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# Attraction by repulsion: compounds with like charges undergo self-assembly in water that improves in high salt and persists in real biological fluids†

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**We report a family of highly anionic calixarenes that form discrete homo-dimeric assemblies in pure water, that get stronger in high salt solutions, and that remain assembled in complex, denaturing solutions like real urine. The results reveal the potential of like-charged subunits for self-assembly in high-salt solutions and biological fluids.**

Most successes in 'bio-supramolecular chemistry' that function in pure water have come from agents that combine rigid, concave structures, a hydrophobic component, and mutual electrostatic attraction between multiple cationic and anionic groups on either partner. Using mutual electrostatic attraction reliably promotes assembly in organic solvents, but has a mixed record of success upon transfer of discrete assemblies to pure water and/or salty, buffered solutions.<sup>1–6</sup> Regardless of the successes or failures of such designs, it is a general feature that the addition of salts (like those found in all biological fluids) will weaken electrostatically driven complexes by increasing the dielectric<sup>6</sup> and/or directly competing for binding sites.<sup>7</sup> Salts also weaken ion binding by neutral hosts like cucurbiturils.<sup>8</sup> Denaturing organic co-solutes (such as urea in urine) create a further challenge for synthetic molecular recognition systems intended to work in real biological fluids. We report here a new assembly motif in which similarly charged building blocks bind to each other faithfully in water, form a stronger complex upon addition of salt, and remain assembled in complex solutions like mock serum and real urine. Our results show the unexpected advantages of using like-charged molecular recognition elements in high-salt solutions.

We previously prepared mono-functionalized, sulfonated calixarenes because the appended group tunes the recognition of methyllysine guests within the binding pocket.<sup>9,10</sup> In the course of this work, we noticed that tolyl-substituted compound **2** (Fig. 1) has an unusual NMR spectrum in water. Resonances for the pendant methyl group are shifted upfield by up to 2.8 ppm in D<sub>2</sub>O and in phosphate-buffered D<sub>2</sub>O, suggesting aggregation. The resonances return to their normal chemical shifts upon dilution (Fig. 2a), showing that intermolecular association is responsible for the observed shifts. No signs of assembly exist in CD<sub>3</sub>OD (Fig. 2a).

We prepared analogs **1–5** bearing a series of alkyl substituents in order to characterize this mode of assembly. Each analog forms assemblies similar to those of the parent. Upfield chemical shifts occur in D<sub>2</sub>O (Fig. 1b) and in phosphate-buffered D<sub>2</sub>O, but not CD<sub>3</sub>OD (ESI†). The shifts are concentration dependent and return to normal values upon dilution (ESI†). The chemical shift perturbations show that the pendant arm in each compound is bound in a calixarene pocket in an end-on manner (Fig. 1b). Molecular modeling suggests this can only be achieved in a homodimer arrangement, and diffusion constants (*D*) obtained for **5** by DOSY-NMR (Table 1) and NOE data (Fig. 1c, ESI†) further support formation of a homodimer. The single crystal X-ray structure of **5**, obtained after slow evaporation of water, revealed a homodimeric structure that is consistent with all solution-phase NMR data (Fig. 1d).‡

The thermodynamics of dimer formation reveal the influence of charge and salt on the assemblies. Isothermal titration calorimetry (ITC) dilution titrations carried out in the same phosphate buffer as NMR experiments show low-mM *K<sub>d</sub>* values (Table 2). The strength of binding increases with increasing hydrophobic surface area. The same ITC experiments were repeated in phosphate buffered saline containing the concentrations of NaCl, KCl, and MgCl<sub>2</sub> found in human serum (Table 2). Each of **1–5** assembled more strongly in the high-salt solution, with the strongest dimer **5** reaching a *K<sub>d</sub>* of 730 μM under these conditions.

The very low p*K<sub>a</sub>*'s of sulfonic acids (p*K<sub>a</sub>* < 0) and of the first phenol OH in a calix[4]arene scaffold (p*K<sub>a</sub>* = 3.26)<sup>11</sup> mean that each monomer **1–5** is tetra-anionic under the buffered conditions

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group takes the place of the alkyl groups in 1–5. Compound 6 shows NMR shifts in water that support assembly as a similar dimer in which the  $\text{CH}_2\text{NH}_3^+$  group interacts with the pocket and sulfonates of its partner ( $\text{ESI}^+$ ). Some aryl–aryl hydrophobic contacts are retained in the dimer of 6, but the mutual electrostatic repulsion is partly replaced with a more conventional cation–anion interaction. Unlike 1–5, ITC shows that the dimer of 6 becomes slightly weaker upon addition of salt, as expected of a dimer that is at least partially driven by electrostatic attraction. Literature examples that involve multiply charged cationic and anionic calix[4]arenes forming heterodimers either precipitate from pure water<sup>4</sup> or have not been studied in high salt,<sup>13,14</sup> making direct comparisons between the salt responses of 1–5 and those of purely electrostatically driven dimers difficult.

Self-assembly in nature is a tug-of-war between electrostatics and hydrophobicity. Lipids self-assemble into polymeric aggregates under the control of charged head groups, lipid surface area, and molecular shape parameters.<sup>15</sup> But the ways that proteins dictate their own discrete assemblies are more subtle and complex. Some have evolved to position charged, repulsive “gatekeeper” residues on aggregation prone surfaces in order to control specificity, maintain solubility, and avoid undesired aggregation—an evolved strategy that has also been called negative design.<sup>16,17</sup>

Compounds 1–5 also use repulsion to temper their mutual hydrophobic attraction, and it occurred to us that this might enable them to remain faithful to each other even in diverse salt conditions and in real biological fluids containing other potential partners for binding and aggregation. Fig. 2 shows the NMR spectra of compound 5 in water, water containing added divalent salts, phosphate buffer, mock serum, mock urine, and real human urine. In each case, the characteristic NMR shifts confirm the presence of the assembly. Therefore, these recognition modules can survive the presence of high concentrations of salts, denaturing solutes (urea, creatinine), hydrophobic metabolites (steroids), proteins (albumin), and other dissolved biomolecules. They remain assembled with each other even though other sulfonated calix[4]arenes are known to bind alkali metal ions,<sup>7,18</sup> steroids,<sup>19</sup> and albumin.<sup>20</sup> The more weakly dimerising tolyl monomer 2 shows similar results ( $\text{ESI}^+$ ), showing that robustness in real biological fluids is a general feature of this recognition motif. Few examples exist of host–guest chemistry in untreated urine, a salty and aggressively denaturing medium.<sup>21–23</sup>

The use of like charges in assemblies that persist in high salt has not previously been highlighted as a self-assembly motif, but we identified some recent literature examples that suggest that it is a general route to achieve molecular recognition in salt water. (1) The ‘octa-acid’ cavitand reported by Gibb bears a (–8) charge, yet binds hydrophobic anions within its interior and shows increased affinity for anion guest binding upon addition of NaCl.<sup>24</sup> (2) A water-soluble bambusuril host has 12 anionic pendant groups, binds hydrophobic anions in its interior, and retains its anion affinity upon the addition of very high concentrations of salt.<sup>25</sup> (3) Anionic calixarene hosts, including a sulfonatocalix[8]arene with a net –8 charge, bind and transport the membrane-impermeable, dianionic dye Lucifer yellow

across a layer of Caco-2 epithelial cells.<sup>26</sup> (4) For good measure, a simple tetracationic oxacalixarene host was recently shown to bind to the dicationic guest paraquat in water, although salt effects were not reported.<sup>27</sup> The motif that is common among these systems is the use of hydrophobic attraction tempered by like-charged electrostatic elements within a rigid, concave molecular host framework.

The emerging picture is that, while approach of like charges is repulsive in the microscopic sense, it can provide net benefits for specific molecular recognition in salty solutions. Where traditional cation + anion designs intrinsically fight against the competing influence of salt, the counterintuitive use of anionic elements to bind other anions is inherently well suited to achieve molecular recognition. Another advantage is that these designs retain excellent solubility in diverse aqueous media, whereas precipitation upon binding and charge neutralization is a common liability for hetero-charged systems of many types. In the face of many results—published and unpublished—of molecular recognition systems that work in polar solvents but fail to survive the move to pure water, added salt and/or dissolution in a real biological fluid, the use of like-charged motifs provides a useful alternative.

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## Notes and references

‡ Crystallographic data for 5:  $\text{C}_{38}\text{H}_{70}\text{Na}_3\text{O}_{32}\text{S}_3$ , ( $\text{C}_{38}\text{H}_{32}\text{O}_{13}\text{Na}_3(\text{H}_2\text{O})_{19}$ ),  $M = 1204.09$ ,  $0.14 \times 0.12 \times 0.09$  mm,  $T = 200$  K, hexagonal, space group  $P\bar{3}1c$ ,  $a = 27.0902(13)$  Å,  $b = 27.0902(13)$  Å,  $c = 30.1891(16)$  Å,  $V = 19186.9(17)$  Å<sup>3</sup>,  $Z = 12$ ,  $D_c = 1.251$  Mg m<sup>–3</sup>,  $\mu = 1.971$  mm<sup>–1</sup>,  $F(000) = 7620$ ,  $2\theta_{\text{max}} = 100.86^\circ$ , 35587 reflections, 6710 independent reflections [ $R_{\text{int}} = 0.0331$ ],  $R_1 = 0.0794$ ,  $wR_2 = 0.2433$  and GOF = 1.110 for 6710 reflections (571 parameters) with  $I > 2\sigma(I)$ ,  $R_1 = 0.0989$ ,  $wR_2 = 0.2670$  and GOF = 1.110 for all reflections, max/min residual electron density +0.443/–0.374 e Å<sup>–3</sup>. Deposited as CCDC 1033560.

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