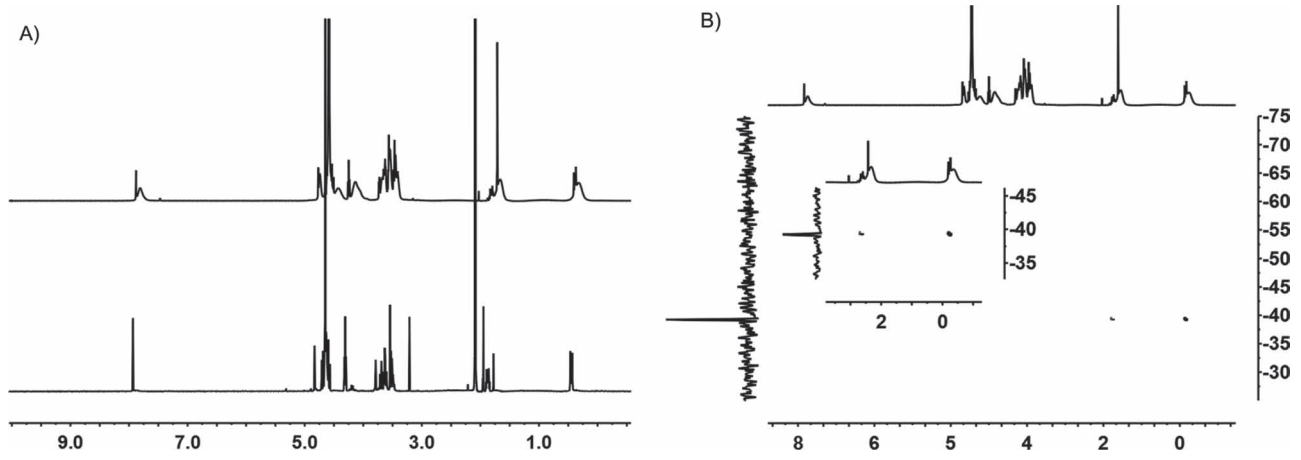


Figure 1. A) ^{1}H NMR spectra (in D_2O) of the product isolated in the CuAAC reaction of **2c** with **1** (top spectrum) and after treatment of the NMR sample with ultrasonic irradiation for 1 h at 40 °C (bottom spectrum). B) ^{29}Si-^{1}H HMBC spectrum of the product isolated in the CuAAC reaction of **2c** with **1**, showing only the signals corresponding to **4c** (insert shows an enlargement of ^{29}Si-^{1}H HMBC spectrum, 0–3 ppm).

a range of magnetically non-equivalent ^{29}Si NMR signals with poor intensities should be expected for this mixture, which would hamper their detection in a reasonable acquisition time.[38]

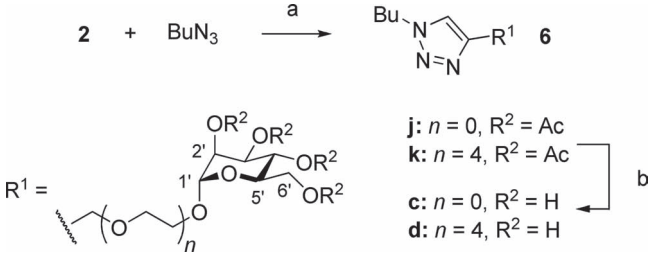
In summary, a successful synthesis of glyco-POSS from **1** requires the use of *O*-protected sugar alkynes that can be subsequently deprotected under non-basic and non-nucleophilic conditions to avoid cleavage of the POSS cage during the CuAAC reaction and during the final deprotection step.

2.2. Binding of D-Mannose Glyco-POSS to Concanavalin A

In order to evaluate the performance of the novel glyco-POSS as multivalent ligands for carbohydrate recognition proteins, we determined the binding interaction of α-D-mannopyranosyl glyco-POSS **3c** and **3d** with the model plant lectin concanavalin A (Con A),[1d,1g,39] which specifically recognizes α-D-mannopyranosides and, to a lesser extent, α-D-glucopyranosides. Con A exists as a homodimer at pH < 6, as a homotetramer above pH 7, and as an equilibrium mixture of dimer and tetramer at pH 6–7. The molecular weight of the monomer is 25.5 kDa, and each monomer contains one carbohydrate recognition domain, a calcium binding site and a transition metal binding site. For this study, we have used an array of binding assays including surface plasmon resonance (SPR), isothermal titration microcalorimetry (ITC), enzyme-linked lectin assays (ELLA), and a turbidimetric assay. Compounds **6c** and **6d**, readily prepared from 1-azidobutane as shown in **Scheme 3**, were also included in this study as monovalent models of **3c** and **3d**, respectively, lacking the POSS cage.

2.2.1. SPR

For the SPR binding assays, Con A was covalently attached to a polycarboxylated sensor chip (CM5) at pH 4.5 (6800 RU) using amine coupling reagents. With the same protocol, toxic shock syndrome toxin 1 (TSST-1) was immobilized at pH 5.5 (5400 RU) as a negative control, since this protein does not recognize α-D-mannopyranosides. Binding was evaluated by sequentially injecting increasing concentrations of compounds **6c**, **6d**, **3c**, and **3d** at pH = 7.4. Compounds **6c** and **6d** exhibited no detectable response signal due to their small molecular weight. Conversely, injections of glyco-POSS **3c** and **3d** (**Figure 2**A) showed clear binding responses. Affinity constants for both ligands were calculated using a general steady-state equilibrium model that assumes that the system achieved equilibrium during



Scheme 3. Reagents and conditions: a) cat. $CuSO_4{\cdot}5H_2O$, sodium ascorbate, CH_2Cl_2/H_2O (1:1), rt, 2 h (**6j**, 75%; **6k**, 77%). b) cat. K_2CO_3, MeOH, rt, 3 h (**6c**, 91%; **6d**, 90%).

sample injections (**Table 1**).[40] A plot of the response signal at equilibrium as a function of concentration for each glyco-POSS was adjusted to a hyperbolic equation from which steady-state association constants (K_A) could be obtained as shown in Figure 2A. The corresponding dissociation constants (K_D) for glyco-POSS **3c** and **3d** (Table 1, entries 2,3) were 3.59 and 3.15 μM, respectively, which are surprisingly similar despite the very different lengths of the linkers[41] connecting the carbohydrate ligand to the silsesquioxane inorganic cage in each case. However, these binding affinities are 25- and 30-fold higher for **3c** and **3d**, respectively, relative to that of methyl α-D-mannopyranoside determined also by SPR,[42] as a result of the multivalent effect.[1]

2.2.2. ITC

In order to derive thermodynamic parameters of the glyco-POSS/Con A binding interaction in solution, we conducted an isothermal titration calorimetry (ITC) study (Figure 2B). For the measurements, model compounds **6c** and **6d** and glyco-POSS **3c** and **3d** were titrated into a solution of Con A at pH = 7.4. ITC data for the binding of glyco-POSS were fitted using a single site model based on monomeric Con A. Figure 2B shows the titrations obtained for the complex formation of ligands **6c**, **6d**, **3c** and **3d** with Con A. The K_D values for the monovalent compounds **6c** and **6d** (**Table 2**, entries 2,3) were similar to those described in the literature[43–44] for methyl α-D-mannopyranoside using this technique (Table 2, entry 1). The stoichiometry (n) determined for the complex of the monovalent ligands with Con A was 1:1 (n = 1; i.e., each monovalent ligand is able to interact exclusively with one unit of Con A), as expected (Table 2, entries 2,3). In contrast, glyco-POSS **3c** showed an 80-fold higher binding affinity for Con A relative to its monovalent analog **6c** (Table 2, entries 2,4). The binding stoichoimetry for the **3c**/Con A complex formation was 1:4 (n = 0.25), indicating that each molecule of **3c** binds four monomers of Con A and, thus, behaves as a tetravalent ligand. On the other hand, glyco-POSS **3d**, with the longest linker between the carbohydrate and the silsesquioxane cage, exhibited a 255-fold higher binding affinity for Con A relative to its monovalent analog **6d** (Table 2, entries 3,5), which is 2.5-fold higher than that of glyco-POSS **3c** (Table 2, entry 4). For the **3d**/Con A complex, the binding stoichiometry was 1:8 (n = 0.12) that corresponds to one molecule of glyco-POSS **3d** binding to eight monomers of Con A, which is full occupancy of the carbohydrate units in the complex with protein molecules. It appears that for this glyco-POSS, the D-mannose units are positioned sufficiently far apart from each other as to accommodate eight complexed units of Con A without significant steric interference.

As in other cases of carbohydrate-protein recognition processes, we observed a linear relationship between the enthalpies and entropies of association determined for all compounds in Table 2, including methyl α-D-mannopyranoside (see Supporting Information). This extrathermodynamic relationship is commonly known as enthalpy-entropy compensation, a general and still poorly understood phenomenon observed in a wide variety of molecular processes including macromolecular interactions.[45] The accepted explanation for this compensation

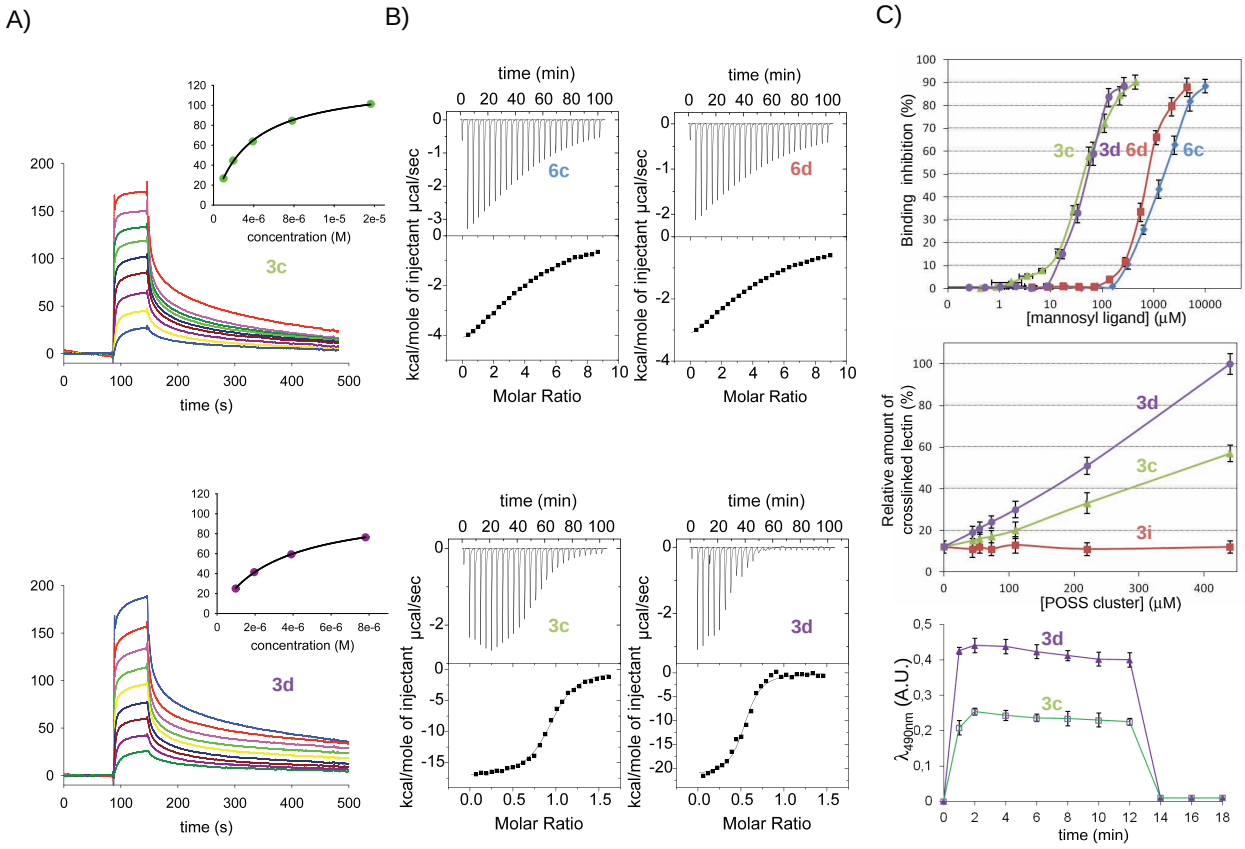


Figure 2. A) SPR sensorgrams of glyco-POSS **3c** (top) and **3d** (bottom) at different concentrations binding to immobilized Con A. Insets: equilibrium response as a function of glyco-POSS **3c** (top) and **3d** (bottom) concentration. B) ITC raw and integrated data for the binding interaction of **6c**, **6d**, **3d**, and **3c** with Con A. The experimental points are represented as filled squares and the best fit of these points to a one-site binding model is represented as a solid curve. C) Top: ELLA plots (logarithm scale) for the inhibition of Con A-HRP binding to yeast mannan by increasing concentrations of **3c** and **3d** in comparison with the monovalent ligands **6c** and **6d**. Values are expressed as mean ± SD (n = 3). Middle: Relative crosslinking efficiencies of **3c** and **3d** at different concentrations. The non-glycosylated POSS derivative **3i** was included as negative control. Bottom: Absorption changes of Con A (1 mg·mL^{-1}) at 490 nm upon addition of **3c** and **3d**. α-D-Mannose (100 mM) was added to both mixtures after 12 min.

Table 1. Binding parameters of glyco-POSS/Con A complexes as determined from SPR measurements.

Entry	Ligand	K_D [µM]	$\chi^{2b)}$
1	α-D-MeMan[a)]	85.5	—
2	**3c**	3.59	0.7
3	**3d**	3.15	0.16

[a)]Ref. [42]; [b)]Pearson's chi-square test.

in the case of macromolecular interactions is that increases in the enthalpy of association necessarily follow a decrease in the degrees of freedom upon formation of the complex, which, in turn, imparts an entropic cost.

2.2.3. ELLA

In order to get further information on the mechanisms involved in the multivalent recognition of glyco-POSS ligands by Con A, we have additionally performed enzyme-linked lectin

Table 2. Binding stoichiometries and thermodynamic parameters of carbohydrate/Con A interactions at 25 °C measured by ITC.

Entry	Ligand	$\Delta H^{a)}$	$T\Delta S^{a)}$	$K_D{}^{b)}$	$n^{c)}$	$\Delta G^{a)}$
1	α-D-MeMan[d)]	−8.4	−2.8	83	1.0	−5.6
2	**6c**	−6.16 ± 0.21	−0.78 ± 0.23	102 ± 0.02	1.00 ±< 0.01	−5.38 ± 0.02
3	**6d**	−5.19 ± 0.02	0.04 ± 0.04	133 ± 0.1	1.01 ± 0.01	−5.15 ± 0.06
4	**3c**	−16.12 ± 1.23	−8.25 ± 1.25	1.26 ± 1.8	0.25 ± 0.03	−7.87 ± 0.02
5	**3d**	−22.32 ± 0.78	−14.09 ± 0.53	0.52 ± 3.3	0.12 ± 0.01	−8.23 ± 0.72

[a)]kcal mol^{-1}; [b)]µM; [c)]Stoichiometry of the glyco-POSS/Con A complex (referred to monomeric Con A); [d)]Ref. [43].

Table 3. ELLA data for binding inhibition of HRP-labeled Con A lectin to immobilized yeast mannan by glyco-POSS **3c** and **3d** in comparison with the monovalent model conjugates methyl α-D-mannopyranoside (α-D-MeMan), **6c** and **6d**.

Compound	IC_{50} [μM]	Relative potency	Man molar potency
α-D-MeMan	851 ± 25	1.0	1.0
6c	1974 ± 30	0.4	0.4
6d	775 ± 15	1.1	1.1
3c	42 ± 3	20	2.5 (5.8)[a]
3d	52 ± 3	16	2.0 (1.8)[a]

[a]Values in parenthesis are referred to the respective monovalent analogue **6c** and **6d**, respectively.

assay (ELLA) experiments. This test measures the ability of a soluble saccharide to inhibit the association between a labeled lectin (here Con A lectin labeled with horseradish peroxidase, Con A-HRP) and a ligand immobilized on the microtiter well (here a yeast mannan). The presence of the relatively large HRP protein label (40 kD) prevents two lectin moieties from approaching each other, resulting in 1:1 binding stoichiometries with the saccharide ligand.[10e,46] To discard interferences arising from the POSS scaffold, an analog sharing the general structure **3** but terminated in tetraethyleneglycol chains (**3i**) was synthesized (Scheme 1) and included in the evaluation experiments as a negative control.

IC_{50} values (**Table 3**), assumed to be proportional to the corresponding binding affinities, were calculated from the percentages of inhibition with up to eleven different concentrations of each saccharide sample (Figure 2C: top). No inhibition of the Con A-yeast mannan association was observed in the presence of the negative control **3i** at concentrations up to 600 μM. Under the same conditions, methyl α-D-mannopyranoside, used as a monovalent reference, gave an IC_{50} value of 851 μM. The monovalent model **6d** essentially matched this value (775 μM). In contrast, **6c** was recognized by Con A with a significantly lower affinity (IC_{50} value 1974 μM) pointing to a detrimental effect of the shorter aglycon. In any case, no significant differences were noted when comparing the octavalent POSS conjugates **3c** and **3d**, which exhibited IC_{50} values of 42 and 52 μM, respectively, meaning binding affinities 20- and 16-fold higher as compared to methyl α-D-mannopyranoside. A more accurate evaluation of the intrinsic multivalent effect can be obtained by taking the respective monovalent model **6c** or **6d** as references and correcting the affinity enhancements on a mannose molar basis. Thus, each mannosyl residue in **3c** is recognized by Con A-HRP with a 6-fold higher efficiency as compared to the mannosyl ligand in **6c**, whereas the ratio is only 2-fold for the **3d**/**6d** pair (Table 3). This result is consistent with a scenario in which the local concentration of the monosaccharide motif, expected to be higher for the more compact arrangement provided by the shorter spacer arm in glyco-POSS **3c**, is the critical parameter contributing to the observed affinity enhancements.

To evaluate the lectin clustering abilities of **3c** and **3d** toward Con A lectin, a two-site "sandwich" ELLA experiment was performed. Unlabeled (therefore crosslinkable) Con A was first adsorbed onto a microtiter well coated with yeast mannan. Pre-formed complexes of **3c** and **3d** at different concentrations with HRP-labeled Con A were then added. The plot of the relative amounts of bound Con A-HRP as a function of glyco-POSS concentration is presented in Figure 2C (middle panel), with the maximum for compound **3d** at the higher concentration (440 μM) set as 100%. The crosslinking ability of compound **3d**, having the longer linker, was found to be approximately twice that of compound **3c**, likely indicative of the tetra(ethylene glycol) chain providing the optimal length to overcome unfavorable steric interactions and to allow two lectin molecules to approach.

2.2.4. Turbidity Assay

Many signaling events are dependent on the rate of ligand binding and the rate of multivalent ligand-induced receptor clustering. To test whether the differences in simultaneously binding two Con A lectin molecules by the octavalent POSS-conjugates **3c** and **3d** as a function of the spacer length correlated with their relative capacity to promote the formation of three-dimensional aggregates, a kinetic turbidimetry assay was carried out. Turbidity measurements can be used to monitor the formation of cross-linked complexes in real time.[47] For that purpose, the ligands were added to a solution of tetrameric Con A in PBS (pH 7.3) and the turbidity of the mixture was scanned.[48] The initial rate of precipitation (V_i) was determined by linear fits of the initial portion of the data (Figure 2C, bottom panel). The obtained values (0.208 and 0.426 A.U.$\cdot$min^{-1} for **3c** and **3d**, respectively) were consistent with roughly the capacity of the conjugate with the longest linker **3d** to aggregate Con A twice as much as that of the analogue **3c**. Control experiments with the monovalent model compounds **6c** and **6d** as well as with the non-glycosylated POSS derivative **3i** showed no precipitation of the lectin under identical conditions. In order to confirm that Con A aggregation by the multivalent conjugates **3c** and **3d** was mannose-dependent, excess α-D-mannose was added to the mixture after 12 min, which led to the instantaneous disruption of the aggregates (Figure 2C, bottom panel).

2.2.5. Structure of Concanavalin A/Glyco-POSS Complexes

We determined the X-ray crystal structure of the glyco-POSS **3d**/Con A complex at 1.7 Å resolution (**Figure 3**A).[49] The asymmetric unit was found to contain tetrameric Con A. Each monomer consists of two antiparallel β-sheets and two metal binding sites, which are necessary for carbohydrate binding. We found one unit of D-mannopyranose (from glyco-POSS **3d**) bound to each monomer (Figure 3B).[50] However, no electron density that would correspond to the silsesquioxane T_8 cage or the tetraethylenglycol linker was observed, either due to flexibility of compound **3d** or to hydrolysis of the cage during the course of crystallization (see below). Comparison of this structure with that of Con A/methyl α-D-mannopyranoside[51] reveals similarities in the position of carbohydrate-protein hydrogen bonds: the primary hydroxyl group OH-6' is hydrogen bonded to Tyr100 and Asp208, OH-4' to Asn14, OH-3' to Arg228, the ring oxygen (O-5') to Leu 99 and OH-2' to Thr226 through a water molecule (Figure 3C).

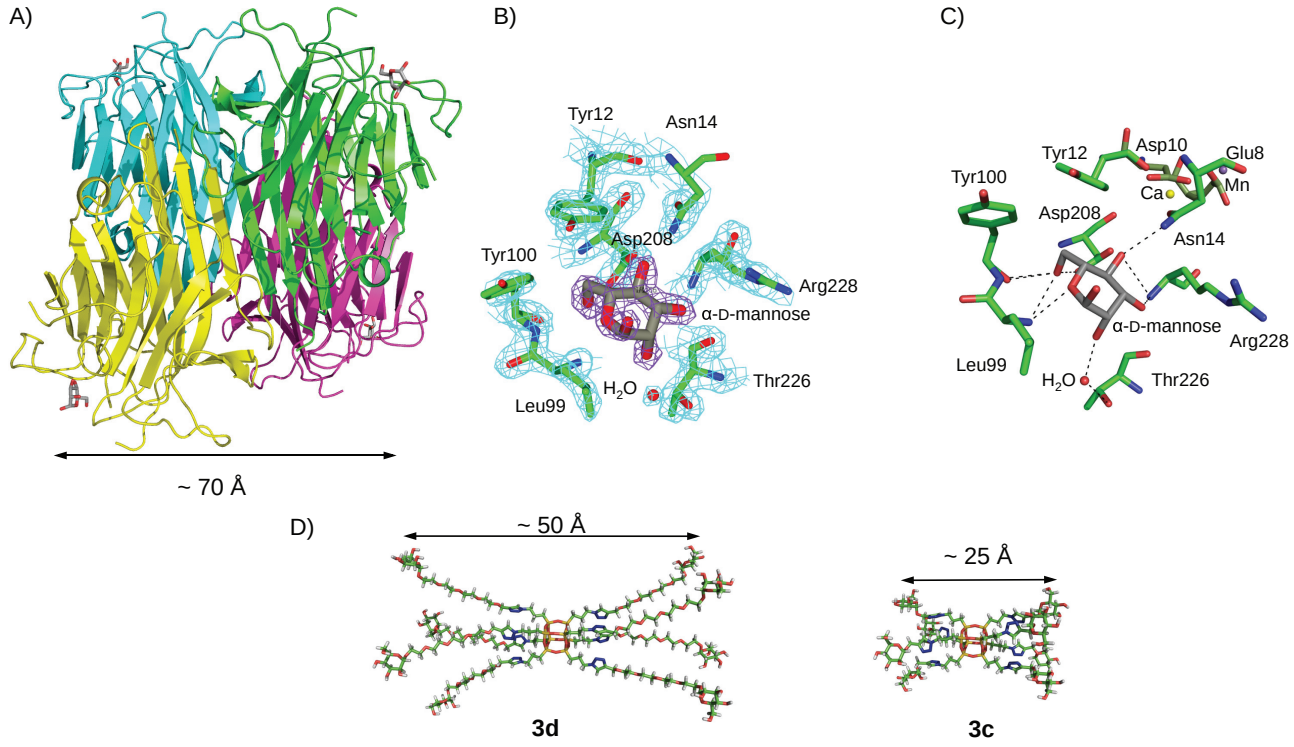


Figure 3. A) Cartoon representation of the tetrameric Con A bound to four mannose moieties of glyco-POSS **3d** (shown as sticks) found in the asymmetric unit of the crystal solved to 1.7 Å. Con A subunits are coloured yellow, green, cyan and magenta while mannose moieties are shown in grey. B) Structure of the Con A-lectin binding site. A 2Fo-Fc σA-weighted electron density map contoured at 1.5 σ of interface residues. C) Stick figure representation of the mannose (grey) binding site on Con A (green). D) Distance between mannoses (measured from the anomeric carbons) in molecular models of glyco-POSS **3c** and **3d** in stretched out conformations calculated using the AM1 semiempirical quantum-mechanical method with full geometry optimization.

2.3. Hydrolysis of Glyco-POSS in Aqueous Media and its Impact on Lectin Binding

As noted, the new glyco-POSS are susceptible to hydrolysis during their synthesis from unprotected sugar alkynes and upon final deprotection under basic conditions. Compound **3d** was chosen as a model to study the stability of glyco-POSS in aqueous media and the implications of hydrolytic processes in the binding interaction with Con A. Solutions of **3d** (8 mM) under different pH conditions and buffer compositions were used to evaluate how these factors affected the hydrolysis process. We used 60 mM phosphate buffer at pH 6.35 and 7.28; and 10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$ at pH 6.55 and 7.20. The samples were analyzed by ^{1}H-^{29}Si HMBC experiments at different time periods after dissolving **3d** in buffer. In phosphate buffer at pH 6.35, the typical ^{29}Si NMR resonance of the T_8 POSS cage of **3d** (–66.6 ppm) was greatly reduced after 24 h incubation at room temperature and a new signal appeared at –39.9 ppm that we assigned to the fully hydrolyzed species **4d** (**Figure** 4A). In the same buffer at pH 7.28, the signal for the T_8 cage diminished much faster, as expected.[34] After 24 h, the T_8 resonance was completely substituted by the resonance at –39.9 and a new smaller signal at –45.9 ppm, which we assigned to silanetriol **4d** and its condensation product disiloxane **5d** (see Scheme 2), respectively, with the two forms in equilibrium (Figure 4B).[30b,36] The experiment was repeated with HEPES buffer collecting spectra at smaller time intervals. Hydrolysis was slower in this buffer than in phosphate buffer at similar pH values (Figure 4A and 4C). The ^{29}Si NMR resonance of the T_8 POSS cage of **3d** (–66.6 ppm) was still the most prominent resonance 12 hours after the preparation of the solution in HEPES buffer at pH 7.20 at 25 °C, which was used in our biophysical studies with Con A. Hence, the octa-triazolyl glyco-POSS are unstable in aqueous media, the stability depending on both the pH (hydrolysis is faster at higher pH values) and the nature of the buffer (hydrolysis is faster in phosphate than in HEPES buffer). Other water soluble POSS compounds[27c,30a,31] have been reported to be more stable toward hydrolysis than our glyco-POSS. We suspect that the triazolyl groups in our glyco-POSS could be responsible for their accelerated hydrolysis. The observed moderate instability of **3d** under physiological conditions and the known low toxicity of monomeric organosilanes suggest the possibility of using these compounds for in vivo applications as multivalent ligands with programmed disassembling properties.

We also assessed the impact of the hydrolytic decomposition of glyco-POSS **3d** and its resulting hydrolysis products, on the interaction with Con A. To this end, **3d** was dissolved in HEPES buffer or PBS (ELLA experiments) at pH 7.4 at 25 °C and ITC, SPR and ELLA measurements were conducted at different incubation

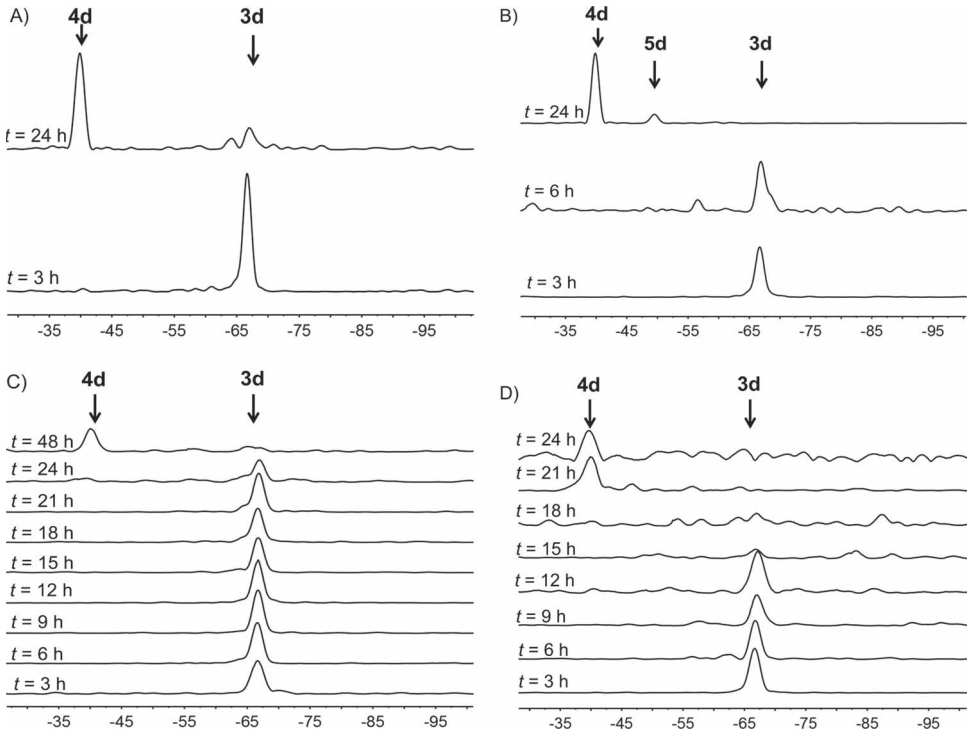


Figure 4. ^{29}Si resonance projections of ^{1}H-^{29}Si HMBC NMR spectra of glyco-POSS **3d** dissolved in phosphate buffer at pH 6.35 (A) and pH 7.28 (B), and in HEPES buffer at pH 6.55 (C) and pH 7.20 (D) as a function of time.

time points (0, 4.5, 9, 24, 48 and 72 h). Using ITC, we observed a marked decrease in binding affinity with time as hydrolysis of the POSS cage progressed (**Figure 5**A). The binding affinity after 48 h was 20-fold weaker than at 0 h and of the same order as those of the monovalent model mannosides **6c** and **6d**, as would be expected for a completely hydrolyzed POSS cage yielding monovalent mannosylsilanetriol **4d** (see Supporting Information). Using SPR, we did not estimate binding affinities at each time point, but we observed a significant decrease in binding response over time (see Supporting Information). The ELLA results exibited a progressive attenuation in the capacity of the samples to inhibit the association of the lectin to yeast mannan, as expected for a gradual decrease in the effective valency of the species in solution. As we observed by ITC, after 48 h the valence-corrected binding affinities relative to the monovalent models **6c** and **6d** were very close to unity, in agreement with the complete transformation of the octavalent glycoclusters **3c** and **3d** into low-valency or monovalent species. Parallel experiments using stock solutions kept at 4 °C for two days showed no differences with the initial capacity of the glyco-POSS to inhibit the association of the lectin to yeast mannan. The decreasing affinity can be traced to the lowering molecular valency of the glyco-cluster as hydrolysis progressed, in parallel with what we observed in the ^{29}Si NMR experiments in the same buffer system (see Figure 4C).

3. Discussion

Multivalency is a complex effect mediated by different mechanisms such as the chelate effect, statistical rebinding and cross-linking.[8,47,52] The biophysical studies presented here provide a structural interpretation of the observed glyco-POSS/Con A binding interaction under various experimental conditions. It is important to note that none of the glyco-POSS is able to simultaneously bind to different binding sites of the same Con A tetrameric assembly, since the distance between sites in the tetramer is larger than the maximum distance that could be attained between mannoses in a stretched out conformation of the glyco-POSS (Figure 3A,D). Thus, chelation cannot explain the observed enhanced binding of **3c** and **3d** to Con A, leaving only statistical rebinding and cross-linking as the likely operating mechanisms for the affinity enhancement[8b] in experiments in solution. The binding affinities of glyco-POSS **3c** and **3d** with Con A measured by ITC in solution were approximately 3- and 6-fold higher, respectively, than those obtained from the SPR analysis with immobilized Con A. Con A was immobilized to the chip as a homodimer at pH 4.5. Even though the SPR binding experiments were performed at pH 7.4, the formation of the homotetramer was likely inhibited by the immobilization process. This is the most probable reason for the higher K_D observed by SPR. Additionally, the difference in binding affinities between **3c** (short connector) and **3d** (long connector) were larger in the ITC experiments in solution than in the SPR analysis. These differences can be explained by the larger contribution of cross-linking phenomena to the multivalent effect in the ITC (lectin and ligand in solution) than in SPR (lectin-coated chip). ELLA data, on the contrary, provides information on the intrinsic multivalent effect for the glyco-POSS systems. That is, the affinity increase in this case is due to the interaction between a multivalent carbohydrate display and a lectin molecule with a

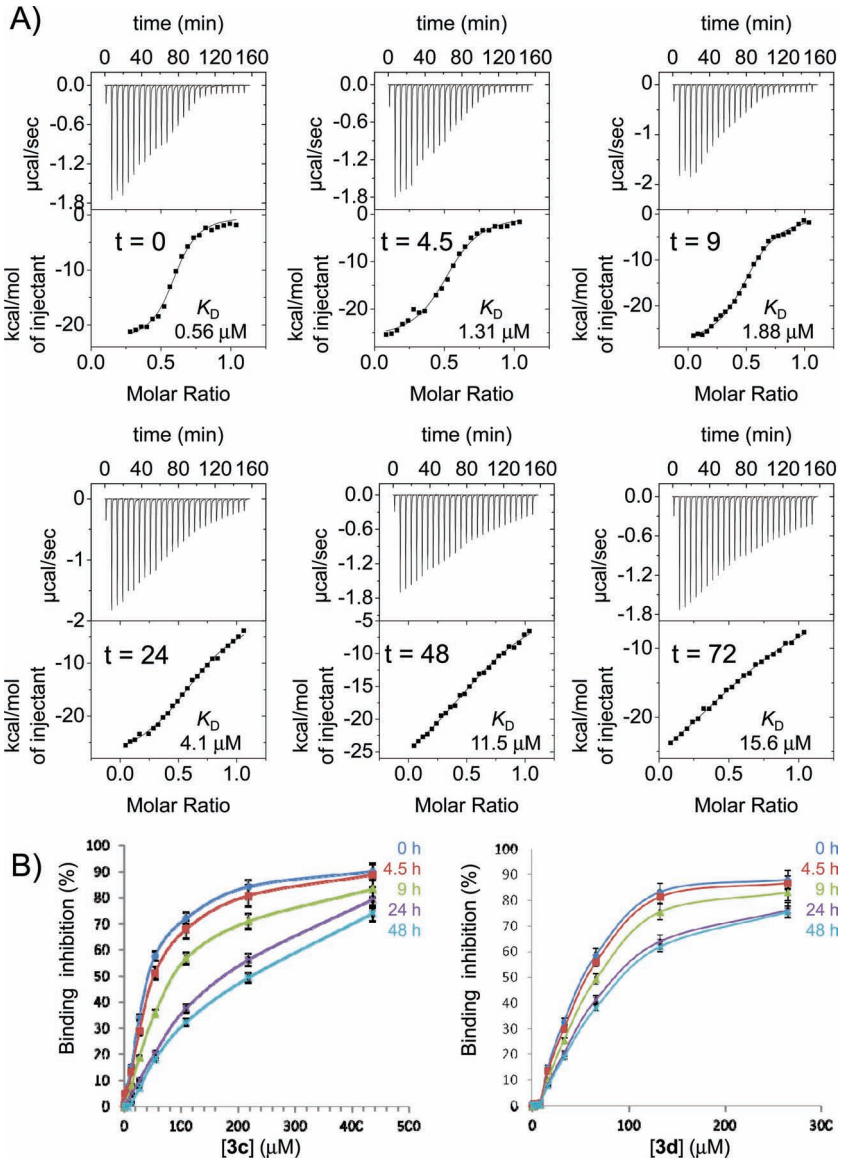


Figure 5. A) ITC raw and integrated data for glyco-POSS **3d** at time 0, 4.5, 9, 24, 48 h, and 72 h after dissolving the compound in HEPES buffer at pH 7.4. The experimental points are represented as filled squares and the best fit of these points to a one-site binding model is represented as a solid curve. B) ELLA plots for the inhibition of Con A-HRP binding to yeast mannan by increasing concentrations of octavalent mannosyl-POSS **3c** and **3d** recorded after stirring in PBS (pH 7.3) for different times. Values are expressed as mean ± SD (n = 3).

single binding site and, consequently, it is devoid of aggregation and lectin association phenomena.[46] The results indicate that the more compact arrangement of mannosyl residues in **3c**, which displays a higher mannosyl density as compared to **3d**, results in higher intrinsic affinities towards Con A. Yet, the capacity of the more flexible glycocluster **3d** to cluster two lectin molecules was higher, as evidenced by the two-site "sandwich" assay. Altogether, these results emphasize the need to combine different evaluation techniques in order to fully assess carbohydrate-lectin molecular recognition processes.[52]

The stoichiometry determined for **3d** in complex with Con A indicates that this compound offers a less sterically hindered binding to Con A than **3c** with its shorter connectors. This allows the formation of an octavalent complex in solution involving eight units of Con A per glyco-POSS, which generates a three-dimensional framework of crosslinked glyco-POSS **3d**/Con A complexes that have all of their binding sites involved in the formation of the network. The ΔG value expected for the octavalent glyco-POSS **3d**/ConA complexation, considering that the eight lectin/mannose interactions are independent of each other, should be approximately eight times that determined for the monovalent model **6d**. However, the theoretically expected value ($\Delta G = 8 \times (-5.15) = -41.2$ kcal·mol^{-1}) is significantly lower than that obtained experimentally for compound **3d** (–8.23 kcal·mol^{-1}). This indicates that glyco-POSS **3d** binds the lectin with negative cooperativity,[1b,53] where each successive binding of a new unit of Con A to glyco-POSS is less stabilizing with increasing degree of complex formation. This phenomenon appears to be general in multivalent complexes in the absence of a chelating effect.[52] The decrease in binding affinity with increasing degree of complexation is due to two factors: 1) the progressive loss of functional valence of the glycocluster (i.e., decrease in the effective concentration of ligand) as the glyco-POSS binds to a higher number of protein molecules and 2) the increase of destabilizing steric interactions in the supramolecular complex with the protein.[53]

As the ^{29}Si NMR experiments showed, unprotected glycosyl-octasilsesquioxanes are not stable in water over time. Our data indicate that the T_8 cage is slowly hydrolyzed to smaller organosilsesquioxane fragments under physiological conditions. Other water-soluble POSS compounds have been reported to be more stable toward hydrolysis than our glyco-POSS.[30b,31] It appears that the hydrolysis of POSS is not only affected by pH and buffer composition, but also by the nature of the organic group attached to the silsesquioxane core, which offers the opportunity to customize POSS systems with programmed half-life in aqueous solution. Despite the observed hydrolysis, the mannosyl-POSS are able to bind Con A with high affinity for a significant time frame, as indicated by our ITC and ELLA studies. Thus, the binding affinity measured by ITC for compound **3d** were 76- and 53-fold higher at 4.5 h and 9 h, respectively, than that of the monovalent analog **6d**. Hydrolytic disassembling is an important design aspect of silsesquioxane-based structures for in vivo applications, since degradation of the silsesquioxane cage into smaller POSS fragments may allow a more rapid clearance from the body of POSS conjugates (or their fragments) avoiding bioaccumulation.

4. Conclusions

Glycosyl-octasilsesquioxanes were readily synthesized from octakis(3-azidopropyl)octasilsesquioxane (**1**) using an efficent CuAAC octafunctionalization process. This approach requires the use of *O*-protected sugar alkynes that can be subsequently deprotected under non-basic and non-nucleophilic conditions to avoid cleavage of the POSS cage during the CuAAC reaction and during the final deprotection step. We have studied the interaction of the obtained mannosyl-octasilsesquioxanes with the model plant lectin Con A using a variety of complementary biophysical techniques including SPR, ITC, ELLA, turbidimetry, and X-ray crystallography. The data obtained from each technique provided unique information with which to understand this complex multivalent interaction. The differences between the K_D values obtained by ITC and SPR suggest that the major contribution to the multivalent effect observed in the ITC experiments in solution is the cross-linking mechanism, which was undetectable in the SPR experiments using the surface-immobilized lectin. The ^{29}Si NMR studies revealed the time-course of the hydrolytic decomposition of these compounds under various aqueous conditions, although this does not prevent their interaction with the lectin, as shown by ITC and ELLA experiments. The observed gradual disassembly of mannosyl-octasilsesquioxanes under physiological conditions and the expected low toxicity of the resulting monomeric organosilanes suggest that these hybrid organic-inorganic multivalent constructs are attractive systems for in vivo applications.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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