Multivalent Interactions between Lectins and Supramolecular Complexes: Galectin-1 and Self-Assembled Pseudopolyrotaxanes

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SUMMARY

Supramolecular chemistry has been employed to develop flexible and adaptable multivalent neoglycoconjugates for binding galectin-1 (Gal-1). Gal-1, a dimeric lectin with two galactoside-binding sites, regulates cancer progression and immune responses. Self-assembled pseudopolyrotaxanes consisting of lactoside-displaying cyclodextrin (LCD) "beads" threaded onto polyviologen "strings" display mobile ligands as a result of cyclodextrin rotation about, and limited translation along, the polymer chain. The pseudopolyrotaxanes rapidly and efficiently precipitate Gal-1 and provide valency-corrected enhancements of up to 30-fold compared to native lactose and 20-fold over free LCD in a T-cell agglutination assay. A supramolecular statistical effect was observed, wherein the efficacy of Gal-1 inhibition correlates with the number of ligands connected to each other solely through mechanical and noncovalent interactions. Such flexible and adaptable self-assembled pseudopolyrotaxanes show promise for the study of multivalent interactions and targeting of therapeutically relevant lectins.

INTRODUCTION

As the static, lock-and-key view of ligand-receptor complexes gives way to more dynamic models [1], the role of flexibility and adaptability in both one-to-one and multivalent binding events has emerged as a major question in the field of molecular recognition. Multivalent interactions [2, 3] are especially prevalent in glycobiology [4–7], where high-avidity interactions take place between multimeric and/or membrane-bound carbohydrate-binding proteins (lectins) and their cognate carbohydrate ligands (epitopes), despite the low affinity provided by a typical mono-

valent interaction between a lectin and its epitope. The fast on- and off-rates of the individual interactions and the fact that many multivalent protein-carbohydrate interactions take place at the fluid and dynamic interface of the cell surface [8] suggest that flexibility and adaptability may play important roles in these interactions.

Glycobiology and, in a more general sense, all of biochemistry are conceptually related to the field of supramolecular chemistry, through the shared focus on molecular recognition and assembly [9, 10]. However, few of the growing classes of synthetic supramolecular assemblies [3, 9–11], such as pseudorotaxanes (ring around a thread) and pseudopolyrotaxanes (many rings around a thread), and related mechanically interlocked molecules [11–13], such as rotaxanes (ring around a dumbbell-shaped thread), catenanes (ring threaded through another ring), and polyrotaxanes (many rings on a dumbbell-shaped component), have been examined in biochemical contexts [14–18]. Recently, we [19–21] and Yui and coworkers [22–25] have used supramolecular chemistry to develop architectures for multivalent binding that are at the flexible and adaptable end of the ligand-display spectrum.

Chemists have synthesized a large variety [26–36] of neoglycoconjugates ranging from small molecules to macro- and supramolecular entities in attempts to understand, mimic, and perturb natural, multivalent lectin-carbohydrate interactions. Factors [30–33] such as rigidity, spacing, topology, and density of saccharides are known to influence the avidity of multivalent, carbohydrate-displaying structures for their respective lectins. Lehn and coworkers have utilized dynamic combinatorial chemistry [34] to select trivalent neoglycoconjugates for the lectin concanavalin A. Other promising approaches that exploit multivalency for lectin binding include neoglycopolymers [4, 27, 29, 31] and dynamic self-assembled systems [26, 35, 36], such as micelles and liposomes. Carbohydrate-displaying (pseudo)polyrotaxanes [19–24] potentially combine advantages from both of these approaches for multivalent ligand presentation, including (1) the ability to span large distances, (2) easily variable ligand densities, (3) adaptability, and (4) ease of synthesis through self-assembly. Both our system [19–21] and those

of Yui and coworkers [22–24] are based on the pioneering work of Harada [37] and Wenz [38] on cyclodextrin (CD)-based (pseudo)polyrotaxanes (recently reviewed in [39]). CDs are naturally occurring, cyclic oligosaccharides comprised of six, seven, or eight glucopyranoside units. Although many of the exotic structures produced by supramolecular chemists are incompatible with aqueous media, CDs are well known to encircle hydrophobic molecules in water. In many cases, the encapsulated products, even large (pseudo)polyrotaxanes [39], retain water solubility. In such "beads-on-a-string" architectures, the CD rings are able to spin around the axis of the polymer chain as well as move back and forth along the polymer backbone. The extent of these movements can be varied by the choice of polymer chain [19, 24, 37–39]. If one considers a ligand attached to each CD ring, the position of this ligand is, to a large extent, independent of the position of a ligand on a neighboring ring; although the ligands are bound via noncovalent interactions of the CD rings with the encircled polymer backbone, they are not directly covalently bound to each other. Both ligands will have considerable freedom, including up to 360° of rotation, to respond to a receptor. Using lactoside-displaying α-CD rings and a polyviologen backbone, we have recently described a self-assembled, multivalent pseudopolyrotaxane [20] that was used to target galectin-1 (Gal-1) [8, 40–44], a bivalent galactoside-binding lectin.

Gal-1 is a soluble, 14 kDa dimeric lectin whose natural ligands typically, but not exclusively [45],reside on cellular membranes. Gal-1 binding mediates the organization [5, 6, 8] of cell-surface glycoproteins through crosslinking of terminal or polymeric N-acetyllactosamine residues. Gal–1 is the prototypical member of the galectins [44–49], a ubiquitous family of galactoside-binding lectins that mediate cell adhesion, signaling, and death. Certain galectins, particularly Gal-1 and Gal-3, are overexpressed in a variety of cancers [43, 44, 48, 49]. In addition to promoting motility and metastasis [46], certain cancer cells are able to use Gal-1 as a self-defense mechanism [42, 43] by taking advantage of the natural function [41, 47] of Gal-1 as an apoptotic signal for T- and other cells of the immune system. Topologically, Gal-1 presents a particularly interesting challenge for targeting with synthetic, multivalent ligands (recently reviewed in [44]) since it is a rigid dimer [40] with two binding sites oriented in opposite directions such that the entrance to each of the binding sites is located 6 nm apart. Since the biological targets of Gal-1 reside on the cell surface, one strategy for targeting Gal-1 is to use structures that mimic the fluidity and adaptability of cellular membranes.

In our original report [20], a pseudopolyrotaxane (Figure 1A) composed of a polyviologen chain with, on average, 17 repeating units, over 90% threaded with lactoside-displaying CDs (**LCD**s), proved effective at targeting Gal-1. The pseudopolyrotaxane displays highly mobile ligands as a result of CD rotation about, and limited translation along, the polymer chain. Multivalent enhancements of 6.7-fold and 10-fold relative to the free lactoside-CD and free lactose, respectively, were obtained for the

inhibition of Gal-1 in a T-cell agglutination assay. This valency-corrected enhancement was greater than that observed for lactoside-bearing, trivalent glycoclusters and a fully covalent (chitosan-based) polymer tested by using the same assay. Herein, we investigate these multivalent interactions between Gal-1 and lactoside-displaying supramolecular assemblies further by using T-cell agglutination and quantitative precipitation assays to evaluate pseudopolyrotaxanes with different degrees of threading and different polyviologen chain lengths.

RESULTS AND DISCUSSION

Supramolecular Assemblies

Polyviologen AB-copolymers, consisting of alternating decamethylene (A) and positively charged bipyridinium (B) segments, form [20, 37–39] stable, water-soluble complexes with α-CD, wherein the positive charges, associated formally with the nitrogen atoms on the bipyridinium segments of the polymer, act as electronic "speed bumps" [39, 50] that reduce the translational motion of the CD rings. In aqueous solution, the α-CD rings thread onto the polymer chain and rest predominantly on its decamethylene segments, stabilized by the hydrophobic interactions inside the cavities of the CDs. By separating the hydrophobic segments into domains flanked by "speed bumps," polyviologens can support high levels of threading at equilibrium, with the timescale of self-assembly being relatively slow (3–30 days), due to the necessity of the α-CD rings passing over multiple bipyridinium segments. For example, in the self-assembly of the pseudopolyrotaxane from our previous report [20], a mixture of 17 equivalents of **LCD** (20 mM) with a polyviologen containing, on average, 17 repeating units (**PV-17**) resulted in over 90% threading. This assembly is henceforth designated **[17:17]**, in which the number to the left of the colon indicates the equivalents of ring to thread in the solution (not necessarily on the polyviologen backbone), and the number to the right of the colon indicates the average number of polyviologen repeating units in the backbone (i.e., 17 for **PV-17**). The actual degree of threading (>90%) was estimated from ^{1}H-NMR spectroscopy and thin-layer chromatography. It was also reported that a 50-fold dilution resulted in no change in the ^{1}H-NMR spectrum even after a week. As described elsewhere [21], there is now reason to believe that the results of this original ^{1}H-NMR dilution experiment are not general. In the course of developing a quantitative precipitation assay (vide infra), we had the occasion to subject these pseudopolyrotaxanes to the Bradford assay [51] (Bio-Rad), a well-known analytical method for quantifying protein concentrations based on a blue shift in the absorption spectra of the dye, Coomassie Blue. This assay resulted in several surprising discoveries [21]: (1) free polyviologens are responsive to the Bradford assay, which is usually highly selective for proteins; (2) for pseudopolyrotaxanes derived from polyviologens, the response of the Bradford assay was dependent on, and thus indicative of, the degree of threading; and using this simple colometric assay
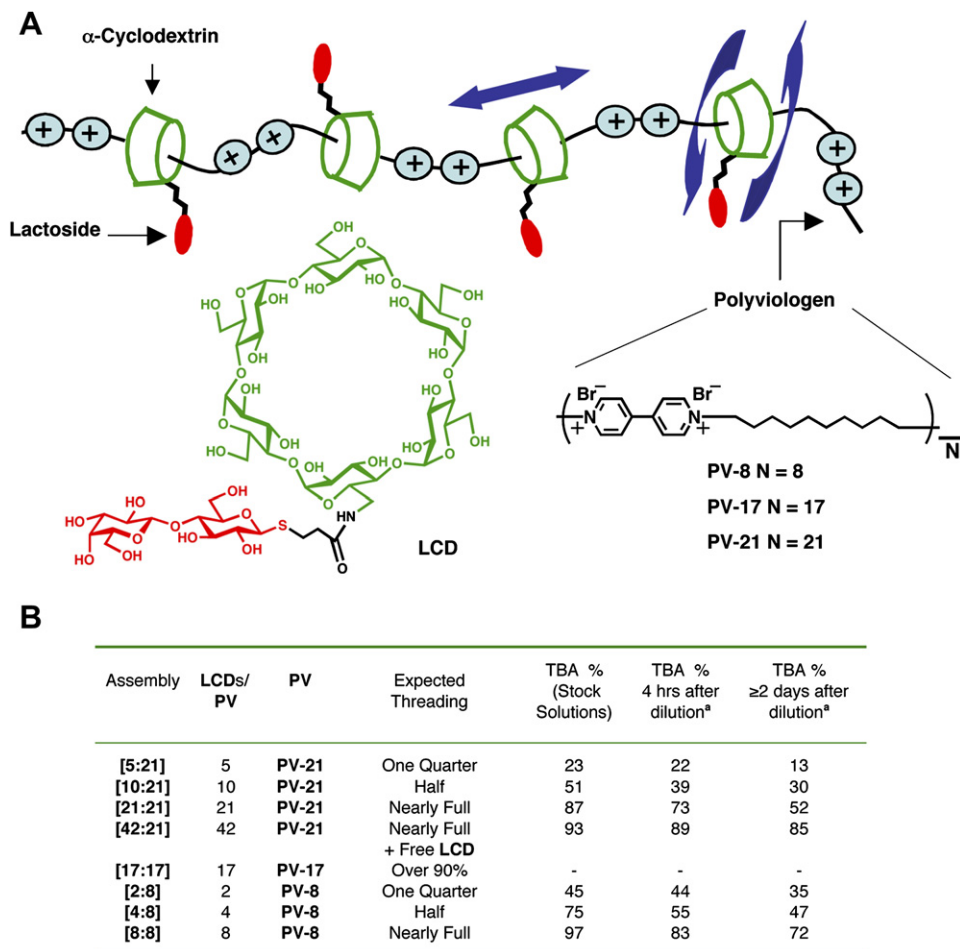
Figure 1. Self-Assembled, Multivalent Pseudopolyrotaxanes

(A) Schematic representation of pseudopolyrotaxanes composed of lactoside-displaying cyclodextrin (**LCD**) rings and polyviologen threads. Chemical structures of the components are also shown.

(B) Table describing the degree of threading for a series of pseudopolyrotaxane assemblies. [a]Dilution to 1 mM polyviologen repeating unit. "TBA %," the degree of threading, as measured by the Bradford assay, is defined as: $100 \times (1 - [OD_{595}$ sample $- OD_{595}$ background]/$[OD_{595}$ free $PV - OD_{595}$ background]). The values in the table are reproduced from a full account [21] of the development of the assay. Using the first entry as an example, this pseudopolyrotaxane was self-assembled from a ratio of five **LCD**s to each **PV-21** thread, hence the nomenclature **[5:21]**. Since the thread has, on average, 21 polyviologen repeating units, this assembly is expected to be quarter-threaded at equilibrium after self-assembly, which is in good agreement with the measured TBA of 23% for the stock solution (20 mM in **LCD**). Upon dilution, equilibrium is reestablished ($\geq$ 2 days) with a TBA of 13% for this assembly, but this dethreading is not yet significant at 4 hr after dilution (22% TBA).

as a reporter, (3) dethreading was observed for diluted solutions of the pseudopolyrotaxanes upon standing. Although these pseudopolyrotaxanes do not appear to be as thermodynamically stable to dilution as previously supposed, they are kinetically stable on the timescale (15 min) of the T-cell agglutination assays. As a result of the electronic speed bumps provided by the viologen units, it can take up to 2 days to reach equilibrium upon dilution; initial dethreading is observed, however, on the timescale (3 hr) of the precipitation experiments presented herein.

The polyviologens **PV-8** and **PV-21** were synthesized according to literature precedent [50]. The number average molecular weights ($M_n = 3650$ and 9581), which were determined (end-group analysis) by ^{1}H-NMR spectroscopy, correspond to an average of 8 and 21, respec-

tively, repeating AB units. **PV-21** is somewhat longer than the previously reported **PV-17**, whereas **PV-8** is considerably shorter. Seven solutions (Figure 1B) were prepared with 20 mM **LCD** in H$_2$O and different amounts of either **PV-8** or **PV-21**. With an equal number of rings to repeating units, solutions **[21:21]** and **[8:8]** are expected to contain nearly fully threaded pseudopolyrotaxanes and few free **LCD**s. With (roughly) half as many rings as repeating units, solutions **[10:21]** and **[4:8]** are expected to be nearly half-threaded, whereas **[5:21]** and **[2:8]**, with (roughly) a quarter of the rings to repeating units, are expected to be quarter-threaded. By contrast, solution **[42:21]** contains twice as many rings as repeating units; thus, it should contain a (very nearly) fully threaded pseudopolyrotaxane and a full equivalent of free **LCD**. Results

from the Bradford assay are shown in Figure 1B as TBA (Threading by Bradford Assay) percentages [21] for these solutions at the concentration (20 mM **LCD**) used for self-assembly. Note that the TBA percentages correlate extremely well with our expectations of threading by the **PV-21**-derived pseudopolyrotaxanes and also report—apparently overestimating—the degree of threading for **PV-8**-derived pseudopolyrotaxanes. The aqueous solutions of self-assembled pseudopolyrotaxanes (20 mM **LCD**) were used as stock solutions for the T-cell agglutination and quantitative precipitation experiments, which were performed immediately after dilution unless otherwise noted. Diluted solutions of the pseudopolyrotaxanes experienced dethreading upon standing, as observed by lower TBA percentages (Figure 1B, dilution to 1 mM polyviologen repeating unit), which correlated with decreased activity in functional assays (vide infra). Note that assembly **[21:21]**, for which this concentration is a 20-fold dilution in both **LCD** and **PV-21**, goes from 87% to 52% TBA and reaches equilibrium in 2 days, whereas assembly **[42:21]**, which contains the full extra equivalent of **LCD**, is much more resistant to dethreading. (Dynamic unthreading and rethreading will occur for all of the assemblies, but for **[42:21]**, the extra equivalent of **LCD** shifts the equilibrium toward the nearly fully threaded state, with 85% TBA observed after 2 days.) Although some dethreading is observed for all pseudopolyrotaxanes after 2 days, **[21:21]**, **[10:21]**, **[8:8]**, and **[4:8]** show noticeable dethreading after only 4 hr.

Quantitative Precipitation Assay

It is well established that multivalent lectins and carbohydrates can induce each other to precipitate [5, 6, 30, 31]. If it is possible to distinguish between the lectin and carbohydrate portion of the precipitate, quantitative precipitations [52] can be performed to gain such information as the ratio of lectin to carbohydrate in the precipitate. The Bradford assay was used as a protein-specific reporter, whereas UV spectroscopy provides the total amount of both protein and polymer in the solution and in the precipitate (see Experimental Procedures for details). The responsiveness of the pseudopolyrotaxanes themselves to the Bradford assay [21] limits our ability to quantify precipitation results by this method to those concentrations and degrees of threading that do not yield a significant Bradford response. However, at concentrations relevant to the precipitation assay, many of the assemblies and, in particular, the highly effective **[21:21]** and **[42:21]** do not yield a Bradford response above background; thus, in these cases, the assay can be used to report solely on protein concentration in the presence of pseudopolyrotaxane.

Neither the pseudopolyrotaxanes, their components, nor recombinant human Gal-1 [53] precipitate on their own, at least during the timescale of these experiments. The individual pseudopolyrotaxane components, **LCD**, **PV-8**, and **PV-21**, do not induce Gal-1 to precipitate. With the pseudopolyrotaxanes, precipitation (Figure 2A) is rapid, as determined visually or by light scattering (see

the Supplemental Data available with this article online). A white precipitate is clearly visible within the first minute of mixing for concentrations such as 4:1 polyviologen repeating unit of assembly **[21:21]**:Gal-1 (50 μM). As it occurs readily in both polypropylene Eppendorf tubes and polysyrene 96-well plates, precipitation was not dependent on a particular type of surface. After mixing, solutions were left for at least 3 hr before the solution was separated from the precipitate (all precipitations are likely to be complete at ~2 hr, based on light scattering). The precipitate was then dissolved in 0.5 M lactose for analysis by the Bradford assay and UV spectroscopy. Lactose was also immediately added to the separated solution, to prevent additional aggregation/precipitation phenomena that would otherwise occur as equilibrium is reestablished after the removal of the precipitate.

The pseudopolyrotaxanes efficiently precipitate Gal-1 (Figure 2A); assembly **[21:21]** induces over 80% precipitation at a 4:1 starting ratio of the polyviologen repeating unit: Gal-1, which results in a 2:1 ratio of polyviologen repeating unit:Gal-1 in the precipitate. This 2:1 ratio (Figure 2B) is relatively constant over a 10-fold titration range of assembly **[21:21]** relative to a fixed concentration of Gal-1. This suggests that a crosslinking Gal-1 occupies every other potential binding site in the precipitated pseudopolyrotaxane-lectin aggregates. See Figure 2C for an idealized model of the precipitate. This ratio of polyviologen repeating unit:Gal-1 in the precipitate can be driven to nearly 1:1, with an excess of Gal-1 relative to the polyviologen repeating units (Figure 2B). Note that this ratio is not expected to hit exactly 1:1, given that not every repeating unit of the polyviologen backbone will be occupied by an **LCD**. Normalized to the concentration of polyviologen repeating units, assemblies **[8:8]**, (Figure 3A) **[10:21]**, and **[5:21]** were all less efficient at precipitating Gal-1 than assembly **[21:21]** (Figure 3B). However, assembly **[42:21]**, which contains an extra **LCD** for every polyviologen repeating unit, was somewhat more efficient than assembly **[21:21]** (Figure 3B). This result was a surprising one, since free **LCD**s were initially expected to detract from precipitation efficiency. The precipitates from assembly **[42:21]** displayed Gal-1:polyviologen repeating unit ratios close to 1:1, even at excess polyviologen repeating unit:Gal-1 ratios. This observation reflects the increased likelihood of each polyviologen repeating unit to be threaded in assembly **[42:21]**, especially considering its resistance to dethreading, compared with the propensity of pseudopolyrotaxane **[21:21]** to dethread over relatively short timescales (Figure 1B). This increased propensity to be threaded most likely accounts for the increased precipitation efficiency of pseudopolyrotaxane **[42:21]**, overcoming, or perhaps even aided by, the equivalent of free **LCD**. While a large excess of free lactoside inhibits and reverses precipitation, one equivalent could aid precipitation by breaking up unproductive aggregates/chelates in favor of those more prone to precipitate.

Precipitation occurs on a timescale that is competitive with at least the beginnings of dethreading. Dethreading prior to exposure to Gal-1 can limit the effectiveness of
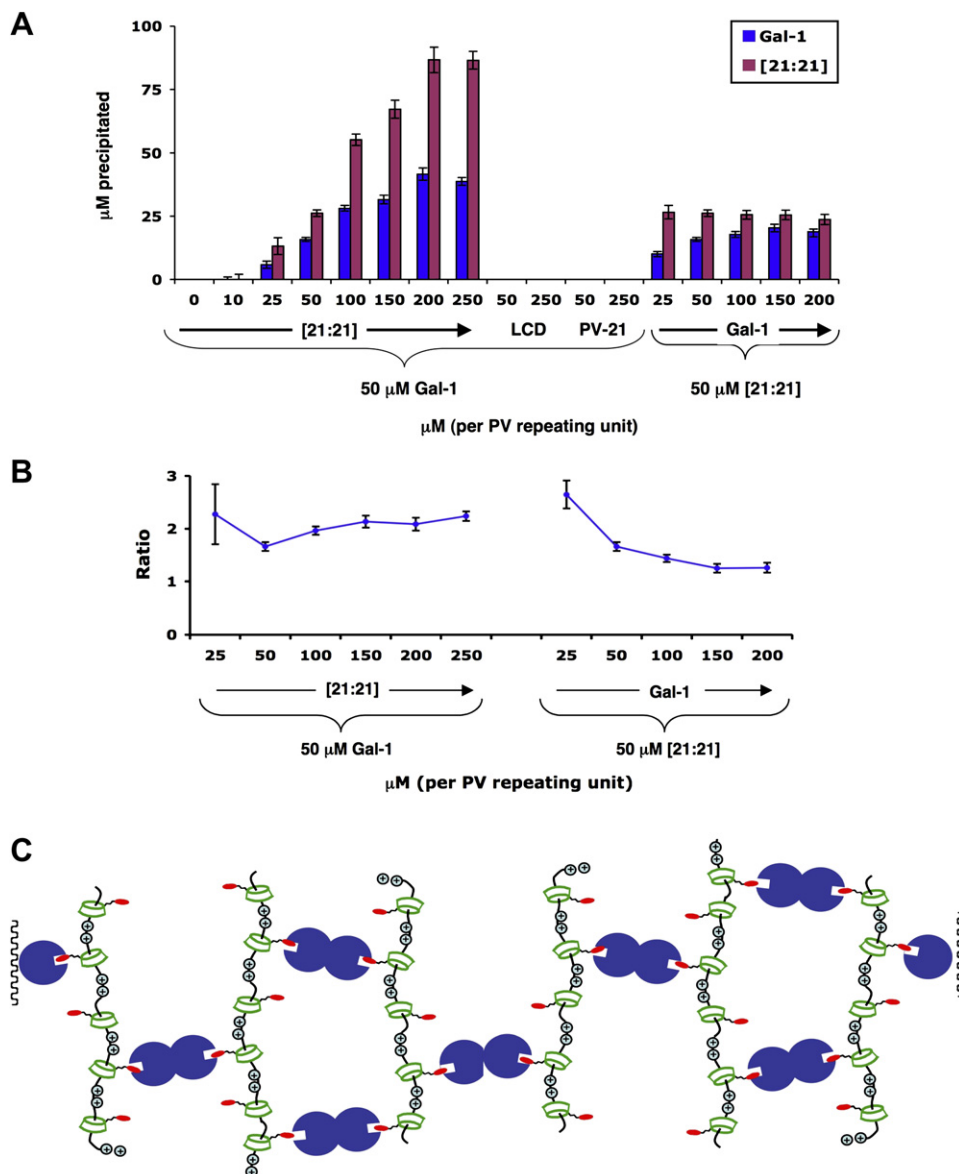
Figure 2. Quantitative Precipitation Assay of Pseudopolyrotaxane [21:21] with Gal-1

(A) Chart describing the concentrations of Gal-1 and pseudopolyrotaxane **[21:21]** in the precipitates obtained from two separate titrations. On the left, increasing concentrations of pseudopolyrotaxane **[21:21]** as well as its components **LCD** and **PV-21** were mixed with a fixed concentration (50 µM) of Gal-1. On the right, increasing concentrations of Gal-1 were mixed with a fixed concentration (50 µM) of pseudopolyrotaxane **[21:21]**. The concentrations of pseudopolyrotaxane **[21:21]** indicated are per polyviologen repeating unit. Error bars indicate the standard deviation.

(B) Chart comparing the ratio of Gal-1 to pseudopolyrotaxane **[21:21]** in the precipitates obtained from both titrations. Error bars indicate the standard deviation.

(C) Idealized model of precipitate highlighting the 2:1 ratio of polyviologen repeating units:Gal-1 found in the precipitate across a 10-fold range of starting pseudopolyrotaxane **[21:21]** concentrations.

precipitation, as was observed when diluted solutions of assembly **[21:21]** were allowed to stand for 3 hr before a precipitation experiment was begun, which resulted in a small, but noticeable, decrease in the amount of Gal-1 precipitated—70% with a 4:1 ratio of polyviologen repeating unit:Gal-1 for **[21:21]** (data not shown). Once exposed to Gal-1, crosslinking almost certainly alters the dethreading kinetics and thermodynamics.

T-Cell Agglutination Assay

A straightforward assay [20] based on the ability of Gal-1 to aggregate T cells was employed to evaluate the pseudopolyrotaxanes. Treatment of CEM cells—cultured human T-leukemia cells—with 10 µM recombinant human Gal-1 [53] for 5 min at 37°C results in large aggregates that resemble tumor emboli, whereas pretreating Gal-1 with lactosides for 10 min at room temperature results in
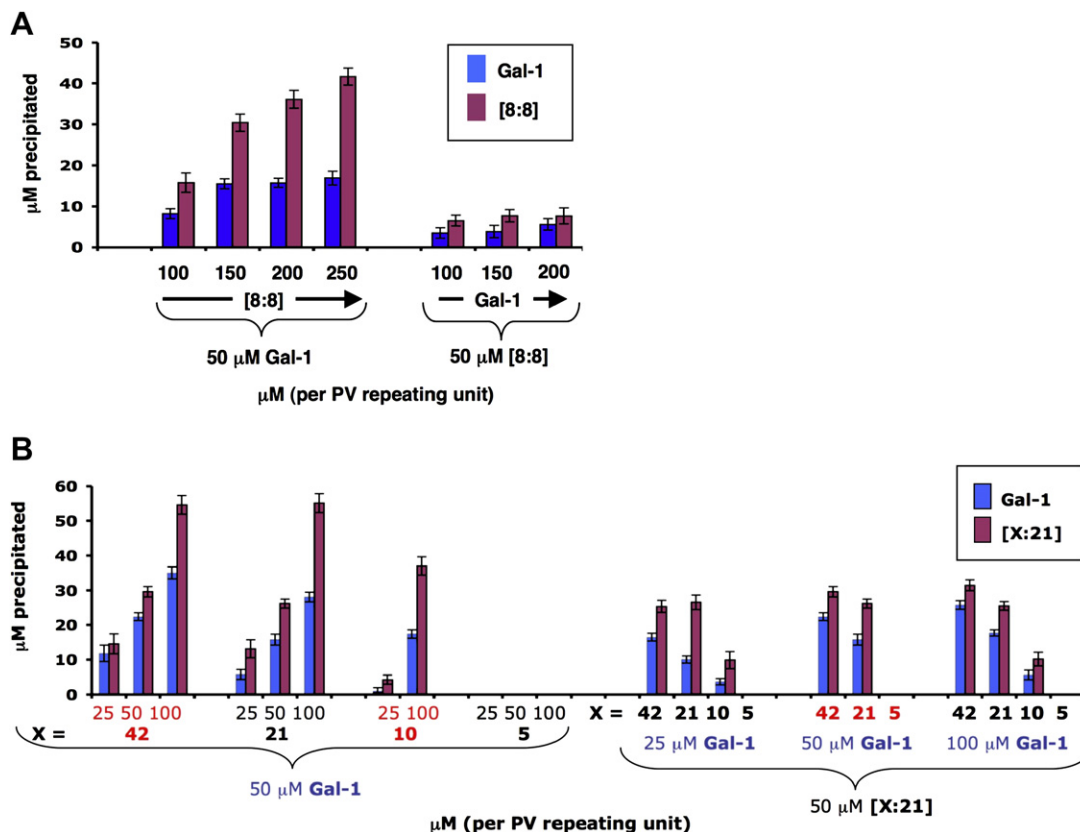
Figure 3. Quantitative Precipitation Assay of Pseudopolyrotaxanes with Gal-1

(A) Chart describing the concentrations of Gal-1 and pseudopolyrotaxane **[8:8]** in the precipitates obtained from two separate titrations. On the left, increasing concentrations of pseudopolyrotaxane **[8:8]** were mixed with a fixed concentration (50 μM) of Gal-1. On the right, increasing concentrations of Gal-1 were mixed with a fixed concentration (50 μM) of pseudopolyrotaxane **[8:8]**. The concentrations of pseudopolyrotaxane **[8:8]** indicated are per polyviologen repeating unit. Error bars indicate the standard deviation.

(B) Chart describing concentrations of Gal-1 and **PV-21**-based pseudopolyrotaxanes in the precipitates obtained from two separate titrations. On the left, increasing concentrations of pseudopolyrotaxanes **[42:21]**, **[21:21]**, **[10:21]**, and **[5:21]** were mixed with a fixed concentration (50 μM) of Gal-1. On the right, increasing concentrations of Gal-1 were mixed with a fixed concentration (50 μM) of each pseudopolyrotaxane. The concentrations of pseudopolyrotaxanes indicated are per polyviologen repeating unit. Error bars indicate the standard deviation.

either similar aggregates, a mixed state of aggregated and dispersed cells, or mostly dispersed cells (Figures 4G, 4D, and 4A, respectively, for example). The lowest value of lactoside for which the mixed state is observed is defined as the minimum inhibitory concentration (MIC). Inhibition has been shown [20] to be lactoside dependent, because free polyviologens or polyviologens threaded with native α-CD are not inhibitors. The **PV-21**- and **PV-8**-based assemblies, always freshly diluted into PBS buffer from the H$_2$O stock solution, were subjected to the agglutination assay (Figures 4–6).

At an intermediate per-lactoside concentration of 300 μM (Figures 4D–4F), assemblies **[4:21]**, **[21:21]**, and **[42:21]** have all yielded the mixed state. At 100 μM (Figures 4G–4I), which is the MIC of assembly **[21:21]** (Figure 5A), the inhibitory affect of assembly **[42:21]**, which contains the extra equivalent of **LCD** and thus might be expected to be half as effective as assembly **[21:21]** on a valency-corrected basis, has indeed been lost. By contrast, the quarter-threaded pseudopolyrotaxane **[5:21]** is still inhibitory. In fact, the pseudopolyrotaxane **[5:21]** displays a valency-corrected MIC of 50 μM (Figure 5A), which is a 30-fold enhancement compared to lactose, a 20-fold enhancement compared to free **LCD**, and 2-fold better than the nearly fully threaded assembly **[21:21]**, despite having only one-quarter the number of available lactoside ligands. The roughly half-threaded assembly **[10:21]** yields the same MIC (100 μM) as the nearly fully threaded assembly **[21:21]**, confirming that, on a per-lactoside basis, it is not necessary to maximize the number of **LCD**s/ thread to attain the mixed state at a low concentration. However, the lower-threaded assemblies **[5:21]** and **[10:21]** attain the dispersed state only at higher concentrations (750 μM, valency corrected) than the more fully threaded assemblies. As portrayed in Figures 4A–4C, **[21:21]** and **[42:21]** have attained the dispersed state at 600 μM (valency corrected), whereas **[5:21]** remains in the mixed state at this concentration. The **PV-8**-based assemblies also show that the more fully-threaded systems attain the dispersed state more readily on a
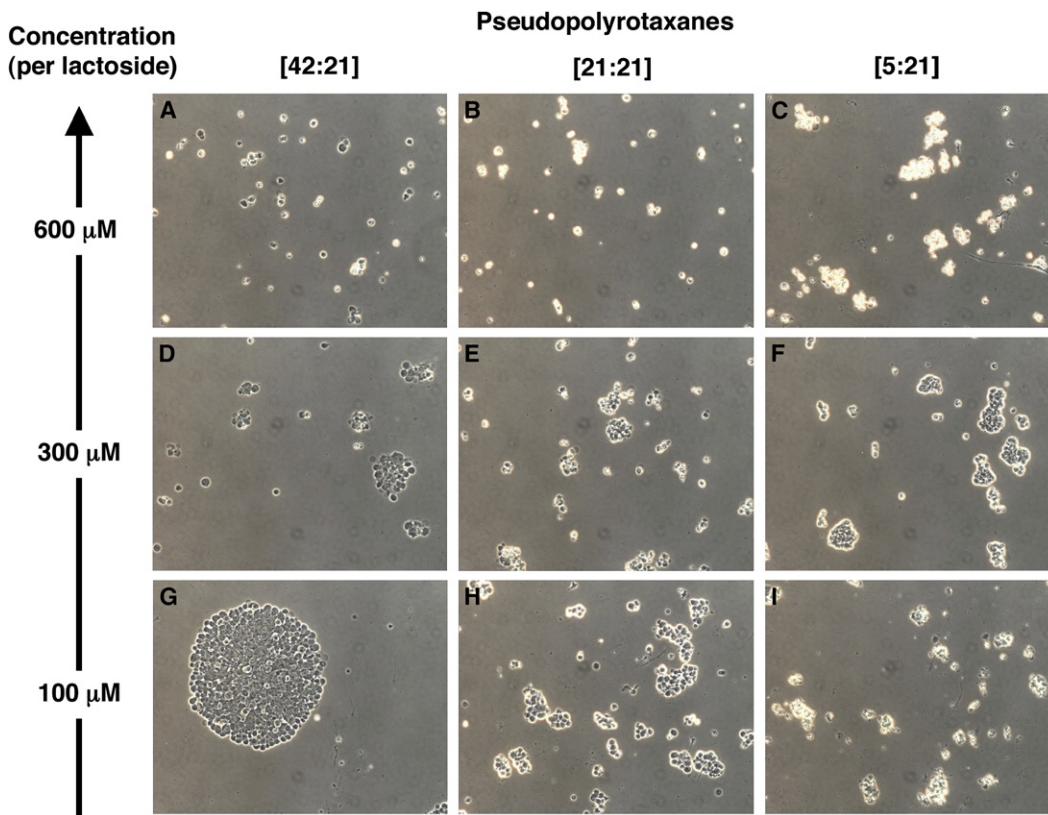
Figure 4. T-Cell Agglutination Assay Light Microscopy, 10×, Images of Titrations of Pseudopolyrotaxanes [42:21], [21:21], and [5:21] in the Presence of CEM Cells and 10 μM Gal-1

(A–I) Valency-corrected lactoside concentrations are shown at the left. At 600 μM lactoside, cells are (A) dispersed for **[42:21]**, (B) dispersed for **[21:21]**, and (C) in the mixed state for **[5:21]**. At 300 μM lactoside, cells are (D) in the mixed state for **[42:21]**, (E) in the mixed state for **[21:21]**, and (F) in the mixed state for **[5:21]**. At 100 μM lactoside, cells are (G) aggregated for **[42:21]**, (H) in the mixed state for **[21:21]**, and (I) in the mixed state for **[5:21]**.

valency-corrected basis, whereas the valency-corrected MIC for each of these assemblies is the same (200 μM). This result represents an enhancement of 7.5-fold versus lactose and 5-fold versus **LCD**, i.e., significant gains, at least for the quarter-threaded assembly **[2:8]**, considering there are only two lactoside ligands (on average) connected in this pseudopolyrotaxane.

The multivalent enhancements (valency-corrected MIC) for the full range of pseudopolyrotaxanes tested versus MIC for free lactose and **LCD** are shown in Figure 5B. The quarter-threaded 21-mer **[5:21]** is the best performer, whereas assembly **[42:21]**, in which half of the **LCD**s are not located on a polymer backbone, is the worst; however, it still generates a 4-fold enhancement compared to free **LCD** or a 6-fold enhancement compared to lactose. The nearly fully threaded **[21:21]** and half-threaded **[10:21]** gave the same enhancement (10-fold versus **LCD**, 15-fold versus lactose); thus, from the "perspective" of lactoside ligand, it does not matter whether the polyviologen backbone is half or nearly fully threaded, although it is more effective to be one-quarter threaded. Indeed, with its valency-corrected enhancement of 20-fold compared to the monovalent ligand (**LCD**), assembly **[5:21]** has the

highest valency-corrected enhancement, of which we are aware, for inhibition of Gal-1 in a cell-based assay. Gabius and coworkers have achieved much greater enhancements for Gal-1 binding, as measured in solid-phase competition assays by using starburst glycodendrimers [54] and wedge-shaped glycodendrons [55], synthesized by Roy and coworkers and by Pieters and coworkers, respectively. At least for the glycodendrons [55], the best efficacy in a cell-based assay was on the same order of magnitude (14.5-fold greater than monovalent lactoside) as seen here for the potent pseudopolyrotaxanes.

There is an interesting progression observed for the nearly fully threaded pseudopolyrotaxanes **[21:21]**, **[17:17]**, and **[8:8]** correlating average polyviologen length with multivalent enhancement (10-, 6.7-, and 5-fold versus **LCD**, respectively), i.e., from the lactoside ligand "perspective," it is more effective to be on a longer polymer thread, which allows for more noncovalently connected ligands and greater total bridgeable distances between the lactosides. As noted, all three of the **PV-8**-based assemblies gave the same multivalent enhancement of 7.5-fold versus lactose and 5-fold versus **LCD**. The **PV-8**-based assemblies differentiate themselves when we consider

A

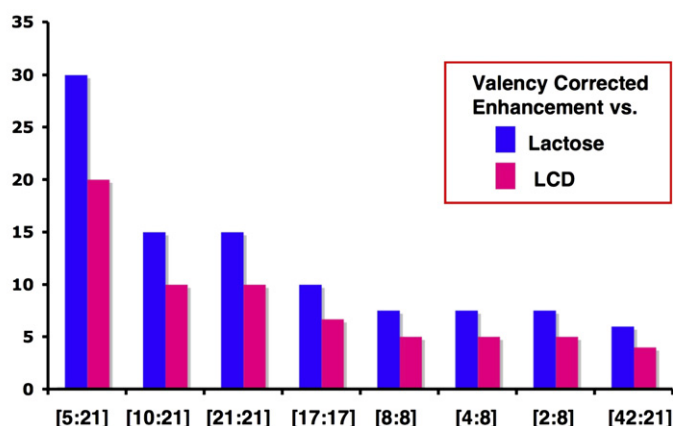|	Lactose[a]	LCD[a]	[5:21]	[10:21]	[21:21]	[42:21]	[17:17][a]	[2:8]	[4:8]	[8:8]
Dispersed (per lactoside)	2000	1500	750	750	600	600	750	800	750	600
Dispersed (per polymer)	N.A.	N.A.	150	75	28.8	14.4	44	400	187.6	75
Mixed (per lactoside)	1500	1000	50	100	100	250	150	200	200	200
Mixed (per polymer)	N.A.	NA.	10.1	10.1	4.8	6	8.8	100	50	25

B

Figure 5. Summary of Agglutination Assays
(A) Table relating the lowest value of inhibitor (in μM) necessary to obtain the dispersed and mixed states (MIC) on both a per-lactoside and per-polymer basis. [a]Values from reference [20]. All titrations were performed at least in triplicate.
(B) Results of the agglutination assay presented as a chart of valency-corrected enhancements, with per-lactoside MIC normalized to the MIC for free lactose (blue) and for free **LCD** (magenta).

the polymer backbone (polyviologen) concentration at the MIC, which ranges from 25 μM for assembly **[8:8]** to 100 μM for assembly **[2:8]**. For the **PV-21**-based assemblies as well, from the "perspective" of the polyviologen backbone, the most effective assembly is the nearly fully threaded **[21:21]**, with an MIC of 4.8 μM on a per-polymer basis. This value is half of the Gal-1 concentration (10 μM) used in these experiments, and it represents an advantage of 357-fold compared to lactose and 208-fold compared to **LCD** on a multivalent, supramolecular complex to monovalent ligand basis.

A comparison of the polymer concentrations at the lowest values of the mixed and dispersed states for all pseudopolyrotaxanes tested reveals a general trend toward increasing efficacy with increased threading. Furthermore, by reorganizing the data in Figure 5A to Figure 6A to highlight the number of ligands (**LCD**s) that may be noncovalently connected to each other in each assembly, regardless of the length of the polyviologen backbone, a clear progression emerges in the values relating the number of potentially connected ligands (average maximum number of **LCD**s/polymer) to the polymer concentration necessary to attain the dispersed state. Indeed, expressed graphically (Figure 6B), the negative log of the polymer concentration versus the log of the average max-

imum number of **LCD**s/polymer yields a reasonable linear fit ($R^2 = 0.965$), indicating that the ability to attain the dispersed state is described reasonably well by this one factor alone. The same trend exists for the mixed state, but the correlation is much worse ($R^2 = 0.87$), an observation that indicates that other factors, presumably including polymer length and ligand spacing, also strongly affect the ability to attain the mixed state. Additionally, the values for the monovalent ligand **LCD**, shown at 0 on the graph (log of 1), fall directly on the line for the dispersed state, and far off the line for the mixed state. By definition, the dispersed state must be at or near saturation of Gal-1-binding sites by the pseudopolyrotaxanes, because there is not enough free Gal-1 to cause substantial aggregation of the T cells. It follows that, on a hypothetical binding curve between Gal-1 and the pseudopolyrotaxanes, the mixed state is a lower point on the curve, perhaps near the hypothetical IC_{50}. While it is notoriously problematic [56] to relate the results of agglutination assays directly to thermodynamic binding parameters, it is not surprising that more subtle factors would be necessary to account for binding near the hypothetical IC_{50} than near saturation. Alternatively, the different dependence on the number of connected ligands could imply a change in the mechanism(s) of binding with increasing pseudopolyrotaxane

A

	[2:8]	[4:8]	[5:21]	[8:8]	[10:21]	[17:17][a]	[21:21]	[42:21]
Avg. (max) # of **LCD**s/polymer	2	4	5	8	10	17	21	21
Dispersed (per polymer)	400	187.6	150	75	75	44	28.8	14.4
Mixed (per polymer)	25	50	10.1	100	10.1	8.8	4.8	6

B

Linear Fit (Mixed):
$y = 1.4989x + 2.3809$
$R^2 = 0.8699$

Linear Fit (Dispersed):
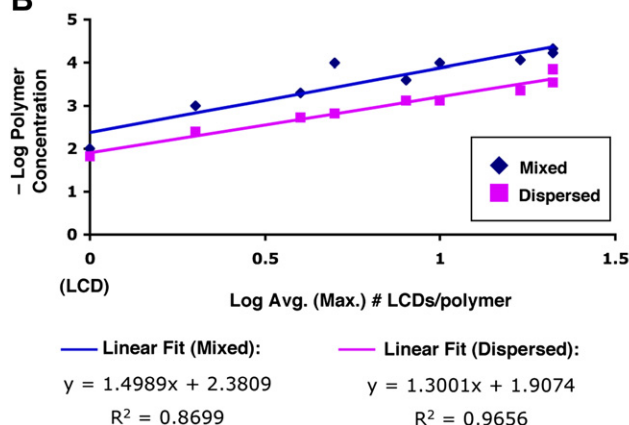$y = 1.3001x + 1.9074$
$R^2 = 0.9656$

Figure 6. Supramolecular Statistical Effect: A Dependence on the Number of Noncovalently Connected Ligands
(A) Table highlighting the agglutination assay results—lowest values of inhibitor (in μM) necessary to obtain the dispersed and mixed states on a per-polymer basis—as a function of the number of noncovalently connected ligands (average maximum number of **LCD**s/polymer).
(B) Graph of the log average maximum number of **LCD**s/polymer versus the negative log of the lowest polymer concentration necessary to obtain the mixed and dispersed states. The linear fits reveal a better correlation for the dispersed state. The large outlier for the mixed state is assembly **[5:21]**.

concentration upon going from the mixed to dispersed states.

A dependence on the number of connected ligands is a hallmark of the statistical effect in multivalent binding [2–7, 30, 31, 52, 56]. Here, the ligands are not connected directly by traditional covalent bonds, but are mechanically connected via the encircled polymer backbone. Hence, we observe a supramolecular statistical effect for these pseudopolyrotaxanes inhibiting Gal-1-mediated agglutination of the T cells, particularly at the higher pseudopolyrotaxane concentrations necessary to obtain the dispersed state. The observation of a supramolecular statistical effect does not, in and of itself, define a mechanism of binding; however, it is consistent with the simplest possible mechanism: one-to-one binding between Gal-1 and a single pseudopolyrotaxane chain, in which only one of the lactoside-binding sites is occupied at a time. Another possible mechanism of binding is chelation, whereby a single pseudopolyrotaxane chain provides lactosides to both Gal-1-binding sites. Aggregation, which, in this context, we explicitly define as crosslinking of different pseudopolyrotaxane chains by one or more Gal-1 dimers, is also likely to be a factor. Although the thermodynamic driving forces behind protein-carbohydrate aggregates are still not fully understood, the formation of such aggregates has been suggested [56] to be a major

factor in agglutination experiments. Furthermore, Gal-1 is an evolutionarily adapted crosslinker [4–6, 40], with a topology that would bias aggregation over chelation. The tendency of Gal-1 and the pseudopolyrotaxanes to aggregate is clear from the light scattering and precipitation experiments, albeit at different concentrations and ratios than are present in the agglutination assay.

It would be particularly interesting to know whether the pseudopolyrotaxanes chelate Gal-1. Chelation is often associated [2–7, 28, 30, 31, 33] with considerably greater multivalent enhancements than are obtained by the statistical effect alone. A traditional view [4, 7, 56] holds that chelation would be disfavored for a flexible system that will freeze out considerable entropy upon chelation, especially given the large distance between, and orientation of, lactoside-binding sites in Gal-1. However, the incompatibility of chelation with flexible systems and long distances between the potentially bridging ligands has been questioned [3, 57, 58] on experimental and theoretical grounds. A definitive answer on the ability of the pseudopolyrotaxanes to chelate Gal-1 and the relative importance of this interaction to the overall binding energy will require a more direct readout for thermodynamic binding parameters. Nevertheless, deviations from the dependence on just the number of connected ligands may reflect some degree of chelation, because certain combinations of

other factors, such as ligand spacing and polymer length, should favor chelation. Considerations of bond lengths and angles in the pseudopolyrotaxanes suggest that chelation could occur for **LCD**s that are a minimum of four polyviologen repeating units apart. This spacing is intriguing because it is reasonable that the **LCD**s of assembly **[5:21]** would be, on average, four repeating units apart. This pseudopolyrotaxane is both the most potent on a valency-corrected basis, and the largest outlier from the trend of simple dependence on the number of connected ligands for the mixed state (Figure 6). Assembly **[5:21]** also maintains the mixed state over a larger concentration range than the more fully threaded pseudopolyrotaxanes (Figures 4 and 5A). Perhaps this range is expanded by access to binding modes, particularly chelation, that are less favored for the other pseudopolyrotaxanes. In comparison with aggregation, for example, it is interesting to consider how poorly pseudopolyrotaxane **[5:21]** performed in the quantitative precipitation assay (Figure 3B). Overall it is likely that the flexible and adaptable pseudopolyrotaxanes exhibit a variety of binding modes with dimeric Gal-1, and that the distribution among these binding modes changes with the degree of threading, which itself is a dynamic quantity.

Conclusions

Biology is replete with examples of individual proteins self-assembling into superstructures wherein the complexes gain or enhance functions with respect to those of the individual components [9, 10]. Here, supramolecular species formed from over 20 (**[21:21]** and **[42:21]**) or as few as 3 (**[2:8]**) self-assembling components have been used to successfully precipitate the bivalent lectin Gal-1 and to inhibit Gal-1 in T-cell agglutination assays. These self-assembled pseudopolyrotaxanes are an example of the kind of flexible and adaptable multivalent ligand display that can be afforded through supramolecular chemistry. The lactoside ligands are able to rotate freely and independently around the polymer backbone, whereas the CDs can translate within the confines of the decamethylene units, which have a length that is twice the depth of a CD cavity. Not only do the lactoside ligands have the ability to "fine tune" their positions without enthalpic penalties having to be paid on account of inducing strain, but the unbound ligands may also reside away from the bulky protein in order to reduce steric clashes. This flexibility and adaptability may contribute to the speed, efficacy, and large amount of Gal-1 captured by the pseudopolyrotaxanes in the precipitations, as well as the supramolecular statistical effect and efficacy observed in the agglutination assay. These flexible and adaptable, multivalent supramolecular complexes show promise both for the study of protein-carbohydrate interactions and the exploitation of multivalency for targeting therapeutically relevant lectins.

SIGNIFICANCE

A key question across many fields of molecular recognition—and, in particular, glycobiology—is the degree to which flexibility and adaptability contribute to multivalent binding. Herein, we have described the interactions of a series of self-assembled multivalent pseudopolyrotaxanes, consisting of lactoside-displaying cyclodextrin (LCD) "beads" threaded onto polyviologen "strings," with a bivalent lectin, galectin-1 (Gal-1). These supramolecular assemblies display highly flexible and adaptable ligands as a result of rotation of the CD torus about, and limited translation along, the polymer chain. Seven assemblies with different ratios of "beads" threading two different length polyviologen "strings" have been prepared. The pseudopolyrotaxanes rapidly and efficiently precipitate Gal-1; over 80% precipitation of Gal-1 is observed. The ratio of Gal-1:polyviologen repeating unit in the precipitate can approach 1:1, i.e., nearly every potential binding site is occupied by a crosslinking Gal-1 in the precipitated pseudopolyrotaxane-lectin aggregates. A supramolecular statistical effect was observed in a T-cell agglutination assay, wherein the efficacy of Gal-1 inhibition correlates with the number of ligands connected to each other solely through mechanical and noncovalent interactions. Also, in this assay, valency-corrected enhancements of up to 30-fold compared to native lactose and 20-fold compared to free LCD, as well as per-polymer enhancements of up to 357- and 208-fold compared to native lactose and free LCD, respectively, were obtained. These results show that synthetic supramolecular complexes are useful tools for the study of multivalent protein-carbohydrate interactions and, more broadly, suggest that the conceptually related, but, in practice, often disparate, fields of biochemistry and supramolecular chemistry have much to offer each other.

EXPERIMENTAL PROCEDURES

General

LCD [19], assembly **[17:17]** (pseudopolyrotaxane **3** in reference [20]), and details of the agglutination assay [20] with CEM cells (subclone of ATCC no. CCL-119) and recombinant human Gal-1 [53] have been previously described. Polyviologens **PV-8** and **PV-21** were synthesized according to literature precedent [20, 50]; for details and characterization, see the Supplemental Data. The use of the Bradford assay to monitor the degree of threading for these pseudopolyrotaxanes has been described in detail separately [21].

Pseudopolyrotaxanes

Stock solutions (20 mM **LCD** in H_2O) of assemblies **[5:21]**, **[10:21]**, **[21:21]**, and **[42:21]** were prepared in 1.5 ml Eppendorf tubes by dissolving **LCD** (10 μmol) and varying quantities of **PV-21** (42, 21, 10, and 5 μmol, respectively) in 500 μl H_2O. Stock solutions (20 mM **LCD** in H_2O) of assemblies **[2:8]**, **[4:8]**, and **[8:8]** were prepared in 1.5 ml Eppendorf tubes by dissolving **LCD** (10 μmol) and varying quantities of **PV-8** (40, 20, and 10 μmol, respectively) in 500 μl H_2O. The pseudopolyrotaxanes self-assembled upon standing (1 month) prior to the initiation of biochemical experiments. The degree of threading in these assemblies was monitored by using the Bradford assay [21].

Quantitative Precipitation

Precipitation reactions were conducted in 1.5 ml Eppendorf tubes and initiated by mixing human recombinant Gal-1 [53] with

a pseudopolyrotaxane assembly in the molar ratios described in Figures 2 and 3, in total volumes of 150 µl PBS-buffered solution. After initial mixing, the tubes were allowed to sit for 3 hr and then centrifuged for 10 min at 14,000 rpm. The 150 µl solutions were then transferred to new 1.5 ml Eppendorf tubes (henceforth S-tubes), and 150 µl lactose solution (0.5 M lactose in PBS) was added to both sets of tubes, which were then vortexed (≥30 s each for the precipitate-containing original tubes, henceforth P-tubes). Aliquots (2 × 10 µl, obtained from dilutions if necessary) were taken from both sets of tubes and analyzed by the Bradford assay [21], by using the appropriate background (0.5 M lactose in PBS for P-tubes and 0.25 M lactose in PBS for S-tubes). Aliquots (50 µl) were taken directly from the S-tubes for analysis by UV-VIS spectroscopy at OD_{263} by using a microcuvette compared to a background of 0.25 M lactose in PBS. Aliquots (25 µl) from the P-tubes were added to 25 µl PBS and analyzed by UV-VIS spectroscopy at OD_{263} by using a microcuvette compared to a background of 0.25 M lactose in PBS.

Control experiments demonstrated that at the concentrations tested, unless otherwise noted, the pseudopolyrotaxanes do not yield a Bradford response above background. Thus, the Bradford response in these samples was attributed solely to Gal-1 and was converted to a molar concentration by comparison with an independently prepared standard curve for Gal-1. Good correlations were obtained for the total measured amounts of Gal-1 from the precipitate and remaining solution compared to the starting amount of Gal-1. While the Bradford assay reported specifically on Gal-1, UV-VIS spectroscopy provided a measure of the total concentration of both Gal-1 and pseudopolyrotaxane in the samples. The concentration of both Gal-1 and the pseudopolyrotaxanes vary linearly with absorbance at 263 nm. The molar absorptivities (ε_{263}) were obtained independently for Gal-1 and each pseudopolyrotaxane. The concentration of Gal-1 in each sample as determined by the Bradford assay was converted to an OD_{263} value that was subtracted from the experimentally measured OD_{263} for each sample. The remaining absorbance was assigned to pseudopolyrotaxane in the sample and was converted to a concentration by using the ε_{263} for that pseudopolyrotaxane. Using this method, good correlations were obtained for the total measured amounts of pseudopolyrotaxane from the precipitate and remaining solution compared to the starting amount of pseudopolyrotaxane. Overall, the amounts of Gal-1 and pseudopolyrotaxane measured independently from the precipitate and the remaining solution agreed well with the total initial concentrations. The results obtained from analyzing the precipitate are presented in Figures 2 and 3. Each independent titration was performed in duplicate, and three independent titrations were performed for each assembly described.

Supplemental Data

Detailed synthetic procedures, characterization of polyviologens **PV-8** and **PV-21**, and an example of a light scattering experiment (Figure S1) are available at http://www.chembiol.com/cgi/content/full/14/10/1140/DC1/.

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