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Cooperative Assembly of Discrete Stacked Aggregates Driven by Supramolecular Host−Guest Complexation

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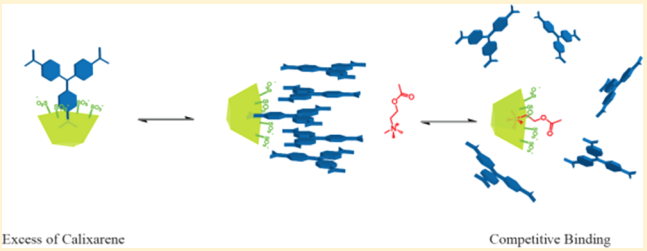
Supporting Information

ABSTRACT: *p*-Sulfonatocalix[4]arene (SC4) interacts with the aromatic dye crystal violet (CV) to form complexes with stoichiometries ranging from SC4:CV = 1:1 up to 1:5 both in solution and in the gas phase. While the 1:1 complex is of the inclusion type, as frequently observed for other guests, in the higher-order complexes the CV molecules interact with SC4 in a peripheral manner. The formation of such complexes is driven by ionic interactions established between the dye and the calixarene and by CV−CV stacking interactions. The application of an advanced fitting procedure made possible a quantitative analysis of the UV−vis data and allowed the determination of the stepwise binding constants. This unprecedented approach provides evidence that the formation of the highest-order complexes occurs through a cooperative mechanism. Moreover, the development of a quantitative analytical model enables the possibility of using this type of system for water-soluble sensing assays, as is also exemplified in the present work.



INTRODUCTION

The formation of self-assembled stacked aggregates of organic dyes is a topic of current interest because of their potential applications in technological fields such as photography, optical recording media, organic photo- and semiconductors, photoelectric cells, sensors, and so on.[1,2] Nature also makes use of the supramolecular organization of dye molecules as a strategy to develop light-harvesting apparatus in photosynthetic organisms or for color development and stabilization in plant cell vacuoles.[1,3,4]

Dye aggregation can be driven by different non-covalent forces, but $\pi-\pi$ interactions are known to play a central role leading to the formation of the stacked aggregates. When two dye molecules aggregate through $\pi-\pi$ interactions, the resulting dimer can further interact with other monomeric or dimeric species present in solution to form higher-order aggregates. This process can lead to stack growth and formation of *infinite* columnar π-stacks.[1] On the other hand, the formation of medium-sized discrete stacks of organic dyes is not so common but is essential to scrutinize the interactions behind the stacking process.[5] Among the several strategies used to control the size and orientation of the aromatic stacks, those based on supramolecular self-assembly are particularly attractive because of their simplicity and effectiveness.

p-Sulfonatocalix[*n*]arenes (SC*n*s) are anionic host molecules that are well-known for their ability to form stable inclusion complexes with cationic and neutral guest species.[6] In addition, SC*n*s also have a particular ability to induce the formation of discrete stacked aggregates of oppositely charged dye molecules by forming peripheral, rather than inclusion, high-order complexes.[7−14] These properties have been elegantly explored to design stimuli-responsive vesicles and multiporphyrin aggregates with programmable stoichiometry and sequence.[8,12,13]

In view of the growing interest and potential of such peripheral complexes, it is fundamental to develop mechanistic models that provide quantitative and deepened understanding of the process. Moreover, such models can be relevant to more complex systems because this class of peripheral complexes have some features that resemble those displayed by dye aggregates formed in the presence of polyelectrolytes (including DNA).[15−20] In this work, *p*-sulfonatocalix[4]arene (SC4) and crystal violet (CV) (Scheme 1) were selected as model compounds to investigate the above-mentioned phenomena. The results indicated the formation of high-order SC4−CV complexes with stoichiometries ranging from 1:1 to 1:5 depending on the concentrations of the species. The initial formation of the 1:1 complex results in a supramolecular host−guest species that has a higher tendency to form stacked aggregates than free CV. Thus, the presence of excess dye leads to the formation of discrete stacked aggregates. However, as the

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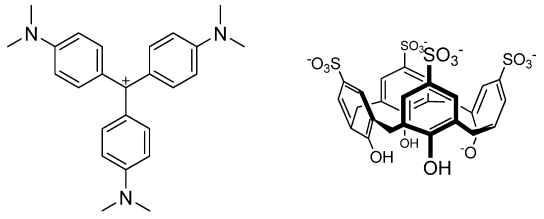
Scheme 1



host concentration is increased, the CV molecules are redistributed and the 1:1 complexes predominate. A mechanism for this process is proposed, and quantitative analysis of the UV–vis spectroscopic data permitted the determination of the microscopic binding constants. In addition, the dynamic nature of the higher- order complexes was proved by competitive binding experiments using the biologically relevant acetylcholine cation, which disrupts the SC4–CV complexes. This further confirmed the proposed mechanism and enabled the use of this system for quantitative sensor applications.

RESULTS AND DISCUSSION

Interpretation of the UV–Vis Data. The absorption spectrum of CV in an aqueous medium can be decomposed into two Gaussian sub-bands centered at 557 and 590 nm. The most accepted interpretation is that the two sub-bands arise from two slightly different conformations of the CV molecule, namely, a planar propeller structure with D_3 symmetry (557 nm) and a pyramidal structure with C_3 symmetry (590 nm).[21,22] The relative intensity of these sub-bands depends on the dye concentration as a result of self-aggregation of CV in aqueous solutions [Figure S1 in the Supporting Information (SI)].[23−25] The overlap of the first two spectra, corresponding to 1×10^{-6} and 1×10^{-5} M, respectively, indicated that aggregation was negligible in this concentration range.

The addition of SC4 to CV (1×10^{-5} M) strongly changed the UV–vis spectrum (Figure 1). Up to [SC4] = 8×10^{-6} M, the intensity at 590 nm decreased and the spectrum became broader. Similar effects were previously reported for the interaction of CV with *p*-sulfonatocalix[6]arene (SC6), but those results were not explored further.[26] Higher concentrations of SC4 led to the recovery of the initial spectrum, although with a slight red shift (7 nm).

Detailed insights into the aggregation of CV induced by SC4 were obtained by plotting the ratio of the absorbance of the two sub-bands ($R = A^{590}/A^{557}$) as a function of SC4 concentration (Figure 2a). The addition of SC4 led to a decrease in *R*, indicating the formation of CV aggregates. Similar observations were previously reported for CV and other triarylmethane dyes in presence of oppositely charged polyelectrolytes.[27] Adjacent binding sites located along the polymeric chain bring together dye molecules, promoting their aggregation at low concentrations. On the basis of the pentaanionic nature of SC4, a

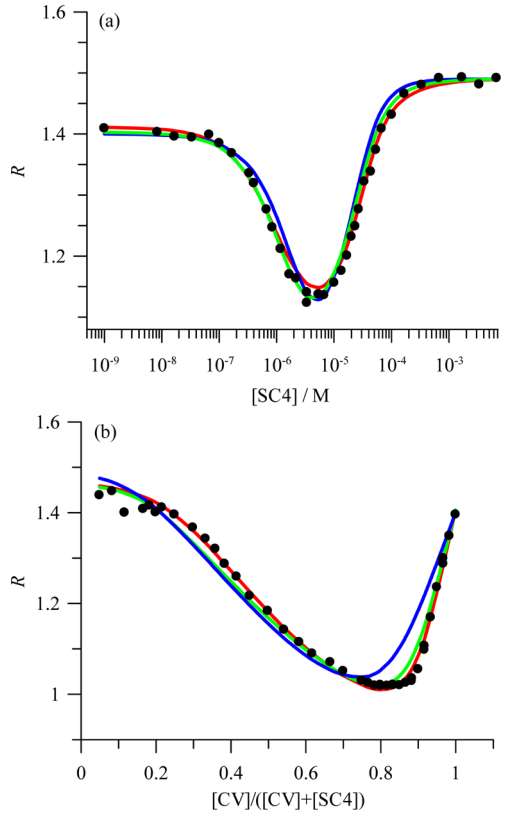


Figure 2. (a) Plot of the absorbance ratio *R* as a function of SC4 concentration at a CV concentration of 1×10^{-5} M. (b) Job plot obtained for a total concentration of [CV] + [SC4] = 2×10^{-5} M. All data were obtained in H_2O at 25 °C. The experimental data (black circles) were globally fitted to 1:3 (blue line), 1:4 (green line), and 1:5 (red line) host–guest binding models.

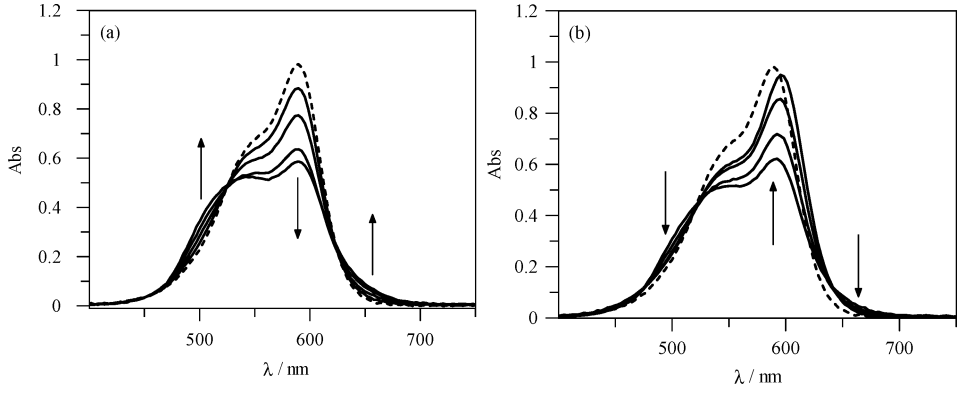


Figure 1. UV–vis absorption spectra of CV in the presence of SC4. (a) [SC4] = 4×10^{-7} to 8×10^{-6} M; (b) [SC4] = 2×10^{-5} to 2×10^{-3} M. The dashed spectra were measured in the absence of SC4. All of the spectra were obtained in H_2O at 25 °C with [CV] = 1×10^{-5} M.

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similar process leading to aggregation and formation of higher-order host–guest complexes would be expected at low concentrations. Further addition of SC4 led to a minimum in *R*, which increased again as the SC4 concentration was further increased. This indicates disaggregation and the formation of 1:1 complexes, which predominate over the higher-order complexes under these conditions.

Control UV–vis experiments performed with 4-hydroxybenzenesulfonate (HBS), the building block unit of SC4, showed that this compound has no considerable influence on *R* for concentrations of HBS up to 0.5 M (Figure S2). This suggests that the recognition ability of the calixarene is critical for triggering the aggregation of the dye. A possible explanation relies on the fact that the dye has more affinity for other CV molecules when it is associated with SC4 than it does as a free molecule. This extra affinity is probably provided by the negatively charged sulfonate groups present in the calixarene framework.

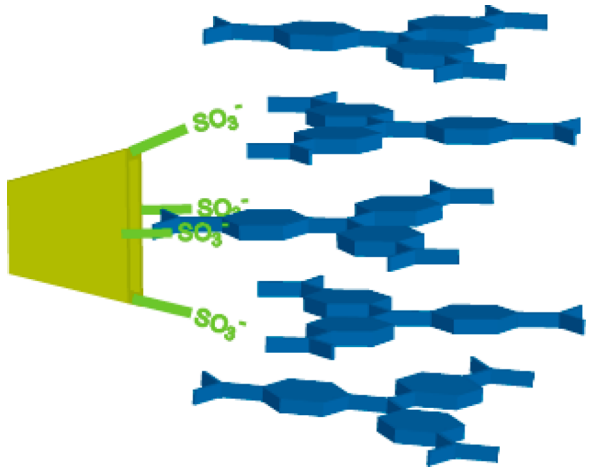
Determination of the Stoichiometry of the SC4–CV Complexes. The possible formation of such higher-order host–guest complexes was confirmed by following the Job plot (Figure 2b). Although the limitations of the method for solutions containing host–guest complexes with different stoichiometries are well-documented,[28] the minimum observed in the molar fraction range 0.80–0.83 suggests the formation of SC4–CV complexes with stoichiometry higher than 1:3. The most likely association models were tested by simulating the UV–vis data presented in Figure 2 (see the SI). As can be observed, the 1:5 model clearly provided the best description of the experimental data. Because the successful simulation of the data in this region is expected to be highly dependent on the stoichiometry of the complexes, the 1:5 binding model is supported at expense of the 1:3 and 1:4 models. The accuracy of the global fits was tested using the normalized root-mean-square deviation expressed as the percent deviation. Normalized deviations of 3.7%, 7.0%, and 12.3% were obtained for complexes with 1:5, 1:4, and 1:3 stoichiometries, respectively, supporting the conclusion that the 1:5 model yields the best description of the system. Moreover, it should be noted that discrepancies between the experimental results and those calculated for the three stoichiometric complexes are most important in the CV-rich region of the Job plot, where high-stoichiometry complexes are allowed.

Additional support for the 1:5 stoichiometry was obtained by electrospray ionization mass spectrometry (ESI-MS) experiments. This technique is recognized to be a powerful tool in supramolecular chemistry, namely in the study of gas-phase host–guest complexes, capsules, and aggregates.[29−31] ESI-MS has been used to characterize calixarene host–guest complexes, heterocapsules, and even interactions between this macrocycle and proteins.[32−34]

When CV was present at equimolar concentration (10 μM), a peak at m/z 1158.2 was observed (Figure S6). Its fragmentation led to the release of fragments of m/z 372 and 452, which correspond to CV and an SO_3–CV pair, respectively (Figure S7). We assign the peak at m/z 1158.2 to the 1:1 complex ($[SC4 + CV + 2Na + H]^-$). Further addition of CV up to a host:guest ratio of 1:4 led to the observation of new signals at m/z 1485.6, 1507.6, and 1856.8 (Figure S8). While the fragmentation of m/z 1485.6 and 1507.6 led to the loss of two CV molecules, that of m/z 1858.8 led to the release of three CV molecules (Figure S9). On the basis of the m/z values and the fragmentation patterns, we assign the peaks at m/z 1485.6 and 1507.6 to the 1:2 complex ($[SC4 + 2CV + 2H]^-$ and $[SC4 + 2CV + Na + H]^-$, respectively) and the signal at m/z 1856.8 to the 1:3 complex ($[SC4 + 3CV + H]^-$). Further addition of CV strongly quenched the ESI-MS signals. This was expected because the addition of CV increases the positive charge of the solution, which makes the formation and detection of negative ions much more difficult. When an aqueous solution containing 5 μM SC4 and 40 μM CV was injected, new signals at m/z 2228.2 and 2635.2 appeared (Figure S10). The former was readily assigned to the 1:4 host–guest complex ($[SC4 + 4CV]^-$) on the basis of its fragmentation pattern (Figure S11). The latter signal was fit to a 1:5 complex with an additional 35 Da. In this structure, the five CV molecules neutralize the five SC4 negative charges, and the additional charge that makes these complexes visible under ESI-MS was provided by Cl^-, the counterion of CV. These results are in agreement with those obtained in solution by UV–vis absorption and support the formation of higher-order host–guest complexes. The observation of aggregates by ESI-MS techniques is quite common. Thus, if the formation of complexes with stoichiometries higher than 1:5 can take place in solution, they should also be observed in the gas phase, which was not the case.

The formation of discrete stacks composed of five tetrapyridylporphyrin molecules in the presence of a cone-shaped *p*-sulfonatocalix[4]arene derivative has been observed in both solution and the solid state. In fact, these stacks are surrounded by four calixarene molecules, since each porphyrin has four cationic pyridyl groups. In addition, the pyridyl arms of the central porphyrin are included within the cavities of the calixarene.[7] By analogy, we propose a 1:5 SC4–CV complex structure inspired by the calixarene–porphyrin system (Scheme 2).

Scheme 2



^{1}H NMR Experiments. To gain further insights into the complex interactions established between SC4 and CV, several ^{1}H NMR spectra of these compounds were acquired (Figure S3). In this experiment, the concentration of CV was kept constant at 5×10^{-5} M and the concentration of SC4 was gradually incremented from 0 to 1.2×10^{-3} M. As can be observed, at low concentrations of SC4 (below 5×10^{-5} M), the intensity of the ^{1}H NMR signals of CV decreased until they became undetectable. This behavior is compatible with the formation of higher-order complexes that eventually further

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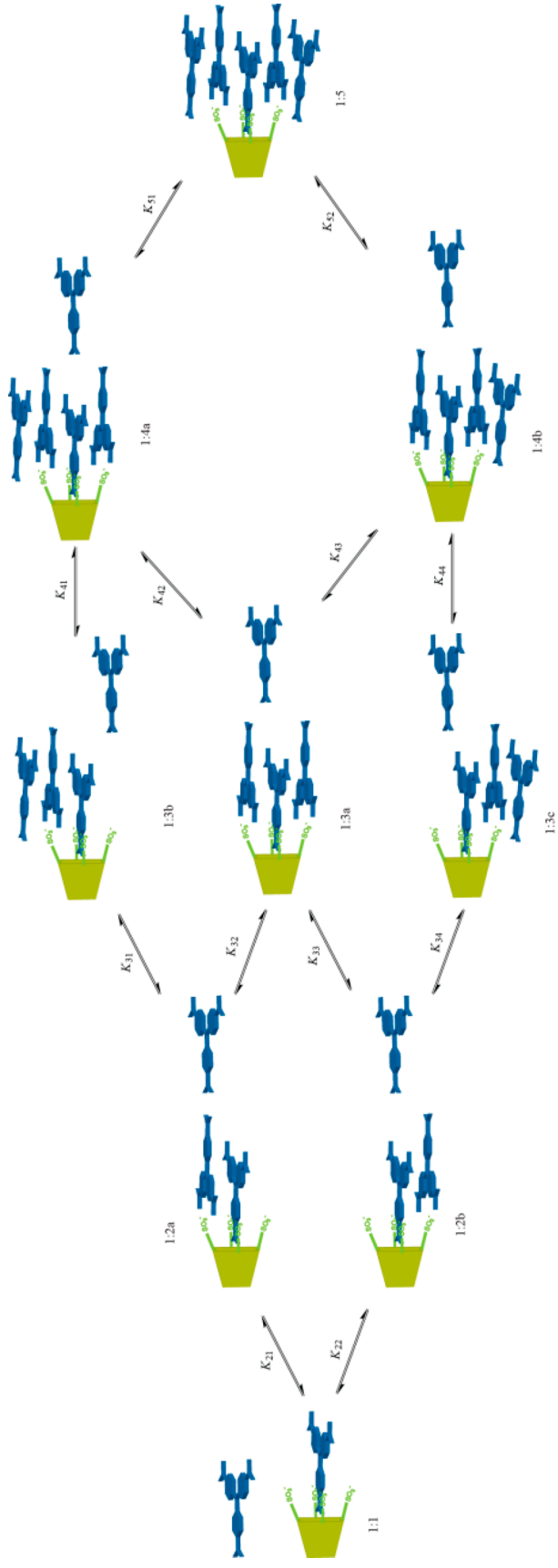

Scheme 3

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aggregate into larger assemblies. In this concentration range, a dusty precipitate could be observed in the NMR tubes, suggesting that the higher-order complexes might form large nonordered structures rather than well-defined assemblies as previously reported for other *p*-sulfonatocalixarene−dye pairs.[12,13] It is worth noting that the formation of precipitates was not observed during the UV−vis experiments, probably because of the lower concentration of CV used.

At concentrations of SC4 above 5×10^{-5} M, the ^{1}H NMR signals of CV reappeared in the spectra considerably displaced to high magnetic field. This behavior is compatible with the partial encapsulation of the CV molecule inside the SC4 cavity, where the guest protons are under the influence of the magnetic ring current of the calixarene aromatic units. This suggests that in this concentration range the 1:1 complex starts to predominate over higher-order complexes. These observations are in good agreement with the UV−vis experiments. The ^{1}H NMR signals of the 1:1 complex also suggested that CV molecule is selectively included in the SC4 cavity through the dimethylamino groups, since the complexation-induced chemical shifts ($\Delta\delta = \delta^{free} - \delta^{complex}$) increased in the order $N(CH_3)_2$ (0.78 ppm) > Ha (0.34 ppm) > Hb (0.07 ppm).[35,36]

Binding Mechanism. Once the stoichiometry of the complex had been established, we decided to investigate the binding mechanism that operates in this involved system. For this purpose, we tested several binding models. It should be noted that for all of the fits presented in this work, the χ^2 value (eq 1 in the Experimental Section) was simultaneously minimized to both the titration and Job plot UV−vis data in order to obtain more reliable combinations of parameters. Even though from a technical point of view it would have been easier to fit each curve independently, it was obvious that the results obtained from the simultaneous (global) fitting of different and independent measurements were much more consistent.

Several statistical binding mechanisms were tested before the following model was proposed (for more details, consult the SI). When statistical binding models are not able to describe the experimental data, one could expect that cooperativity effects are involved in the binding process.[37] If the CV molecules are assumed to interact only with binding sites adjacent to others that are previously occupied in order to favor CV−CV stacking interactions, then a scheme that accounts for all of the sequential binding possibilities can be proposed (Scheme 3). The most simple and intuitive mechanism that accounts for cooperative effects in the peripheral binding of CV to SC4 considers that the microscopic equilibrium constant for the formation of the 1:2 complex (K_{m1}) is different from that associated with the higher-order complexes (K_{m2}). In this case, $K_{m1} = K_{21} = K_{22}$ and $K_{m2} = K_{31} = K_{32} = K_{33} = K_{34} = K_{41} = K_{42} = K_{43} = K_{44} = K_{51} = K_{52}$, while the relations between the apparent and microscopic binding constants are $K_2 = 2K_{m1}$, $K_3 = (3/2)K_{m2}$, $K_4 = (2/3)K_{m2}$, and $K_5 = (1/2)K_{m2}$. As can be observed in Figure S15, the results obtained by applying this model are equivalent to those obtained by the unrestricted reference model (where all of the binding constants are allowed to vary independently). This analysis supports a cooperative binding mechanism at the expense of the described and tested statistical binding mechanisms (see the SI).

Final Equilibrium Association Constants Obtained from the UV−Vis Measurements and Interpretation of the Results. Using the fitting methodology described above, 30 local minima were found within the 2×10^{-4} to 3×10^{-4} standard deviation range for the cooperative model. Since it is evident that the absorbance ratio R decreases when the CV molecules stack together and adopt the planar geometry, all of the minima corresponding to sets of parameters with $R_{1:n}$ values higher than 1.4 for $n > 1$ were discarded, allowing the local minima to be reduced from 34 to 13 (see Table S1 in the SI). The resulting average values of the equilibrium constants are $K_1 = (8.6 \pm 1.0) \times 10^4$ M^{-1}, $K_{m1} = (2.1 \pm 1.8) \times 10^3$ M^{-1}, and $K_{m2} = (8.3 \pm 3.4) \times 10^5$ M^{-1}. These values can be used to compute the concentrations of the various CV species present in solution as a function of the SC4 total concentration (Figure S16)

While K_1 is in the range of values usually observed for the formation of 1:1 complexes between SC4 and organic cations, the values found for K_{m1} and K_{m2} indicate the existence of positive cooperativity.[6] This can be ascribed to the fact that the formation of the 1:2 species may involve an unfavorable free energy contribution associated with the conformational reorientation of two CV molecules from the pyramidal to the planar geometry and reorientation of the cavity-included CV. In contrast, for the formation of higher-order complexes (1:3, 1:4, and 1:5), only the incoming CV molecule has to change its conformation in order to optimize the stacking interactions. In practice, this means that the 1:2, 1:3, and 1:4 complexes have a higher affinity for free CV molecules than the 1:1 complex does.

The equilibrium constants for dimerization and trimerization of CV in the absence of SC4 were found to be 680 and 720 M^{-1}, respectively.[24] These values suggest that the aggregates of CV undergo isodesmic self-assembly (also known as the equal K model).[1] On the basis of these values, it is obvious that the calixarene plays a significant role in the stabilization of the aggregates. These results also suggest that the reason that K_{m1} is lower than K_{m2} may be related to an unfavorable reorientation of the partially included CV molecule in the host cavity during the formation of the 1:2 complex, since in the case of the self-aggregation of uncomplexed CV the dimerization and trimerization equilibrium constants seem to be equivalent. It is worth noting that the cooperative model suggested in this work bears an obvious resemblance to the modified isodesmic model proposed for the aggregation of dye molecules.[1]

Displacement Assay. To test the reversibility of the higher-order complexes and the potential sensing applications of this system, to a solution containing 5×10^{-6} M SC4 and 1×10^{-5} M CV (the conditions required to observe the minimum value in the R vs [SC4] plot in Figure 2a) were successively added increasing concentrations of acetylcholine (ACh). This biologically relevant cation was expected to compete with CV for binding of SC4 since it is known to form stable 1:1 complexes with this host molecule.[38] As can be observed in Figure 3, the R value increased with increasing ACh concentration, indicating that SC4−CV complexes were disrupted by the competitive complexation of ACh (Scheme 4). Eventually, at high concentrations of the competitor, the R value reached that observed in the absence of SC4 (~1.4), indicating that under these conditions CV was mainly present in solution as free molecules rather than in complexes, while on the other hand the SC4 was saturated with ACh cations.

The validity of this new displacement assay was quantitatively tested using a simulation procedure analogous to that described above (see the SI). By fitting the experimental data in Figure 3 to the corresponding equation, the value of K_{ACh} can be readily determined. The fitting procedure was applied to each set of parameter values corresponding to the minima obtained before, and an average K_{ACh} value of $(3.2 \pm 0.5) \times 10^5$ M^{-1} was

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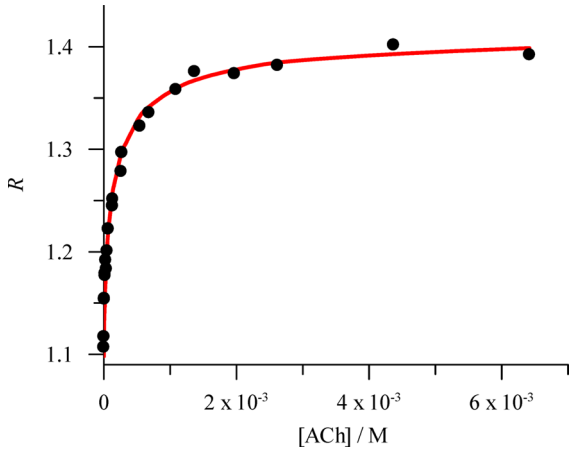
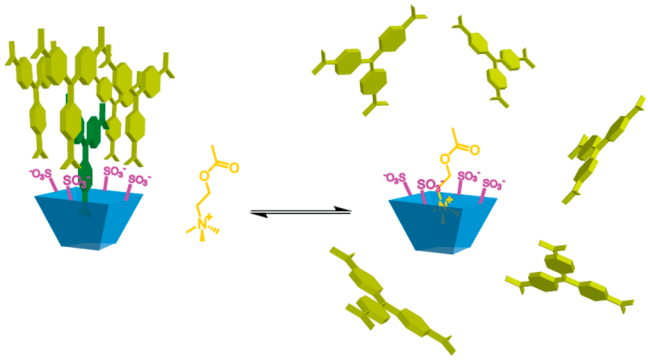


Figure 3. Increase in R for 1×10^{-5} M CV upon successive addition of acetylcholine (ACh) competitor in the presence of 5×10^{-6} M SC4. All of the experiments were performed in H_2O at 25 °C. The red line was calculated by considering a sequential binding model that accounts for the binding of 1 to 5 CV molecules to SC4 in the presence of a competitive binder (see the text for details).

Scheme 4



determined. This value is 3-fold larger than that determined in a previous work [$K_{ACh} = (1.0 \pm 0.1) \times 10^5$ M^{-1}].[38] However, it must be noted that as reported before, the binding constants determined for SC4–ammonium guest complexes depended on the concentration of calixarene used in a given experiment. This dependence resulted from the competitive binding of SC4 counterions (usually sodium ion), and thus, the apparent binding constant decreased as the concentration of SC4 increased.[39] Since the apparent binding constant exhibited a 2-fold decrease as [SC4] increased from 1×10^{-4} to 1×10^{-3} M and in the present work a lower concentration of SC4 was used (5×10^{-6} vs 1.6×10^{-3} M), the discrepancy between the result presented here and that reported previously can be partially justified.

It is also important to emphasize that this competitive binding experiment supports the model proposed above. The complete disruption of the aggregates in the presence of ACh also reinforces the suggestion that the formation of the 1:1 SC4–CV complex is critical for the subsequent formation of higher-order complexes. Otherwise, it would be reasonable that the CV molecules could also bind peripherally to the SC4–ACh complex, in which case the effective disruption would not be observed.

CONCLUSIONS

The present study demonstrates that SC4 can form complexes with CV having different stoichiometries that range from the archetypical value of 1:1 up to 1:5. The association process is initiated by the formation of an 1:1 host–guest inclusion complex between the two species that results in a supermolecule with enhanced affinity for other CV molecules, leading to the formation of 1:2, 1:3, 1:4, and 1:5 peripheral complexes. This extra affinity probably results from the polyanionic nature of SC4, which screens the ionic repulsions between the CV molecules. From the analysis of the results obtained in this work, several possible models were proposed and tested, and it was found that the peripheral binding of CV molecules to the SC4 host (formation of the higher-order complexes) probably occurs through a cooperative mechanism. This mechanism is characterized by two microscopic binding constants K_{m1} and K_{m2} associated with the formation of the 1:2 complex and the other higher-order complexes, respectively. The observed positive cooperativism ($K_{m2} > K_{m1}$) is probably related to the reorientation of the two CV molecules from the pyramidal to the planar geometry in the 1:2 complex, which facilitates the formation of the remaining complexes since the planar molecules are expected to present a higher tendency to form stacked aggregates.

The dynamic nature of the present two-component system allows the distribution the different complexes to be controlled by varying the concentration of SC4. While the 1:1 complex is favored in the presence of excess host, the higher-order complexes are favored at low concentrations of SC4. More importantly, the degree of aggregation can be also controlled by addition of a competitive guest, allowing the application of this type of system in the development of sensors for displacement assays with the predicted advantages of working at low SC4 concentrations and exploring, for example, fluorescence enhancement or quenching effects resulting from dye aggregation.

EXPERIMENTAL SECTION

Materials and Methods. Crystal violet was obtained from a commercial supplier and used without further purification. *p*-Sulfonatocalix[4]arene was available from our previous work.[39] All solutions were prepared with Milli-Q water. UV–vis absorption spectra were recorded on a spectrophotometer equipped with a cell holder thermostatted at 25.0 ± 0.1 °C. The ESI–ion trap mass spectrometric experiments were performed with an ultra mass spectrometer equipped with an ESI source that utilized a nickel-coated glass capillary with an inner diameter of 0.6 mm. Ions were continuously generated by infusing the aqueous solution samples into the source with a syringe pump at a flow rate of 4 μL/min.

Fitting Procedure. The adjustable parameters of the proposed model, R_{CV}, $R_{1:n}$, and $K_{1:n}$ (with $R = A^{590}/A^{557}$) were determined by minimizing the following objective function:

$$\chi^2 = \frac{\sum (R_{obs} - R_{calc})^2}{n - p} \quad (1)$$

where n and p represent the numbers of experimental data points and fitted parameters, respectively. The simulated annealing (SA) algorithm was employed for this aim, as it is well-suited to finding the global minimum by efficiently exploring the multidimensional space generated by the fitting parameters.[40,41] The concentration of the unbound (free) species for each SA iteration during the minimization process was also numerically determined by using a Newton–Raphson algorithm combined with Armijo's rule to improve convergence.[42] In view of the relatively large number of parameters

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involved in the model (see eqs 9−17 in the SI), the presence of local minima was expected. Thus, approximately 1000 independent minimizations of all the experimental data starting from different random seeds were performed. All of the calculations were carried out using our own code developed in the C++ language.

■ ASSOCIATED CONTENT

Supporting Information

UV−vis control experiments, ESI-MS spectra, derivation of equations, and additional simulations of the UV−vis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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