Monitoring cyclodextrin–polyviologen pseudopolyrotaxanes with the Bradford assay†

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Self-assembled multivalent pseudopolyrotaxanes, composed of lactoside-bearing cyclodextrin (CD) rings threaded on linear polyviologen polymers, have been introduced recently as flexible and dynamic neoglycoconjugates. In the course of this research, it was found that polyviologens are responsive to the Bradford assay, which is traditionally highly selective for proteins. The response of the pseudopolyrotaxanes to the Bradford assay was dependant on, and thus indicative of, the degree of threading of the CD rings onto the polyelectrolyte. The assay was then used to report on the threading and dethreading of native and lactoside-bearing α-CD rings onto and off of polyviologen chains, a phenomenon which demonstrates the utility of biochemical assays to address problems unique to supramolecular chemistry.

Introduction

Biochemistry and supramolecular chemistry are both sciences that relate to molecular recognition[1] and self-assembly.[2] Yet, despite their conceptual similarities[3] and the biomimetic roots of supramolecular chemistry,[4] few synthetic supramolecular assemblies[5] and related mechanically-interlocked molecules[6] have been examined in biochemical contexts. Prominent exceptions include complexes[7] and interlocked molecules[8] with cyclodextrins[9] (CDs) as components, a consequence most likely of the water solubility, inherent biocompatibility, and hydrophobically-driven complexation behavior of the CD torus. Indeed, pseudopolyrotaxanes[10] and polyrotaxanes[11] formed from CD rings threaded onto linear polymer backbones are of considerable interest[12] for biological applications, such as drug delivery and tissue engineering. Based on the pioneering supramolecular chemistry of Harada[13] and Wenz,[14] we[15] and others[16] have developed these systems as dynamic multivalent neoglycoconjugates. They offer a variety of potential advantages for multivalent ligand presentation, including (1) the ability to span large distances, (2) the ease of varying ligand densities, (3) their adaptability, and (4) the ease of their synthesis by means of self-assembly.[2] Recently, we targeted[17] Galectin-1,[18] a soluble dimeric lactoside-binding lectin, which is involved in a variety of cancers.[19] Using a self-assembled pseudopolyrotaxane, composed of lactoside-bearing CD (**LCD**) rings and a polyviologen thread (**PV-17**, Fig. 1), multivalent enhancements of 6.7-fold and 10-fold relative to the free **LCD** and free lactose, respectively, were obtained[17] for the inhibition of Galectin-1 in a T-cell agglutination assay. In the

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† Dedicated to Peter Dervan, a pioneer in the use of biological assays to solve chemical problems, and *vice versa*, on the occasion of his 60th birthday.

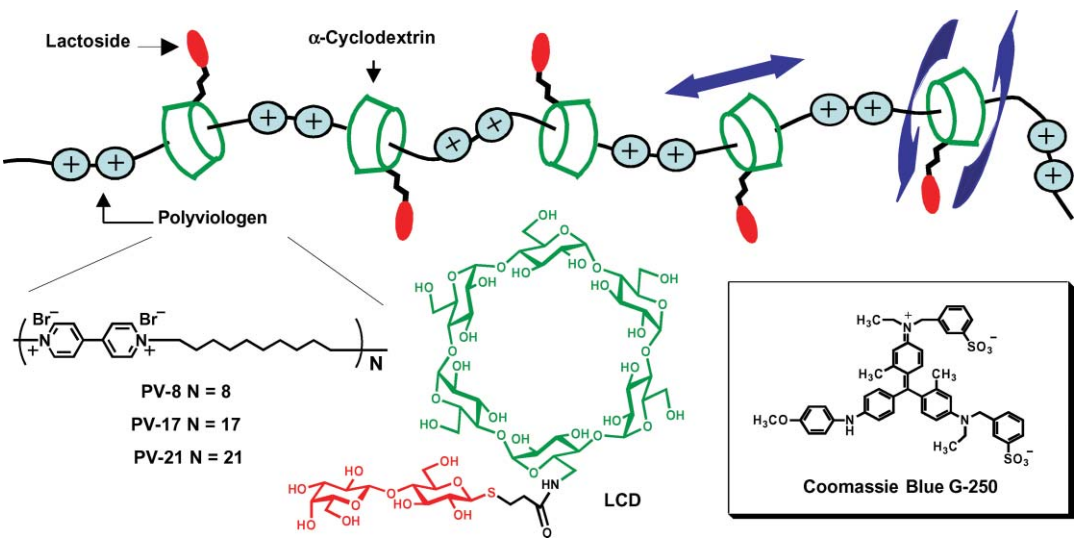

Fig. 1 Schematic representation of pseudopolyrotaxanes composed of lactoside–cyclodextrin (**LCD**) rings and polyviologen threads. Chemical structures of the components and Coomassie Blue G-250, the dye used in the Bradford assay, are also shown.

course of developing a precipitation assay[20] to complement this agglutination assay, we discovered a surprising use of the Bradford assay,[21] a biochemical assay that is typically highly selective for proteins, as a reporter on the degree of threading in cyclodextrin–polyviologen pseudopolyrotaxanes. Thus, in addition to using supramolecular assemblies to address biochemical problems—*e.g.*, the recognition of Galectin-1—assays that are thought to be idiosyncratically biochemical, such as the Bradford assay, can be used to address idiosyncratically supramolecular problems, *i.e.*, the degree of threading in pseudopolyrotaxane complexes.

Results and discussion

Pseudopolyrotaxanes

Polyviologen AB-copolymers, comprised of alternating decamethylene (A) and positively-charged bipyridinium (B) segments, form[22] stable, water-soluble complexes with α-CD. In aqueous solution, the α-CD rings thread onto the polymer chain and rest predominantly on its decamethylene segments,[17] stabilized by the hydrophobic interactions inside the cavities of the CDs. The positive charges, associated formally with the nitrogen atoms on the bipyridinium segments of the polymer, act as electronic "speed bumps" which reduce[14,22] the translational motion of the CD rings. By separating the preferred guest into domains flanked by "speed bumps," polyviologens can support high levels of threading at equilibrium, with the time-scale of self-assembly being relatively slow (≥3 d), because of the necessity for the CD rings to have to pass over multiple bipyridinium segments. For example, in the self-assembly of pseudopolyrotaxane [**17:17**], a mixture of 17 equivalents of **LCD** (20 mM) with a polyviologen containing, on average, 17 repeating units (**PV-17**) resulted[17] in over 90% threading. The designation [**17:17**] is meant to reflect the equivalents of ring to thread in the mixture (not necessarily on the polyviologen backbone):the average length of the polyviologen backbone. The actual degree of threading following self-assembly for 4 d (>90%) was estimated[17] from ^{1}H NMR spectroscopy and TLC. Such mixtures retain the same degree of threading (*vide infra*) and show no signs of chemical degradation of the components, even after months of storage at room temperature. It was also noted that a 50-fold dilution resulted in no change in the ^{1}H NMR spectrum even after a week.

In order to investigate the interactions of lactoside-bearing pseudopolyrotaxanes further with the lactoside-binding protein Galectin-1, the assemblies shown in Table 1 were self-assembled from **LCD** (20 mM) and equivalents of polyviologens of average length 8 (**PV-8**) or 21 (**PV-21**) were varied.[20] 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, while [**5:21**] and [**2:8**], with roughly a quarter of rings to repeating units, are expected to be quarter-threaded. By contrast, solution [**42:21**] contains more rings than repeating units, twice as many, so that it should contain a very nearly fully threaded pseudopolyrotaxane and a full equivalent of free **LCD**. Self-assembly (4 d) in pure H_2O was monitored by ^{1}H NMR spectroscopy, a procedure which confirmed threading at equilibrium in qualitative accordance with these expectations, although the complexity of the spectra preclude an exact numerical determination of the degree of threading.

Bradford assay

In order to develop a quantitative precipitation assay[23] for Galectin-1 with the pseudopolyrotaxanes, an analytical method for distinguishing the protein from the pseudopolyrotaxanes was needed. The Bradford assay (Bio-Rad) is a well-known method for quantifying protein concentrations based on a blue-shift in the absorption spectra of the dye Coomassie Blue G250 upon binding proteins.[21,24–29] The blue-shift to 595 nm develops quickly upon exposure of a protein-containing solution to the dye solution, and the increase in absorbance at OD_{595} can be related directly to protein concentration. Thus, the assay is fast, coloromet ic, and amenable to high throughput and parallel detection-using, for example, 96-well UV-Vis plate reader technology. The Bradford assay is generally highly selective for proteins: other common biochemicals, such as carbohydrates, generally do not yield a response above solution background. Initially, we were pleased to find that, at concentrations relevant to the precipitation experiments, pseudopolyrotaxane [**21:21**] did not give any reading in the Bradford assay above solution background. However, when the individual components of the assembly were included as controls during subsequent precipitation experiments, while **LCD** was not above background, the polyviologens displayed high responses in the Bradford assay. Response curves (Fig. 2) are linear for **PV-8** and **PV-21** in a limited concentration range. When the pseudopolyrotaxanes were subjected to the Bradford assay at 1 mM polyviologen repeating unit, the results (Table 1) for **PV-21**-based assemblies are remarkably similar to our expectations

Table 1 Response of pseudopolyrotaxanes to the Bradford assay[a]

Assembly	LCDs/PV	PV	Expectation	Response *vs.* free **PV** at the same concentration (%)[a]	Threading by Bradford assay [TBA (%)][b]
[**5:21**]	5	**PV-21**	1/4 Threaded	77	23
[**10:21**]	10	**PV-21**	2/4 Threaded	49	51
[**21:21**]	21	**PV-21**	Nearly fully threaded	13	87
[**42:21**]	42	**PV-21**	Nearly fully threaded + 1 equiv. free **LCD**	7	93
[**2:8**]	2	**PV-8**	1/4 Threaded	55	45
[**4:8**]	4	**PV-8**	2/4 Threaded	25	75
[**8:8**]	8	**PV-8**	Nearly fully threaded	3	97

[a] The pseudopolyrotaxanes (20 mM in **LCD**) assembled from different ratios of **LCD** rings to **PV** thread yield responses in the Bradford assay that reflect their expected degree of threading when compared to the response of the corresponding free polyviologen when tested at 1 mM polyviologen repeating unit. [b] TBA defined in eqn 1.

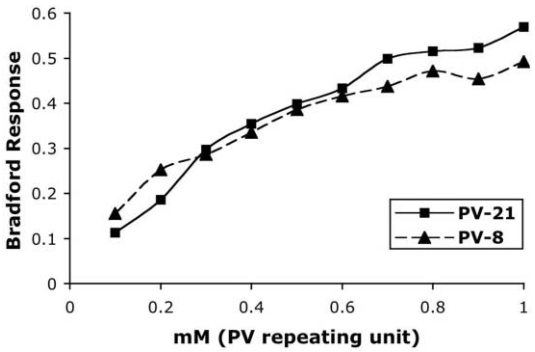

Fig. 2 Plot of response in the Bradford assay (OD_{595} sample − OD_{595} background) *vs.* polyviologen concentration for free polyviologens **PV-21** (squares, solid line) and **PV-8** (triangles, dashed line).

for the degree of threading. The concentration of 1 mM polyviologen repeating unit is convenient to normalize between the polyviologens of different lengths, and yields Bradford assay readings well within the reliable range for UV-Vis detection prior to substraction of the solution background. At this concentration, the assemblies gave readings that were above solution background, in the suggestive qualitative order [**42:21**] < [**21:21**] ≪ [**10:21**] ≪ [**5:21**]. Considered quantitatively, assembly [**5:21**] yields a value (OD_{595} [**5:21**] − OD_{595} background) that was 77% the value (OD_{595} **PV-21** − OD_{595} background) of the corresponding free polyviologen, in excellent accordance with our expectation of one-quarter threading. Similarly, assembly [**10:21**] yields a value of 51%, the response of free **PV-21**, and [**21:21**] yields a value 13%, the response of free **P-21**, close to the >90% threading we estimated for [**17:17**] from ^{1}H NMR spectroscopy and TLC. In order to be able to correlate threading positively with the Bradford response, we have defined the quantity "threading by Bradford assay" (TBA) as:

$$100 \times \left(1 - \frac{(OD_{595}\ \text{Sample} - OD_{595}\ \text{Background})}{(OD_{595}\ \text{FreePV} - OD_{595}\ \text{Background})}\right) \qquad (1)$$

such that the TBA percentages for assemblies [**5:21**], [**10:21**], and [**21:21**] are 23, 51 and 87%, respectively. Assembly [**42:21**], which contains an extra **LCD** ring for every polyviologen repeating unit, is more threaded (93% by this assessment) than assembly [**21:21**]. For the **PV-8**-based assemblies, the Bradford assay over-estimates the presumed degree of threading, especially for [**2:8**] and [**4:8**].

Even regarding proteins, it is not clear[24–26] what Coomassie Blue is binding to that induces the blue shift, although basic,[25–28] aromatic,[28] and hydrophobic[25,29] residues have all been implicated. Since the bipyridinium portion of the polyviologens is both positively charged and aromatic, and viologens are known[30] to bind anionic dyes in aqueous solution, we tested methyl viologen in the Bradford assay. However, methyl viologen (20 mM) did not produce a response above background. It is thus likely that binding to the polyviologens is primarily hydrophobic in nature, augmented by electrostatic interactions between the negatively charged dye and the positively charged backbone. Consistent with the fact that short peptides do not produce a Bradford response,[27] a relatively large binding surface is probably necessary: a cut-off[24,25] of >3000–5000 MW has been suggested for an adequate Bradford response. Despite their extensive charge, unthreaded polyviologens are collapsed hydrophobic coils in aqueous solution.[31] Threading leads to a more extended conformation,[31] and it is clear that "sugar-coating" the decamethylene segments, especially with CDs displaying bulky, hydrophilic lactosides, will reduce the overall hydrophobic surface area. It is likely that a low degree of threading is more of a perturbation to a smaller polyviologen, a likelihood which suggests why the assay appears to describe more accurately the degree of threading for **PV-21**, and to over-estimate the degree of threading for **PV-8**.

Dethreading of pseudopolyrotaxanes

Having uncovered a simple colorometric assay related to the degree of threading, we noticed that diluted phosphate-buffered saline (PBS) solutions[32] of the pseudopolyrotaxanes left standing gave a higher response in the Bradford assay than freshly diluted solutions of the same concentration. We suspected that this observation could be a result of dethreading, a kinetically slow process of re-equilibriating to a lower degree of threading upon dilution. It is important to note, that—in contrast to diluted solutions left standing—freshly diluted samples from the stock solutions (20 mM in **LCD**) give the same TBA even after months of standing at room temperature, and that the TBA percentages of solutions undergoing self-assembly from the free components increase over time (*vide infra*). Both of these observations suggest that the Bradford assay is reporting on the solutions themselves and not, for example, on chemical degradation upon standing or induced by exposure to the dye and its acidic buffer. Thus, we began to study dethreading systematically by taking samples from standing solutions and performing the Bradford assay at various points in time (Table 2 and Fig. 3) following dilution to 1 mM polyviologen repeating unit from pure H_2O stock solutions into PBS buffer. Because the pseudopolyrotaxanes are each self-assembled from 20 mM **LCD**, but have different ratios of ring-to-thread, the concentration of 1 mM polyviologen repeating unit represents a different dilution for each assembly, ranging from 10-fold for [**42:21**] to 84-fold for [**5:21**]. Nevertheless, at this concentration, dethreading can occur to a significant extent in 4 h, particularly for assemblies with ≥50% threading. An equilibrium is reached in *ca.* 2 d, in which assembly [**21:21**], originally 87% threaded by the Bradford assay, is only 50% threaded, and assembly [**10:21**], originally 50% threaded becomes 25% threaded. As expected, assembly [**42:21**], which contains an extra equivalent of **LCD** for every polyviologen repeating unit, is the least affected; it dethreads to the starting level of the 1 : 1 assembly [**21:21**]. While it is less straightforward to relate the Bradford response of

Table 2 Dethreading after dilution[a]

	TBA (%) after dilution[a]		
Assembly	≤15 min[b]	4 h	≥2 d
[**5:21**]	23	22	13
[**10:21**]	51	39	30
[**21:21**]	87	73	52
[**42:21**]	93	89	85
[**2:8**]	45	44	35
[**4:8**]	75	55	47
[**8:8**]	97	83	72

[a] Dilution to 1 mM in polyviologen repeating unit from the stock solutions at 20 mM in **LCD**. [b] Values also reported in Table 1.

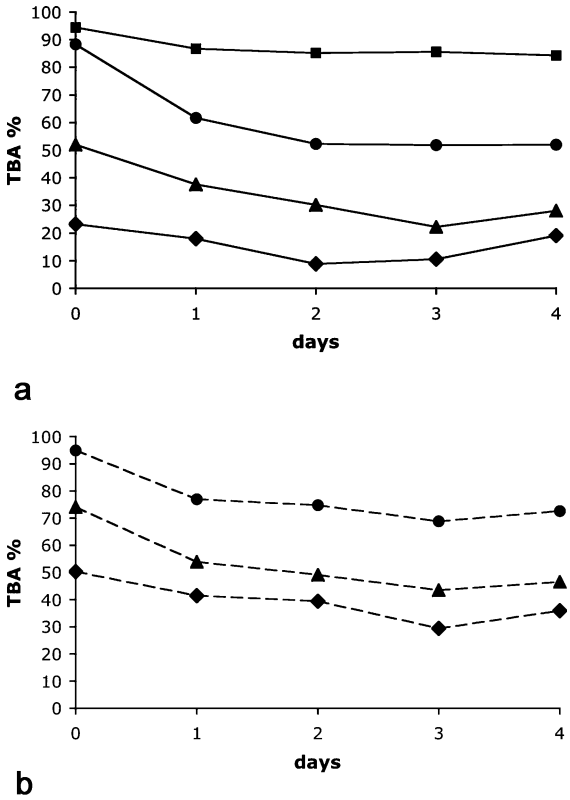

Fig. 3 (a) Time course following dilution to a concentration of 1 mM polyviologen repeating unit from the stock solutions (20 mM in **LCD**) of **PV-21**-based assemblies (solid lines) [**42:21**] (squares), [**21:21**] (circles), [**10:21**] (triangles), and [**5:21**] (diamonds). (b) Time course following dilution to a concentration of 1 mM polyviologen repeating unit from the stock solutions (20 mM in **LCD**) of **PV-8**-based assemblies (dashed lines) [**8:8**] (circles), [**4:8**] (triangles), and [**2:8**] (diamonds).

the **PV-8**-based assemblies to an actual degree of threading, the trend (Fig. 3b) towards dethreading is clearly similar.

These results appear to contradict our earlier finding[17] with pseudopolyrotaxane [**17:17**] that a 50-fold dilution resulted no change in the ^{1}H NMR spectrum even after a week. One potential difference is the dilution into PBS buffer rather than into pure D_2O. However, we discovered little to no difference for diluting stock solutions of the **PV-21**-based assemblies into pure H_2O or PBS buffer, at least according to the Bradford assay (Fig. 4). Thus, after ruling out degradation or enhanced dethreading in PBS buffer, it is hard to conceive of any phenomena other than dethreading that accounts for these results. Indeed, dethreading as measured by the Bradford assay correlates with our observations of diminished efficacy of diluted solutions left standing in functional assays with Galectin-1.[20] The discrepancy with the original NMR dilution experiment may be a consequence of to the complexity of the ^{1}H NMR spectra, particularly the overlap of resonances assigned to complexed and uncomplexed species.[17] Indeed, self-assembly as measured by the Bradford assay (*vide infra*), is a slower process than we expected from ^{1}H NMR spectroscopy, with small changes happening in the slow final stages (>3 d) which may not be readily visible by ^{1}H NMR spectroscopy. This situation produces two problems: imprecise readout, and, because the sample was diluted at 4 d, the spectrum, if precisely

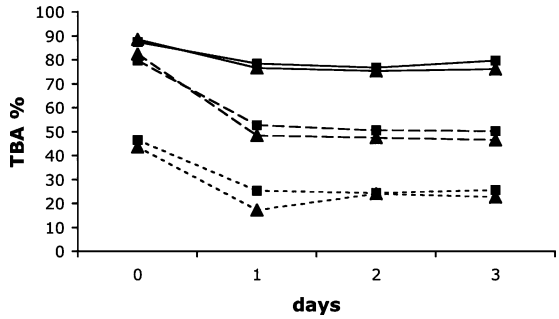

Fig. 4 Time course following dilution into either pure H_2O (squares) or PBS buffer (triangles). Stock solutions (20 mM in **LCD**) of assemblies [**42:21**] (solid lines), [**21:21**] (dashed lines) and [**10:21**] (dotted lines) were diluted to 1 mM polyviologen repeating unit.

read, would represent less of a difference upon re-equilibrating than if equilibrium would have been fully established.

α-CD pseudopolyrotaxanes

The lactoside portion of **LCD** is not required for responsiveness to the Bradford assay. Following self-assembly from 20 mM native α-CD (no lactoside) and **PV-21**, four aqueous solutions with α-CD-to-polyviologen repeating unit ratios of roughly 1 : 4, 1 : 2, 3 : 4, and 1 : 1, the TBA percentages for the resulting pseudopolyrotaxanes α-[**5:21**], α-[**10:21**], α-[**15:21**], α-[**21**:**21**], respectively (the designation "α" is used to represent native α-CD as opposed to **LCD**), were obtained (Table 3). The **LCD**-based assembly [**15:21**]—not yet tested in biological assays—was also self-assembled for comparison. The measured degrees of threading were quite similar for both **LCD**- and native α-CD-based assemblies in the case of **PV-21**. Thus, functionalization of α-CD has little effect on the degree of threading at equilibrium, at least by this measure. (In addition to potentially affecting the actual degree of threading, the bulky hydrophilic lactosides could have an additional effect on the binding/blue shift of Coomassie Blue with the polyviologen backbone.)

Using the Bradford assay as a simple spectroscopic readout, the time course of self-assembly of native α-CD and **PV-21** at two concentrations—20 mM in lactoside (aliquots freshly diluted for Bradford assay) and 1 mM in polyviologen repeating unit (aliquots taken directly from standing solution)—was monitored (Fig. 5, Tables 3 and 4) at the four different ratios representing α-[**5:21**], α-[**10:21**], α-[**15:21**], and α-[**21:21**] in pure H_2O. We were surprised to find that the rate of the final stages of self-assembly is much slower than expected from ^{1}H NMR spectroscopy, perhaps indicating that this spectroscopic technique is not sensitive enough to distinguish

Table 3 LCD and α-CD pseudopolyrotaxanes[a]

LCD Assembly	TBA (%)	α-CD Assembly	TBA (%)
[**5:21**]	23[b]	α-[**5:21**]	29
[**10:21**]	51[b]	α-[**10:21**]	50
[**15:21**]	66	α-[**15:21**]	71
[**21:21**]	87[b]	α-[**21:21**]	88

[a] Aliquots (1 mM polyviologen repeating unit) tested immediately following dilution from stock solutions (20 mM in **LCD** or α-CD). [b] Values also reported in Table 1.

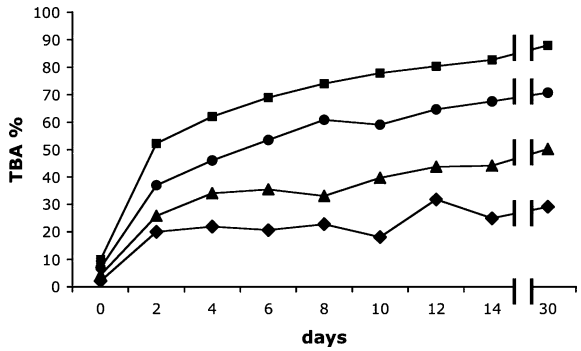

Fig. 5 Time course for self-assembly of α-CD-based pseudopolyrotaxanes at 20 mM in α-CD monitored by the Bradford assay for assemblies α-[**21:21**] (squares), α-[**15:21**] (circles), α-[**10:21**] (triangles), and α-[**5:21**] (diamonds).

these slow final stages of threading. After 3 d, by which time each of the assemblies has reached *ca.* 70% of its TBA value at equilibrium, continued threading slows down considerably, with this final equilibrium position taking more than two weeks to attain! By contrast, the **LCD**-based pseudopolyrotaxanes, while still slower to become fully threaded than initially expected, are faster than the α-CD-based pseudopolyrotaxanes. For example, [**15:21**] reaches equilibrium (66% TBA) in *ca.* 6 d, and all are complete within two weeks. Dethreading of the α-CD-based pseudopolyrotaxanes is also slower than that observed for the corresponding **LCD**-based assemblies since the re-establishment of equilibrium upon dilution to 1 mM polyviologen repeating unit took *ca.* 4–6 d (Fig. 6) for these α-CD-based assemblies rather than *ca.* 2 d (Figs. 3 and 4) for the corresponding **LCD** pseudopolyrotaxanes. The TBA values at the re-established equilibrium are again quite similar—compare the corresponding assemblies between Tables 4 and 2. One possible reason for the faster kinetics of assembly and disassembly with the more bulky, substituted CD ring is that, at equilibrium, the flexible and adaptable nature of the pseudopolyrotaxane scaffold avoids steric clashes that emerge for the bulkier **LCD**-based assemblies when they are shifted out of—or shifted toward—equilibrium. These negative steric interactions could allow **LCD**-based pseudopolyrotaxanes to escape from kinetically trapped states more easily than with the smaller native α-CD rings. A true equilibrium should be approachable from both sides. Self-assembly (21 d) from the individual components (α-CD, **PV-21**) at the lower concentration of 1 mM polyviologen repeating unit (Table 4), yields equilibrium TBA values that are in close accordance with those obtained by diluting pre-assembled pseudopolyrotaxanes to this concentration, confirming that the degree of threading in these pseudopolyrotaxanes is truly under thermodynamic control.

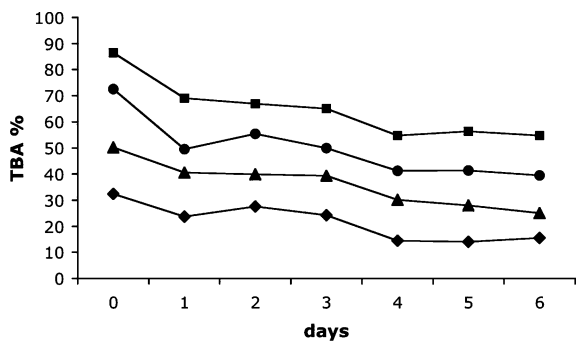

Fig. 6 Time course following dilution to a concentration of 1 mM polyviologen repeating unit from the stock solutions (20 mM in α-CD) of α-CD-based assemblies α-[**21:21**] (squares), α-[**15:21**] (circles), α-[**10:21**] (triangles), and α-[**5:21**] (diamonds).

Table 4 Equilibrium from both sides[a]

α-CD Assembly	Threading[b] TBA (%, 21 d)[d]	Dethreading[c] TBA% (%, 4 d)[d]
α-[**5:21**]	16	17
α-[**10:21**]	28	28
α-[**15:21**]	38	41
α-[**21:21**]	51	56

[a] Similar TBA percentages upon reaching equilibrium are obtained for the assemblies at 1 mM polyviologen repeating unit. [b] Those self-assembled at the above concentration. [c] Those diluted from stock solutions (20 mM in α-CD). [d] Time to reach equilibrium.

Conclusions

Beside their use in supramolecular chemistry,[31] polyviologens are of technological interest as components of electrochemical sensors[33] and (bio)-electronic devices.[34] In these contexts, the Bradford assay could be useful for determining polyviologen concentration and/or average chain length, and may be able to report on the noncovalent association of other kinds of molecules that block or modify hydrophobic portions of the polymer. In the context of supramolecular chemistry, a simple, coloremetic assay for analyzing the degree of threading in pseudopolyrotaxanes is highly desirable. As noted, there is considerable interest[12] in biological applications of CD-based (pseudo)polyrotaxanes, and the Bradford assay is likely to be able to report on the degree of threading in many of these systems. Indeed, if the hypotheses that (1) large hydrophobic surfaces in aqueous solution are needed for the binding/blue shift of Coomassie Blue,[25,29] and (2) threading of species with hydrophilic surfaces onto hydrophobic segments of polymers can diminish this binding/blue shift are accurate, then the Bradford assay may be applicable for monitoring threading/dethreading in a wide range of aqueous supramolecular systems. For our system in particular, the realization, courtesy of the Bradford assay, that dethreading can occur in **LCD**-polyviologen pseudopolyrotaxanes more readily than we originally supposed from NMR spectrosoptic analysis, and indeed responds on the time-scale of precipitation experiments,[20] has considerable implications for our understanding and future development of these self-assembled multivalent neoglyconjugates.

Although supramolecular chemistry and biochemistry have many similarities, a considerable difference is the huge amount of effort in analytical chemistry that has been devoted to biochemical problems. Here, we show that an idiosyncratically biochemical assay, the Bradford assay, can be applied to an idiosyncratically supramolecular problem—namely, the threading and dethreading of pseudopolyrotaxanes. Indeed, major techniques, such as MALDI TOF mass spectrometry[35] and gel electrophoresis,[36] which were developed for the analytical characterization of biomolecules are being applied to supramolecular systems. Electrophoretic gels have been employed to separate carbon nanotubes

via their noncovalent association with DNA,[37] and even to study wholly artificial self-assembling polymers.[38] The drive to study larger and larger biomolecules and their complexes by mass spectrometry[39] is having a revolutionary affect on supramolecular chemisty.[40] However, in addition to these major techniques, biochemistry is replete with simple, often colometric,[24] analytical tools[41] such as the Bradford assay. We suggest that these may be more broadly useful to supramolecular chemists than previously recognized.

Experimental

General experimental

α-CD was purchased from Wacker. The syntheses of **LCD**,[15] **PV-8**,[20] and **PV-21**[20] have been described elsewhere. Initial stock solutions of pseudopolyrotaxanes [**2:8**], [**4:8**], [**8:8**], [**5:21**], [**10:21**], [**21:21**], and [**42:21**] were self-assembled as described[20] at a standard concentration of 20 mM **LCD** in pure H_2O. Additional stock solutions (20 mM **LCD** in pure H_2O) of assemblies [**5:21**], [**10:21**], and [**21:21**] and new assemblies [**15:21**], α-[**5:21**], α-[**10:21**], α-[**15:21**], and α-[**21:21**] were prepared in 1.5 mL Eppendorf tubes from aliquots of freshly prepared aqueous solutions of **PV-21** (8 mM) and either **LCD** (40 mM) or α-CD (40 mM) with added H_2O as necessary to a standard final concentration of 20 mM in either α-CD or **LCD** and variable concentrations of **PV-21** according to the ratios indicated, with final volumes of 200 or 400 μL. To follow self-assembly at the concentration used to study dethreading, stock solutions (1 mM polyviologen repeating unit in pure H_2O) of α-[**5:21**], α-[**10:21**], α-[**15:21**], and α-[**21:21**] were prepared in 1.5 mL Eppendorf tubes from aliquots of freshly prepared aqueous solutions of **PV-21** (8 mM) and α-CD (40 mM) and diluted with H_2O to a final volume of 400 μL and a standard concentration of 1 mM polyviologen repeating unit with variable concentrations of α-CD according to the ratios indicated. All solutions were extensively vortexed at the time of mixing, and vortexed briefly prior to removal of aliquots for dilution or the Bradford assay.

Bradford Assay

Bio-Rad Protein Assay dye reagent concentrate solution containing Coomassie Blue G-250 in 2 : 1 : 1 phosphoric acid–water–methanol was purchased from Bio-Rad and used according to the specifications provided. This solution was stored at 4 °C and aliquots were diluted 1 : 4 with water to form the stock solution for the Bradford assay. Stock solutions were stored at room temperature in the dark and freshly prepared at least once every two weeks. The assay was performed in 96-well plates using a Bio-Rad Benchmark Plus microplate UV-Vis spectrophotometer. 200 μL aliquots of the Coomassie Blue G-250 stock solution were added to 10 μL aliquots of sample solution. The 96-well plates were then shaken on an orbital shaker for 10 min prior to spectroscopic analysis at OD_{595}. Color development was rapid and judged to be stable in this time frame. Each data point was performed at least in duplicate. The assay was performed with at least two background wells containing either pure water or PBS buffer (in the case of dilutions into PBS), and at least two wells containing the relevant free polyviologen, either **PV-8** or **PV-21**. For pseudopolyrotaxanes, all data points were taken at the concentration of 1 mM polyviologen repeating unit, either from solutions at this concentration or by freshly diluting aliquots from the stock solutions (20 mM in **LCD** or α-CD) to this concentration.

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