Intrinsic and extrinsic control of the pK_a of thiol guests inside yocto-liter containers

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Abstract

Despite decades of research there are still many open questions surrounding the mechanisms by which enzymes catalyze reactions. Understanding all the non-covalent forces involved has the potential to allow *de novo* catalysis design, and as a step towards this, understanding how to control the charge-state of ionizable groups represents a powerful yet straightforward approach to probing complex systems. Here we utilize supramolecular capsules assembled via the hydrophobic effect to encapsulate guests and control their acidity. We find that the greatest influence on the acidity of bound guests is the location of the acidic group within the yocto-liter space. However, the nature of the electrostatic field generated by the (remote) charged solubilizing groups also plays a significant role on acidity, as does counter-ion complexation to the outer surfaces of the capsules. Taken together, these results suggest new ways in which to affect reactions in confined spaces.

Introduction

Many different non-covalent interactions come together in an enzyme to contribute to its catalytic properties, but at a general level it is currently believed that of all of these,[1] Coulombic interactions reign supreme.[2] This is perhaps not surprising; in vacuum, two mono-valent ions will experience an attraction (or repulsion) greater than the thermal energy kT at a distance of ~56 Å. Even in a high dielectric medium such as water (ε_r = 78.4 versus 1 for vacuum), this Bjerrum length is relatively long; ions must be within approximately 7 Å to feel each other above the energy fluctuations of the solution. Hence an electrostatic potential field (EPF) emanating from a charged group in a protein can extend some distance along the surface, and more deeply through the lower dielectric of the inner structure.[3]

One way to try and understand enzymes is to mimic them, and over the last decade or so the field of supramolecular chemistry has made considerable advances in this regard.[4] Thus, supramolecular containers assembled via metal-ligand coordination have proven to be successful catalysts and stoichiometric reagents,[5] as have hydrogen-bonded containers,[6] covalent hosts,[7] and containers assembled via the hydrophobic effect.[8] These many advances noted, because of the overall complexity of chemical transformations, this body of work tends to identify new and exciting chemical conversions rather than reveal the inner workings of enzymes.

To better parse out the non-covalent contributions to enzyme catalysis, more straightforward metrics are preferable. One such metric is the acidity of an ionizable group. Thus, in studying how context affects acidity, the pK_a values of protonated lysine residues in engineered variants of *staphylococcal nuclease* have been found to vary from the typical value of 10, to a value as low as 5.3 depending on their position in the protein.[9] Similarly, the pK_a values of the carboxylate groups of aspartic and glutamic acid have been found to be shifted up to 4-5 units depending on their environment,[10] whilst similar variations in the pK_a of cysteine resides are intimately linked to their redox and signaling properties.[11] Complementary to these types of studies, considerable effort has also gone into the modelling the effects of electrostatic charges in proteins,[12] and taken together with the empirical studies, this work reveals subtle contextual effects on the ionization-state of functional groups.

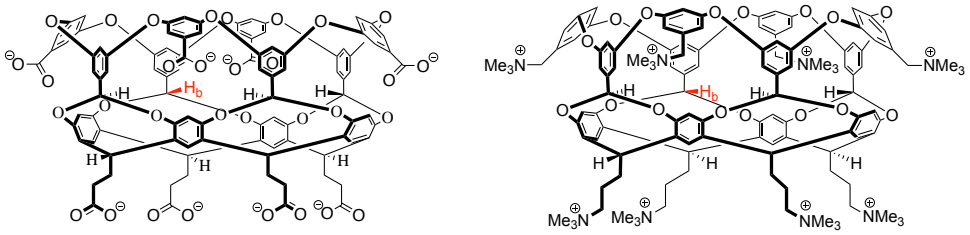
The relatively simple structures of macrocyclic hosts also offer considerable opportunity to investigate how local environment affects the pK_a of an ionizable group (and hence any property that is itself controlled by ionization state).[13] For example, in the presence of the weak dipole of cyclodextrins, it is typically observed that the pK_a shifts for bound guest are < 1 unit.[14 15] In contrast, the larger quadrupole of cucurbiturils can shift the pK_a of bound ammonium dyes[16] by 2-3 units.[17] Moreover, shifts as large as ~5 pK_a units have been observed in selected examples of 2:1

complexes between CB7 and coumarin dyes. In such cases, it appears that bringing two quadrupoles to bear on the central protonated N-atom of the bound dye induces the relatively large observed shift in acidity.[17b, 18]

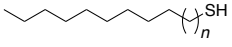
To build on our understanding of how local charges and electrostatic potential fields (EPFs) can affect acidity constants, we report here on the acidity of guests encapsulated within the two supramolecular capsules formed by octa-carboxylate **1a** and positand **1b** (Figure 1).[19] These hosts dimerize around guests, internalizing them within identically shaped non-polar nano-spaces. The only significant difference between the inner space of each capsule is that one is located within a negative EPF created by sixteen remote negative charges (**1a**$_2$), whilst the other resides within a positive EPF emanating from trimethyl ammonium solubilizing groups (**1b**$_2$). Figure S1b shows the EPF for each host. We report here on the acidity of a range of thiol guests (**2**-**10**, *n* = 1-9, Figure 1) within these supramolecular capsules. Our acid-base titrations of bound guests reveal how the EPF generated by the arrays of charge groups affects acidity, and how acidity is also intimately tied to the precise position of the thiol group in the inner space. Finally, we demonstrate how extrinsic factors – salts dissolved in the outer aqueous medium – can bind to the outer surface of the host, modulate the EPF of the capsule, and hence change the acidity of the bound guest. Taken together, these studies reveal new details of the extent to which different factors can influence the acidity of internalized guests.

Results and Discussion

Hosts **1a** and **1b** were synthesized as previously described.[19] Guests **3**, **4**, **6**, **8** and **10** were commercially available, whilst guests **2**, **5**, **7** and **9** were synthesized from the corresponding 1-bromoalkane, via formation of the *S*-alkyl ethanethioate (KSAc in THF) and acid hydrolysis (HCl in MeOH). Details of the guest syntheses are given in the Supporting Information.



1a **1b**



2-**10**: *n* = 1-9

Figure 1: Hosts **1** and **2**, and guests **2**-**10** (*n* = 1-9 respectively) used in this study.

A combination of signal integration in the ^{1}H NMR spectrum of each complex, and DOSY NMR spectroscopy of the free host and the different complexes, confirmed 2:1 stoichiometries for each combination of host and guest. Each complex was typified by significant shifts in the host aromatic signals (Figure S3 and S4, Supporting Information), and by bound guest signals between –1 and –4 ppm (Figures S5-S10). By recording the NMR spectra in buffered (8 mM NaOH) 95:5 H_2O:D_2O it was possible to observe the SH signal from the bound thiol group for the majority of complexes, and in most cases COSY NMR spectroscopy allowed full peak assignment (Figures S11 to S36). Figure 2 shows the guest binding region for three guests within the negative **1a**$_2$ capsule: *n*-decyl thiol **2**, *n*-tetradecyl thiol **6**, and *n*-octadecyl thiol **10**. Peak assignments allowed the calculation of the signal shift data of the guest between the bound and the free state ($\Delta\delta = \delta_{bound} - \delta_{free}$), which revealed the approximate location of each group within a host and hence the overall preferred binding motif of each guest.[20] In general, we found (Figure 3 and Figure S12) that the smaller guests such as **2** and **3** adopted an extended motif in which the two termini of the guest bound to the two 'poles' of the capsule, whilst guests such as **8** or **10** that were too long for the nano-space were forced to bind in a J-shaped motif.

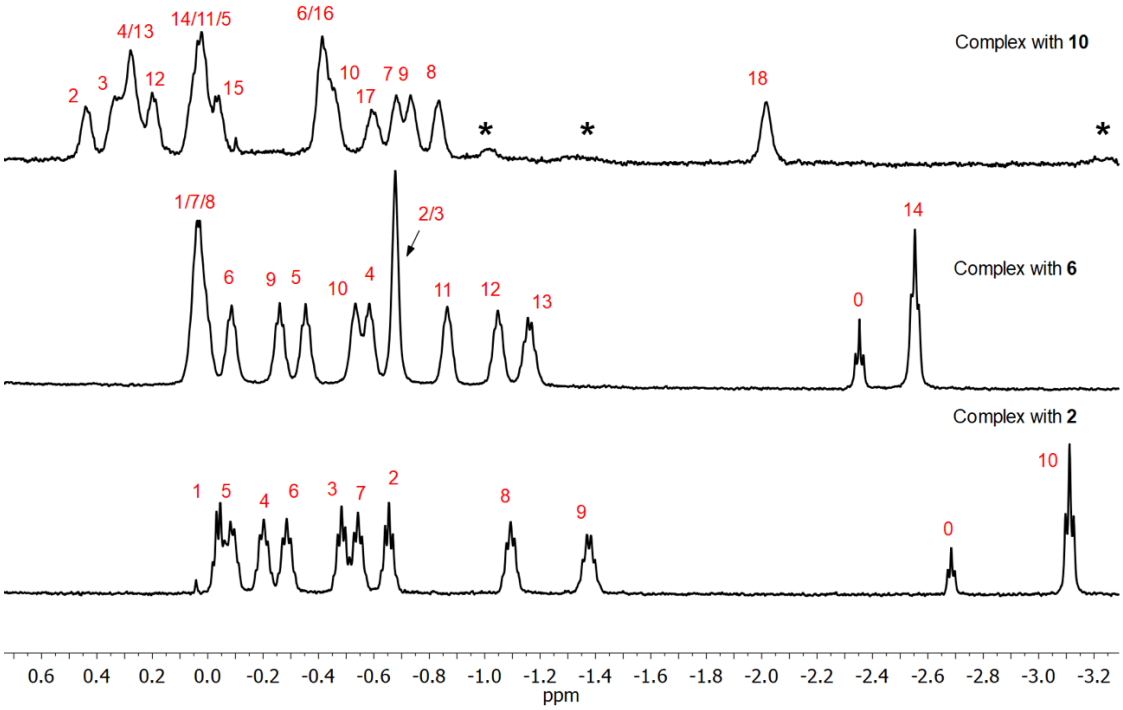


Figure 2: Guest binding region of the ^{1}H NMR spectra of the capsular complexes of **1a** with thiols **2**, **6** and **10** (all 1.0 mM concentration, in 8 mM NaOH 95%H_2O/5%D_2O). Numbering is from the thiol (0) to the methyl terminus (10, 14 or 18 respectively). The indicated (*), broad signals in the complex with **10** are attributed to a minor guest motif (see main text).

Returning to Figure 2, wide signal anisotropy is typically observed with guests such as **2** that adopt an extended motif.[21] This arises because the Δδ values for the two termini are large, whereas the Δδ value for the mid-section of the bound guest is small. Specifically, in the case of **2** inside capsule **1a**$_2$ the Δδ values of the thiol S–H, the mid-section methylenes (C4/C5), and the C10 terminal methyl signals were –3.98, –1.32/–1.29, and –3.92 ppm respectively. With increasing size of guest, so there is a transition from an extended motif, to an extended motif with a kink, to motifs possessing a full reverse turn.[20a] This is exemplified by the smaller upfield shifts of the termini regions of guest **6** inside negative **1a**$_2$ (Figure 2); the Δδ values of the thiol S–H, C7/C8 methylenes and C14 terminal methyl signals were –3.65, –1.17/–1.17, and –3.37 ppm respectively. Interestingly, guest **8** ($C_{16}H_{33}SH$) lay at the transition between the extended and J-motifs. This guest had a mostly unresolvable COSY NMR (Figure S26). To probe this further we examined the ^{1}H and COSY NMR of the complex of the corresponding alcohol ($C_{16}H_{33}OH$); reasoning that the equatorial region of the capsule is more polar/hydrophilic (*vide infra*) and that therefore the alcohol guest would be more predisposed to form the J-motif(*Me*) with its polar OH group at the equator (c.f Figure 3, *right*). This was indeed the case; the bound ^{1}H NMR signals were exceptionally well resolved and indicated a well-defined J-motif(*Me*) with the OH at the polar region of the host (Figure S27 and S28). For thiol **8** however, there is evidently no such driving force helping define its motif, and as a result the extended motif and both J-motifs must be approximately isoenergetic. Finally, the ^{1}H NMR signals of bound **10** showed a wider anisotropy but were relatively broad (Figure 2). Additionally, the signal from the thiol group was not evident. Nevertheless, the relatively large upfield shifts of the C8/9 signals (approximately –2 ppm, Figure S32) suggest a J-motif in which one of the cavitand hemispheres is occupied by a turn in the guest mainchain. However, this data alone is not sufficient to determine if the J motif has the Me at the base of one cavitand and the thiol at the equatorial region of the capsule (J-motif(*Me*), Figure 3 *right*), or the 'opposite' motif with thiol group at the base of the pocket and the Me at the equator (J-motif(*SH*)). However, a key clue to the preferred binding motif of this guest is that the ^{1}H NMR spectrum for the complex with **10** also revealed the presence of small, broad signals suggestive of a minor secondary motif (Figure 2). Considering the positional similarity of the most upfield signal of this minor motif to the Me signal of complexed **3** and **4**, we tentatively attribute this minor motif of **10** to the J-motif(*Me*) with the thiol at the equatorial region of the capsule. To support this hypothesis, we formed the complex between negative **1a**$_2$ and the corresponding C18 alcohol. The guest binding region of the ^{1}H NMR spectrum of the complex revealed sharp, well resolved signals with the terminal methyl group at –3.25 ppm. Moreover, the corresponding COSY spectrum allowed full signal identification (Figures S35 and S36) and conformation of a J-

motif(*Me*). Thus bound **10** primarily adopts a J-motif(*SH*) with the thiol group bound into the base of a cavitand and the Me nearer the equator. But considering that both motifs can be observed for bound **10**, there can be little energy difference between them.

The well-defined ^{1}H NMR signal from the thiol S–H allowed acid-base titrations with sodium hydroxide to determine the pK_a of each bound guest (Figures S42-S64) for all host guest pairs, except those involving guests **9** and **10**.[22] During these titrations, other than the disappearance of the S–H signal, we saw only small changes to the guest binding region of the ^{1}H NMR spectrum.[23] Specifically, there were slight shifts in all the bound guest signals, and changes to the (poorly defined) signal splitting of the bound C1 methylene (e.g., compair Figures S15 and S45). We conclude that, at least as measured by 500 MHz ^{1}H NMR, deprotonation did not significantly change the guest binding motif.[23] This tells us that alkyl group solvation (for a free guest) is more energetically costly than binding the thiolate in a non-polar pocket. But what is the nature of the bound thiolate? There are four possibilities: not solvated and not ion-paired; not solvated and ion-paired; solvated and not ion-paired; and solvated and ion-paired. We have previously demonstrated that partially solvated, polarizable anions have an affinity for the pockets of hosts **1a** and **1b**,[24] and therefore discount the first two of these options. In other words, we believe the bound thiolate is stabilized by one or two waters of solvation. However, the location of the counter cation (outside or co-encapsulated) is unclear. However, the fact that the guest motif does not change much upon deprotonation, and the fact that the concentration of the guest in the capsule is ~3.2 M, suggests a bound (but solvated) RSNa group with little in the way of charge separation. Further efforts may ultimately discern between all of these possibilities.

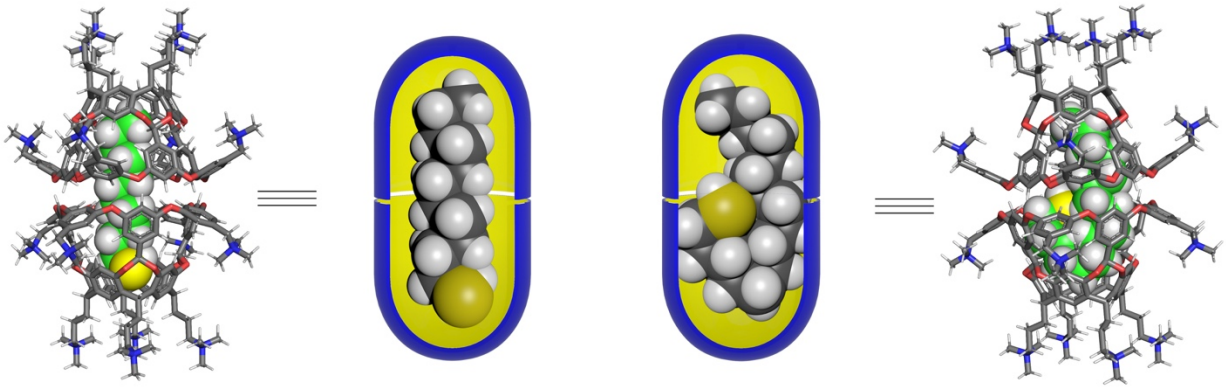


Figure 3: Representative examples of the guest binding-motifs observed for the complexes formed between the hosts and guests in this study. Shown are molecular models of the complexes formed by positively charged **1b** and the C11 thiol guest **3** in the extended conformation (*extreme left*), and the J-motif for the C16 thiol guest (**8**, extreme *right*), as well as their corresponding schematic representations (*center*).

By way of example, Figure 4a shows the titration data for bound guest **6** inside the positively and negatively charged capsules. Figure 4b shows the obtained pK_a values for each thiol guest in the different host-guest combinations (estimated errors: < ± 0.1 pK_a units for guests **2**-**7**, and < ± 0.2 pK_a units for guest **8**; see Table S4). These values compare to the typical pK_a of a (free) thiol in aqueous solution of approximately 10-11. The data suggests that for both capsules there is a maximal pK_a value for guest **3**. With increasing guest length, the pK_a of the thiol group decreases: in the case of negative capsule **1a**$_2$ the pK_a range varies from 13.0 to 8.4 (ΔpK_a = 4.6), whilst in the case of positive capsule **1b**$_2$ the range is 12 to 6.3 (ΔpK_a = 5.7). We attribute this general trend to the position of the thiol group in the complex. Thus, for the shorter guests the thiol group is located deep in the end of the capsule in a relatively non-polar environment (Figure 3, *left*). As a result, its acidity is lower than that of a free thiol in solution. However, with longer guests that energetically prefer to adopt a J-motif, the thiol group is at least in part located near to the equatorial region of the capsule (Figure 3, *right*). Considering the propensity of these capsules to "breath", i.e., partially open to allow the entry and egression of small molecules,[25] this equatorial region would be expected to be more hydrophilic, and the trends in acidity constants shown in Figure 4b support this notion. Thus, in the case of the capsule formed by positive **1b**, the position of the thiol group can alter its acidity by almost 6 pK_a units.

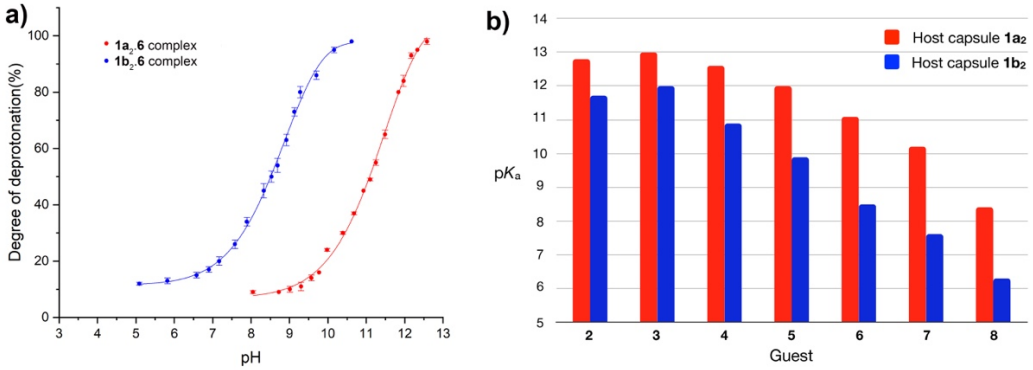


Figure 4: a) pH titrations for guest **6** encapsulated within negative and positive capsules **1a**$_2$ and **1b**$_2$. Individual data points are shown as circles with attendant error bars, whilst data fitting (Supporting Information) is shown as the corresponding lines. Each titration was recorded in at least duplicate. The degree of deprotonation was determined by integrating the thiol proton signal relative to the terminal methyl signal of the guest as a function of added NaOH. b) Bar graph of pK_a values for complexes of guests **2**-**8** inside the capsules formed by **1a** and **1b**. Estimated errors are less than ± 0.1 pK_a units for guests **2**-**7**, and ± 0.2 pK_a units for guests **8** (see Table S4, Supporting Information).

The difference in the pK_a of individual guests (ΔpK_a) in the two capsules ranges from 1.0 to 2.6 units, which corresponds to 6.3 to 14.8 kJ mol^{-1} of stabilization in the positive capsule ($\Delta\Delta G = 2.3RT\Delta pK_a$). Previous electrostatic potential and charge stabilization calculations suggest

that a negative charge should be stabilized in the positive EPF of **1b**$_2$ by ~13 kJ mol^{-1} relative to the negative EPF of **1a**$_2$.[26] Moreover, these calculations also suggest that the EPFs in the inner-spaces of the capsules are rather homogeneous (Figure S1b). Hence the relatively small ΔpK_a between the two capsules for the shorter guests is quite surprising. Why is this? Coulombic interactions are non-directional, whereas ion-dipole interactions very much are. Thus, our tentative hypothesis is that the latter is key to explain the small difference in pK_a of small guests. For example, the positive and negative charges of the two hosts may establish or alter (sub)dipoles within the two hosts such that their H_b protons (Figure 1) form hydrogen bonds of different strength to the bound thiol/thiolate. Alternatively, the opposite charges of the hosts may alter the position and orientation of any co-encapsulated water molecules within the two inner-spaces of the capsules. Thus, if the inner space of negatively charged host **1a**$_2$ was more strongly solvated, then any bound water molecule co-encapsulated with a thiol would enhance the acidity relative to a dryer pocket. Our data cannot of course differentiate between these or other scenarios, but it seems quite likely that the inherent asymmetry of positive ion-dipole versus negative ion-dipole interactions (e.g., $M^+\cdots OH_2$ versus $X^-\cdots HOH$) is somehow a pK_a equalizer with thiol groups at the base of the pocket. It will be interesting to see in future work if the same applies to other ionizable groups.

How does the ionic strength of the external aqueous solution affect the acidity of internally bound guests? We have previously shown that anions bind more strongly to the outer surface of positive **1b** than cations are attracted to the surface of negative **1a**.[27] For example, ClO_4^- binds to the inner pocket and outer crown of four, trimethylammonium pendent groups of **1b** with affinities of 4,300 and 2,400 M^{-1} respectively. This means that perchlorate can bring about charge neutralization and precipitation of the host at ~ 10 mM.[24a] In contrast, the alkali metal ion that binds most strongly to **1a**, Cs^+,[28] fails to induce the precipitation of this host even in the hundreds of mM concentration range.[27] Being the strongest binding alkali metal ion, we selected Cs^+ (as the chloride salt) to examine the effects of cation binding to the pK_a of guest **5** bound to **1a**$_2$. As anticipated, binding was weak; the addition of 200 equivalents of the salt reduced the negative EPF of the capsule somewhat and shifted the pK_a of bound **5** from to 12.0 to 11.8 (Figures S65-67).[29] In contrast, the addition of 200 equivalents of NaBr to the complex of guest **5** inside positive capsule **1b**$_2$ led to a larger pK_a shift: from 9.9 to 10.4; this despite Br^- being a relatively weakly binding anion to **1b** (K_a for binding to the crown of four trimethyl ammonium pendent groups = 710 M^{-1}).[24a]

Larger and more polarizable anions bind even more strongly to positive **1b** (see, for example, the aforementioned ClO_4^- affinity data) and induce precipitation via charge

neutralization at millimolar concentrations.[24a] Thus, we observed that more than 10 equivalents of $NaClO_4$ caused aggregation of the **5**.**1b**$_2$ complex. Correspondingly, to examine the effects that this stronger binding anion has on the pK_a of a bound guest, we carried out acid-base titrations of the complex in the presence of 0, 1, 2, 3, 4, 6, and 8 equivalents of $NaClO_4$ (Figures S73-S83). During each ^{1}H NMR spectroscopy titration we saw no evidence of either co-encapsulation or the anion displacing the large thiol/thiolate guest (Figure S73),[24a] only signal shifts indicative of binding to the crowns of four trimethylammonium pendent groups at the 'poles' of the capsule.

Figure 5 shows a plot of the measured pK_a value of bound guest **5** as a function of different equivalents of $NaClO_4$. As this figure reveals, the acidity of the guest decreased from pK_a = 9.9 to ~10.4 over the salt concentration range examined (ΔpK_a = 0.5). Thus, perchlorate had the same net effect as bromide, but at much lower concentrations.

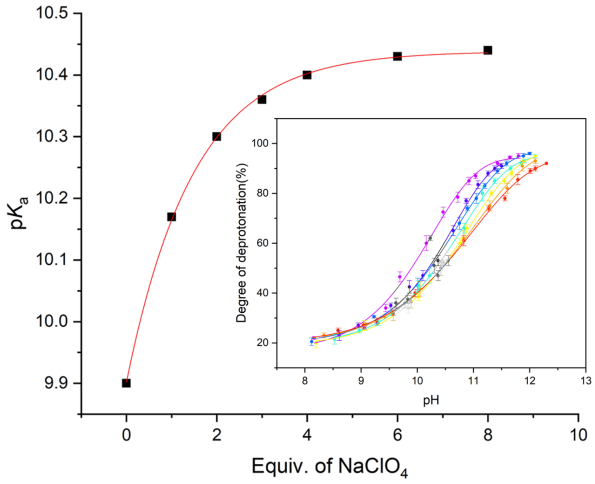


Figure 5: The obtained pK_a values from pH titrations of bound **5** within the **1b**$_2$ capsule, plotted against the equivalents of $NaClO_4$ present in each titration. The red line is a fit to 1:1 binding model. Inset: the source pH titration curves for guest **5** encapsulated within capsule **1b**$_2$. Each titration was recorded in at least duplicate.

Fitting the data in Figure 5 to a 1:1 model for ion complexation gave a K_{app} = 1,553 M^{-1}, Figures S81 and S82). As discussed above, the binding constant of ClO_4^- to the crown of four trimethyl ammonium pendent groups of **1b**, corresponding to the 'poles' of the capsule 'was found to be 2,400 M^{-1}.[24a] We suspect that the K_{app} obtained here represents the average affinity for anion binding to the capsule surface. For example, in addition to the 'pole' binding sites, in the positive **1b**$_2$ capsule, it may be the case that there are weaker binding sites between pairs of equatorially-located benzyltrimethylammonium groups on opposing hemispheres. Interestingly, that saturation of the change in pK_a of bound guest **5** occurred close to the aggregation point of the complex suggests that many of its sixteen positive charges are neutralized by binding ClO_4^-.

To confirm this, we utilized zeta potential measurements (Figure S84). Thus, in the presence of 60 equivalents of NaCl (to ensure good conductivity) the zeta potential (ζ-potential) of the **1b**$_2$ capsular complex was measured to be approximately +30 mV, whereas in the presence of 50 equivalents of NaCl and 10 equivalents of $NaClO_4$, the ζ-potential of the complex was only +10 mV. This compares to the capsular complex **5**.**1a**$_2$ in the presence of 60 equivalents of NaCl, where the ζ-potential was –60 mV. This last point again illustrates the lower degree of counter-ion binding to negatively charged **1a**$_2$ versus **1b**$_2$. Overall, these results demonstrate that ion binding to the outer surface of the capsule is greater in the case of **1b**$_2$, but that the effect of this on the acidity of the bound guest is relatively small compared to the effect of the location of the thiol group within the inner space.

In conclusion, despite the relative remoteness of the water-solubilizing groups of the supramolecular capsules **1a**$_2$ and **1b**$_2$, the acidity of guests within their inner yocto-liter spaces is considerably influenced by the electrostatic field generated by the solubilizing groups. In these capsules however a bigger influence is the position that the acidic thiol group adopts within the inner-space; pK_a measurements suggest that acidity can be altered by up to almost 6 pK_a units depending on whether the acidic group is located in the pole or equatorial region of the capsule. (Counter) ion binding to the outside of the capsule also affects the pK_a of internalized guests, especially in the case of the positive capsule **1b**$_2$. However, the extent to which the acidity can be modulated appears to be relatively small and is bounded by aggregation effects at low ζ-potential values. Overall, these results help define the impact-potential of different factors in reactions in confined spaces, and suggest new subtleties for controlling chemical conversions within such inner-spaces.

Acknowledgements

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Supporting Information. Synthesis of guests **2**-**10**, ^{1}H, COSY and DOSY NMR characterization of host-guest complexes, pK_a determinations in the absence and presence of added salts, and zeta-potential measurements of selected complexes.

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22. There are multiple equilibria involved in the deprotonation of the bond guest. Specifically, 1:1 complex formation, capping of this to make the 2:1 capsular complex, and potentially the equilibrium between the 2:1 complex and the empty capsule and the free guest. However, we see no evidence of any of these equilibria and therefore, in defining pK_a, assume that the 2:1 complex is an integral molecule.

23. An exception to this was guest **10**. Although in this case there was no apparent SH to titrate with base, the addition of excess base to the complex (to give a pH = 12.3 solution) led to significant changes in the guest binding region of the ^{1}H NMR spectrum (Figure S33 shows the COSY NMR spectrum of the complex with host **1a**). At this high pH, the signals attributed to the J-motif(SH) disappeared, whilst those corresponding to a J-motif(Me), with the thiolate at the equatorial region, increased (e.g., the terminal Me signal at approximately –3.25 ppm; for Δδ vaules of all signals see Figure S34). This further supports the assignment of the motif of this guest at close to neutral pH (Figure 2 and accompanying text). However, at pH 12.3 other broad signals indicative of a fourth motif also appeared. Unfortunately, COSY NMR (Figure S33) could not resolve most of the signals in this motif and its identity is unknown.

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29. We also carried out a titration using CsOH instead of NaOH as base (Figure S68 and S69). This revealed a shift in pK_a for guest **5** in the capsule formed by **1a** from 11.97 to 11.73 when NaOH was replaced by CsOH.

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