EDGE ARTICLE

Catenanes from catenanes: quantitative assessment of cooperativity in dynamic combinatorial catenation†

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A new azobenzene-based dithiol building block was developed which, upon oxidation, forms predominantly a [2]catenane consisting of two interlocked trimers. In the presence of cyclodextrin templates a series of [2] and [3]catenanes was formed instead. We developed a method that enabled estimating the equilibrium constants for catenation in all of these systems. The formation of the [3] catenanes is either cooperative or anticooperative, depending on the nature of the cyclodextrin. Using molecular dynamics simulations, we linked positive and negative cooperativity to, respectively, burying and exposure of hydrophobic surfaces upon catenation. Our results underline the importance of directly quantifying noncovalent interactions within catenanes, as the corresponding pseudo-rotaxane model systems were found to be poor predictors of binding interactions in the actual catenane.

Introduction

Catenanes represent an important class of mechanically interlocked molecules and are popular motifs for the construction of molecular machines.[1] Higher catenanes are especially promising topologies for unidirectional machines.[2] Catenane synthesis is increasingly performed using reversible chemistry, which ensures that catenane production occurs under thermodynamic control, allowing for error-correction to enhance the yield of the catenation step.[3] Dynamic combinatorial chemistry[4] is a particularly powerful tool for the discovery and synthesis of new catenanes.[5] Dynamic combinatorial libraries (DCLs) are mixtures of interconverting molecules produced by linking building blocks together using reversible chemical bonds. The distribution of all molecules in such a network is typically governed by thermodynamics. Addition of template molecules can influence the stability of the library members and thereby alter the composition of DCLs. In the case of applying this strategy for exploring new catenanes, the non-covalent interactions lead to catenation and shift the equilibrium of the mixture towards the formation of the catenanes. The production of catenanes relies in nearly all cases on non-covalent interactions that drive the self-assembly of the

components prior to the ring-closing reaction that produces the catenane.

Given the importance of these noncovalent interactions in catenation, methods that allow the direct quantitative assessment of their strength are desirable. Insight into interaction energies is essential for applications that involve the movement of catenane rings with respect to each other.[2,6] For catenanes containing three or more rings, catenane synthesis may exhibit positive or negative cooperativity.[7] While positive allosteric regulation in biological systems has been well studied, design of supramolecular systems characterized by positive allosteric cooperativity has seen relatively slow progress.[8,9] Although cooperative effects could improve the synthesis of higher catenanes, so far they have not been explored. The problem is rooted in the lack of a general method for quantifying cooperativity for higher catenanes. In fact, catenation equilibria are only rarely measured even for [2]catenanes. This may stem from the fact that the most common methods for determining binding interactions in supramolecular chemistry (NMR, UV or ITC titrations) cannot be used to determine binding interactions in catenanes as the mechanical bond between the rings in a catenane do not allow for typical titration experiments.

We now show that a global analysis of product distributions obtained for reversible catenation reactions performed at different concentrations allows the quantification of binding interactions within the catenanes, even when the concentration of some of the components involved in catenation cannot be directly quantified. We demonstrate the use of this methodology for two different cyclodextrin–azobenzene-based[10] [3]catenane systems: one which exhibits positive cooperativity and one where catenation is negatively cooperative. This

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† Electronic supplementary information (ESI) available: Details of materials, synthesis of building block **1**, identification of library members, ¹H NMR spectra of catenanes, evaluation of equilibrium constants by DCLFit, UV-vis titrations of building block **1** to β-CD and γ-CD, molecular dynamics simulations and HPLC analysis of libraries without/with α-CD. See DOI: 10.1039/c4sc01998a

methodology should be applicable to any thermodynamically controlled catenation reaction.

Results and discussion

Preparation of DCLs and analysis of their compositions

DCLs were prepared from azobenzene derivative **1** (Scheme 1). This building block contains two thiol groups which can be oxidized to form a small DCL of macrocyclic disulfides.[11] It also features two carboxylic acid groups for water solubility. Its synthesis is described in the ESI.†

Stirring an aqueous solution of dithiol **1** in the presence of oxygen from the air gave rise to a small dynamic combinatorial library of disulfides. Disulfide exchange in these systems takes place through reaction between the disulfides and residual thiolate anion[11] and the equilibrium distribution is reached after 4 days. The resulting mixture was analysed by HPLC-MS (Fig. 1) which revealed a dominant peak with mass 1994.3, corresponding to six units of **1**. MS-MS analysis of this peak (inset in Fig. 1a) showed the trimer of **1** as the only fragmentation product, suggesting that this compound is [2]catenane **2** made from two interlocked timers of **1**. Cyclic trimer **3** and tetramer **4** were also detected as minor constituents of the small DCL.

We then prepared the same DCL, but this time added 0.25 equivalents (with respect to **1**) of one of the cyclodextrin (CD) homologues (α-CD, β-CD or γ-CD), respectively, as a template. Upon addition of α-CD the library composition remained unchanged (see ESI†), but in the presence of β-CD or γ-CD new peaks appeared in the HPLC chromatograms. Upon addition of β-CD three new peaks appeared, as shown in Fig. 1b. MS analysis (see ESI†) revealed that these correspond to isomeric
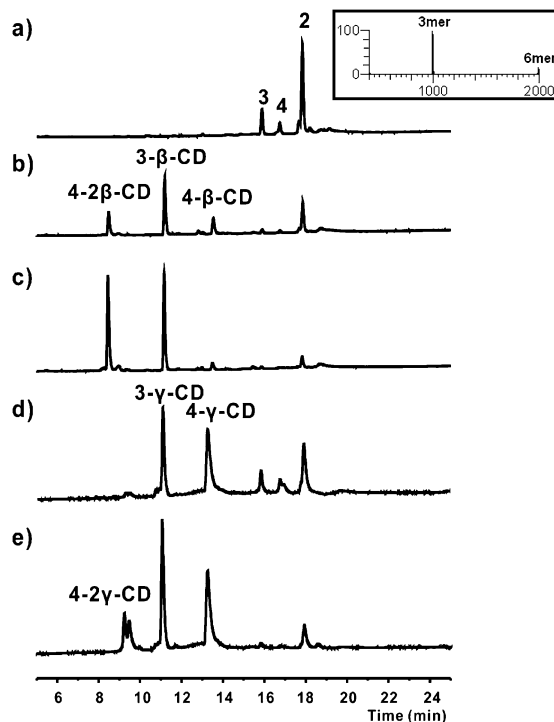

Fig. 1 HPLC-MS analysis of DCLs made from 2.0 mM building block **1** in aqueous borate buffer (50 mM, pH 8.2) (a) without β-CD; (b) with 0.25 eq. β-CD; (c) 1.0 eq. β-CD; (d) with 0.25 eq. γ-CD and (e) 1.0 eq. γ-CD. The inset shows the MS-MS fragmentation spectrum of [2] catenane **2**.

[3]catenanes **4**-2β-CD consisting of one tetrameric macrocycle interlocked with two β-CDs, and [2]catenanes **3**-β-CD and **4**-β-CD, consisting of one trimeric or one tetrameric macrocycle, respectively, interlocked with one β-CD.[12] When γ-CD was used as a template instead of β-CD, analogous [2]- and [3]catenanes were obtained, but in markedly different ratios (Fig. 1d). Formation of the γ-CD [3]catenanes was less favorable than for β-CD, suggesting differences in the cooperativity of catenation between the two cyclodextrin homologues. When the amount of cyclodextrin was increased to one equivalent compared to building block **1**, α-CD still did not amplify any species (see ESI,† Fig. S46). However, for the libraries in the presence of β-CD or γ-CD, the concentration of the [3]catenanes increased at the expense of the other catenanes (Fig. 1c and e).

We have isolated the two [3]catenanes **4**-2β-CD as a mixture of isomers and [2]catenane **3**-β-CD using preparative HPLC. The ¹H-NMR spectrum of **4**-2β-CD showed broad peaks of the azobenzene units, whereas the [2]catenane **3**-β-CD gave sharp signals, in which all three azobenzene units were indistinguishable (see ESI†). Stoddart[12b] has observed such desymmetrisation in cyclodextrin catenanes with two chemically different stations to which the cyclodextrin can bind, but not in an analogous catenane with three degenerate stations. Catenane **3**-β-CD contains three degenerate stations; the fact that no desymmetrisation is detected is therefore not unexpected. These results suggest that the [3]catenanes **4**-2β-CD have slower rotation kinetics, presumably because the motions of the two β-
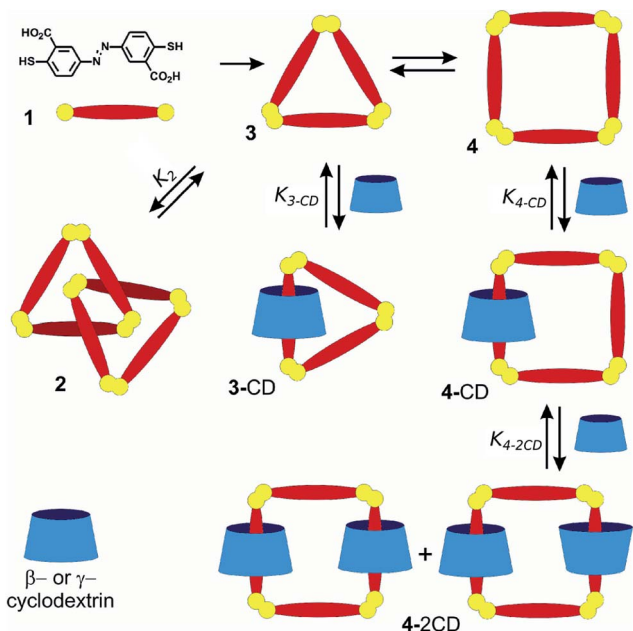
Scheme 1 DCLs made from building block **1** in the presence of β-CD or γ-CD.

CD rings are coupled. This notion was supported by inspection of CPK models, which revealed that it is sterically not feasible to have two cyclodextrin rings occupy adjacent azobenzene stations in catenanes **4**-2β-CD, hence the cyclodextrin rings cannot change stations independently.

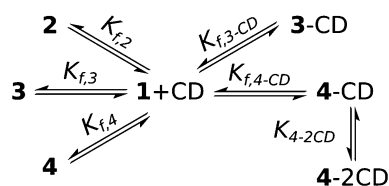### Determination of equilibrium constants for catenation

Establishing the equilibrium constant for catenation for **2** is trivial. The catenane and the macrocycle from which it is constituted appear as separate peaks on the HPLC trace and the concentration of both species can be quantified‡ (see ESI†). This then directly gives the equilibrium constant from eqn (1):

$$K_2 = [\text{catenane } \mathbf{2}]/[\text{trimer } \mathbf{3}]^2 \qquad (1)$$

The equilibrium constant for catenation obtained for this system is $\log K_2 = 3.63 \pm 0.18$.

Having established the nature of the main species in the DCL in the presence of the cyclodextrin homologues, we proceeded with the determination of the binding affinities. Note that the concentration of free cyclodextrin is not readily measurable in the present system. Thus, we cannot use eqn (1) and we had to resort to an approach that relies on fitting the concentrations of the various species that can be directly quantified to an equilibrium model that explicitly includes the catenanes and the corresponding non-catenated macrocycles. The model is shown in Scheme 2. The only specific equilibrium constants that are fitted include those that relate library members to their appropriate building blocks (termed formation constants: K_f).[13] The building block represents a common reference state through which the relative stabilities of all the species in the mixture may be compared. Note that these equilibria do not represent realistic reaction pathways, since disulfide reduction does not occur under the conditions of our experiments. Therefore the values of the equilibrium constants that relate library members to their building blocks have no physical meaning. Nevertheless the differences between these values can be used to assess the other equilibria in the system. For example, the equilibrium constant ($K_{4\text{-CD}}$) for [2]catenane formation from **4** and a CD equals to $K_{f,4\text{-CD}}/K_{f,4}$. This methodology has previously been validated in other DCLs of host–guest systems.[14]

In order to obtain accurate estimates of the equilibrium constants in the system, it is necessary to accumulate a data set for a number of different DCLs under a range of experimental conditions (*i.e.* different building block and cyclodextrin concentrations). Thus two series of eight DCLs were set up, with

concentrations of building block **1** and β-CD or γ-CD in the range of 0 to 5 mM, respectively (Fig. 2).

The concentrations of the DCL members in the presence or absence of β-CD or γ-CD were determined by analytical HPLC.‡ Fitting the corresponding dataset to the model in Scheme 2 may be performed with the help of established multivariate analysis algorithms, which iterate and adjust the values of the equilibrium constants until the error between fitted and observed data is minimal. We have previously implemented these in our DCLFit software.[13] As with any multivariate analysis, it is important to ensure that the fitting converges on an error value that corresponds to the global minimum, rather than a local minimum. Thus, we ran the fitting procedure starting from 500 different estimates of initial equilibrium constants and found the same minimum error in more than 99% of runs, each time giving closely comparable values for the equilibrium constants. The entire analysis was repeated twice for two separately prepared sets of DCLs, and the averaged results are shown in Table 1.

Scheme 2 Model used to fit the library distributions obtained for different concentrations of cyclodextrin template. Stoichiometries omitted for clarity.
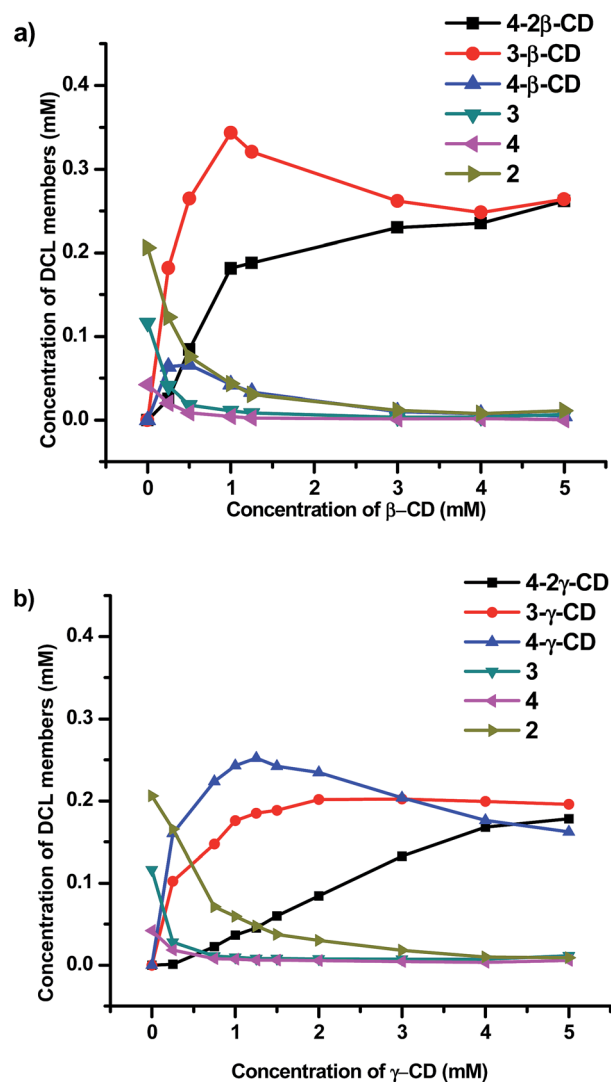

Fig. 2 Product distribution in the DCL made from building block **1** as a function of the concentration of (a) β-CD and (b) γ-CD template.

We also measured the equilibrium constant between building block **1** and β-CD and γ-CD by UV titration (see ESI†). These results are also included in Table 1. In order to compare the values for binding of the CDs to building block **1** and the various catenation equilibria, we corrected the catenation equilibrium constants for the number of azobenzene binding sites.§ The thus corrected equilibrium constants for binding β-CD within [2]catenanes **3**-β-CD and **4**-β-CD are similar to binding of β-CD to monomer **1** (see Table 1). However, the equilibrium constant for incorporating the second β-CD to yield the [3]catenanes is an order of magnitude larger, indicating that the formation of **4**-2β-CD exhibits strong positive cooperativity.

Repeating the same analysis for γ-CD reveals a very different behaviour. The binding affinity between **1** and γ-CD is weaker than the one between **1** and β-CD. Yet, the catenation of the first γ-CD is somewhat more favourable than the corresponding process for β-CD, especially for the tetramer. Note that for both γ-CD [2]catenanes the equilibrium constant for catenation is substantially larger than the equilibrium constant for binding of **1** to γ-CD. However, the incorporation of the second γ-CD to yield [3]catenanes **4**-2γ-CD is two orders of magnitude weaker than the binding of the first γ-CD, hence [3]catenation with this cyclodextrin homologue shows strong negative cooperativity.

It is interesting to note how the concentrations of the two [2]catenanes depend on the cyclodextrin concentration. At low cyclodextrin concentrations the catenated tetramer **4**-γ-CD dominates, while at higher concentrations the catenated trimer **3**-γ-CD is present at higher concentrations, despite the fact that **4**-γ-CD exhibits the highest binding affinity (Table 1). This behaviour is a consequence of the system maximizing binding energy. At high cyclodextrin concentrations, when building block **1** is limiting, it is possible to harvest more binding energy by making more copies of a relatively weakly binding catenane. At lower cyclodextrin concentrations, when the cyclodextrin is limiting, the system will preferentially catenate the macrocycle that has the highest affinity. Similar systems effects have been observed previously in dynamic combinatorial libraries.[15]

The quantitative data in Table 1 indicates that [3]catenanes based on β-CD are more stable than those based on γ-CD. This prediction was verified in a competition experiment in which both cyclodextrins were added together (alongside α-CD) to the same DCL. HPLC analysis confirmed that [3]catenanes **4**-2β-CD dominate the mixture (Fig. 4a). Interestingly, while a small amount of **4**-2γ-CD was observed, no mixed catenane **4**-β-CD-γ-CD could be detected, indicating that the catenanes are self-sorting.

Molecular dynamics (MD) simulation of the catenanes

In order to obtain insight into the origins of the markedly different cooperativity of the β- and γ-CD systems, we decided to use molecular dynamics (MD) simulations, which have been successfully utilized to study cyclodextrin–azobenzene complexes.[16] We employed the Amber 11 program package,[17] the GAFF force field parameters[18] for molecule **4**, torsional azobenzene parameters proposed by Duchstein et al.[19] and GLYCAM04 (ref. 20) for the cyclodextrins. Partial atomic charges of the azobenzene monomers were obtained using a restrained electrostatic potential fit (RESP) as implemented in the R.E.D.-III.5 tools.[21] All simulations have been performed in a periodic box of TIP3P water[22] (see ESI† for the detailed methodology). Representative 3-D structures are shown in Fig. 3, stripped trajectories, and resulting raw geometric data were deposited to figshare.[23]

We simulated all systems involved in the equilibria leading to the formation of both [3]-catenanes (**4**, CD, **4**-CD, **4**-2CD for both β- and γ-CD) for 40 ns. After an initial equilibration phase we observed that all systems converged to stable trajectories. Visual inspection of equilibrated trajectories revealed that in all cases the systems evolved towards conformations minimizing exposed hydrophobic surfaces (Fig. 3). Both cyclodextrins and macrocycle **4** contain relatively large hydrophobic surfaces. Formation of inclusion complexes with azobenzene leads to a decrease of the hydrophobic hydration[24] of both the cyclodextrin and the azobenzene macrocycle. We estimated the change in water-accessible surface area upon catenation, averaged over the last 20 ns of the simulations. The resulting data for each of the catenanes are shown in Table 2.

Table 1 Equilibrium constants for binding of **1** to β-CD and γ-CD and for catenane formation[a]

Templates	log K_1	log $K_{3\text{-CD}}$	log $K_{4\text{-CD}}$	log $K_{4\text{-2CD}}$[b]
β-CD	3.22 ± 0.15	3.88 ± 0.12	3.65 ± 0.13	4.05 ± 0.22
Corrected[c]	3.22 ± 0.15	3.41 ± 0.12	3.05 ± 0.12	4.05 ± 0.22
γ-CD	2.45 ± 0.08	4.19 ± 0.08	4.71 ± 0.12	2.46 ± 0.11
Corrected[c]	2.45 ± 0.08	3.71 ± 0.08	4.13 ± 0.08	2.46 ± 0.11

[a] The corresponding binding constants K are in M^{-1}. [b] $K_{4\text{-2CD}}$ refers to the equilibrium constant for the formation of [3]catenane from [2]catenane **4**-CD. [c] Equilibrium constants to which a statistical correction has been applied to account for the number accessible azobenzene sites.§ No statistical correction was applied for the formation of the [3]catenanes, since the [2]catenanes have only a single accessible cyclodextrin binding site.
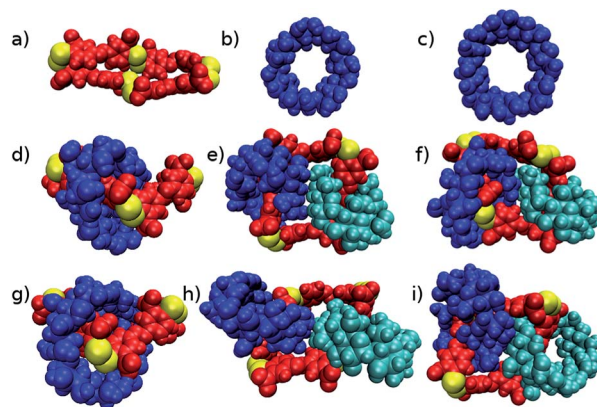

Fig. 3 Representative structures obtained using molecular dynamics: (a) **4**; (b) β-CD; (c) γ-CD; (d) **4**-β-CD; (e) **4**-2β-CD in antiparallel and (f) parallel orientation; (g) **4**-γ-CD; (h) **4**-2γ-CD in antiparallel and (i) parallel orientation.
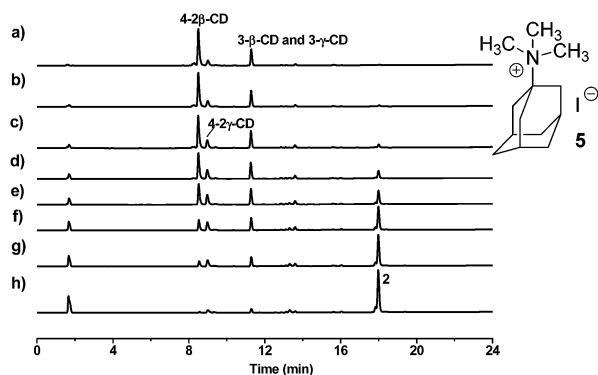
Fig. 4 HPLC-MS analysis of DCLs made from 2.0 mM building block **1**, α-CD, β-CD and γ-CD (2.0 mM each) in aqueous borate buffer (50 mM, pH 8.2) (a) without guest and in the presence of (b) 0.50 mM; (c) 1.0 mM; (d) 1.5 mM; (e) 2.0 mM; (f) 4.0 mM; (g) 8.0 mM and (h) 16 mM of guest **5**.

The flexibility of macrocycle **4** within the [2]catenanes allows it to wrap tightly around the outer part of the cyclodextrins, which, while less lipophilic than its cavity,[25] is still capable of screening hydrophobic azobenzenes from contact with water. Such adaptation is capable of offsetting hydration arising from partial opening of the macrocycle (Table 2). The structures of both [2]-catenanes, while relatively similar, show a few significant differences. The larger size of γ-CD allows it to encompass a larger fraction of the macrocycle **4**, so that the catenane can assume conformations in which more hydrophobic surface area is buried. Fig. 3g shows that γ-CD partially encapsulates two azobenzene units and one disulfide bond within its cavity, while β-CD can efficiently interact with only one azobenzene unit (Fig. 3d). This difference may explain why binding between **4** and γ-CD is almost two orders of magnitude stronger than with **1**, whereas for β-CD the corresponding affinities are relatively similar. Moreover, by localizing the cyclodextrin around only one azobenzene station, **4**-β-CD remains flexible and prone to temporary opening of the conformation of macrocycle **4** and exposing hydrophobic azobenzene surfaces to water (see ESI Fig. S21 and S28†). Additionally, γ-CD, on average, forms more

hydrogen bonds with macrocycle **4** than β-CD (see ESI Table S5†). These factors are main contributors to the more favourable formation of **4**-γ-CD over its β counterpart.

When the second cyclodextrin units are bound, **4** adopts an even more open conformation allowing two cyclodextrins to fill its interior completely (Fig. 2e). Somewhat similar adaptation was observed by Severin *et al.* for flexible coordination cages capable of encapsulating two coronene molecules, likely in a cooperative fashion.[9a] Formation of **4**-2β-CD does not result in any major changes to the interactions between **4** and the cyclodextrin cavity. However, the azobenzene units which are not directly involved in binding are screened from interacting with water by the rims of both β-CDs. These rims are not hydrophobic; in fact they are composed of hydrophilic hydroxyl groups. Nevertheless, the contact between them and the azobenzenes is more favourable than hydrophobic hydration of the latter, resulting in positive cooperativity.

In case of **4**-2γ-CD, the situation is drastically different. One of the cyclodextrins occupies a more central position within macrocycle **4**, interacting stronger with the azobenzene(s), but also pushing the other cyclodextrin outwards (Table 3). This behaviour is especially pronounced in the parallel isomer, where one CD is still tightly bound to two azobenzene units, pushing the second cyclodextrin outwards and exposing its hydrophobic interior (Fig. 2i). For the antiparallel isomer, both CDs are struggling to fill their insides with azobenzenes, which leads to even more unfavourable exposure of their cavities to water. The hydrophobic solvent accessible surface area (SASA) difference between these two isomers seems to be balanced by a higher number of intermolecular hydrogen bonds which can be formed within the antiparallel isomer (see ESI†).

Nevertheless, for both isomers the buried hydrophobic area per cyclodextrin is significantly smaller than for **4**-γ-CD (Table 2), leading to strong anticooperativity.

Guest-triggered catenane interconversion

The responsiveness of this system was investigated by introducing adamantane derivative **5**, known to bind β-CD (log K = 3.99 ± 0.02) and to a lesser degree γ-CD (log K = 3.5 ± 0.06).[26] DCLs were analysed at increasing concentration of guest **5**. When the amount of **5** was increased to 1.5 mM, the concentration of the catenanes made from β-CD decreased while the quantity of the [3]catenanes **4**-2γ-CD increased slightly. Guest **5** acts as a competitive binder for β-CD allowing more γ-CD to catenate the azobenzene macrocycles. Upon further increasing

Table 2 Changes of hydrophobic solvent-accessible surface area upon catenane formation for the different rings of the catenane[a]

Catenane	**4**[a]	CD1[b]	CD2[c]	Total[d]	Per CD[e]
4-β-CD	−75 ± 22	−187 ± 15	n/a	−262 ± 37	−262 ± 37
4-γ-CD	−82 ± 13	−203 ± 6	n/a	−285 ± 19	−285 ± 19
4-2β-CD$_{ap}$	−198 ± 18	−184 ± 2	−183 ± 3	−564 ± 23	−282 ± 12
4-2β-CD$_{p}$	−190 ± 22	−178 ± 3	−174 ± 6	−542 ± 31	−271 ± 16
4-2γ-CD$_{ap}$	−113 ± 10	−169 ± 4	−144 ± 4	−425 ± 19	−213 ± 10
4-2γ-CD$_{p}$	−146 ± 23	−192 ± 6	−161 ± 2	−499 ± 32	−249 ± 16

[a] Changes in hydrophobic SASA of [a]macrocycle **4** and CDs relative to free **4**; [b]CD1 (less hydrated) and [c]CD2 (more hydrated) relative to free CD; [d]the total hydrophobic SASA difference between the catenanes and free **4** and CDs [e]divided by number of CDs. Values were averaged over the last 20 ns of the simulations. The "ap" and "p" suffixes stand for antiparallel and parallel, respectively. All values given in Å².

Table 3 Average distances between the centres of mass of the cyclodextrins and **4**[a]

	4-2β-CD$_{ap}$	**4**-2β-CD$_{p}$	**4**-2γ-CD$_{ap}$	**4**-2γ-CD$_{p}$
CD1	7.2 ± 0.0	7.0 ± 0.2	7.7 ± 0.2	6.9 ± 0.3
CD2	7.0 ± 0.1	7.2 ± 0.2	8.9 ± 0.2	8.8 ± 0.2

[a] Distances listed in Å, averaged over the last 20 ns of simulations. The "ap" and "p" suffixes stand for antiparallel and parallel, respectively.

the concentration of **5**, the concentration of species catenated by both β-CD and γ-CD decreased liberating the azobenzene moieties for incorporation into catenane **2** (Fig. 4). These results show that it is possible to switch between different catenanes by addition of a competitive guest molecule.[27]

Conclusions

In summary, we have demonstrated a method for determining binding constants of topologically bound molecules. Such methodology is important given that binding affinities within catenanes may differ substantially from the more readily accessible affinities of their pseudorotaxane counterparts (up to two orders of magnitude difference in equilibrium constants in the present case). The dynamic covalent nature of the catenanes is essential for such analyses. We discovered that formation of the [3]-catenanes changed from exhibiting pronounced cooperative to anticooperative behaviour upon changing β-CD to γ-CD. MD simulations suggest an important role for the conformational flexibility of the tetrameric azobenzene macrocycle, allowing it to open to various extents to accommodate up to two cyclodextrins. In contrast, the relatively rigid cyclodextrins have a fixed cavity size, leading to a tight fit for β-CD and a mismatch for γ-CD resulting in partially exposed hydrophobic interiors. Finally, we could exploit the dynamic nature of the system by switching heterocatenanes back into homocatenanes by adding a guest. We believe that this study not only introduces a useful tool for analysing responsive interlocked molecules at systems level, but also deepens the understanding of the relationship between structure and cooperativity of such systems.

Acknowledgements

Notes and references

‡ Control experiments confirmed that the azobenzene unit has the same molar absorptivity independent of the compound into which it is incorporated. See section 5 of the ESI† for details.

§ In order to be able to compare the values for the binding constants of the CDs to building block **1** with the various catenation equilibrium constants, we corrected the latter for the number of azobenzene binding sites. The corrected binding constants of $K_{3\text{-CD}}$ and $K_{4\text{-CD}}$ are calculated from $K_{3\text{-CD,corr}} = K_{3\text{-CD}}/3$ and $K_{4\text{-CD,corr}} = K_{4\text{-CD}}/4$ because there are three and four binding sites in [2]catenanes **3-T** and **4-T**, respectively.

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