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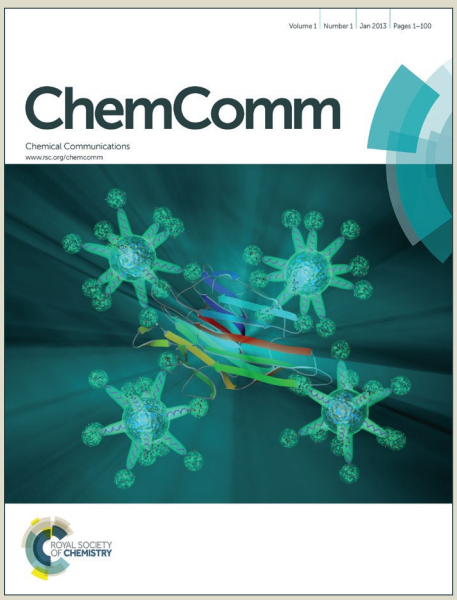

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Enhancing Binding Affinity and Selectivity through Preorganization and Cooperative Enhancement of the Receptor

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When direct host–guest binding interactions are weakened by unfavorable solvent competition, guest-triggered intrareceptor interactions could be used to augment the binding. This strategy of cooperative enhancement, when combined with the principle of preorganization, yielded a strong and selective receptor for binding citrate in water.

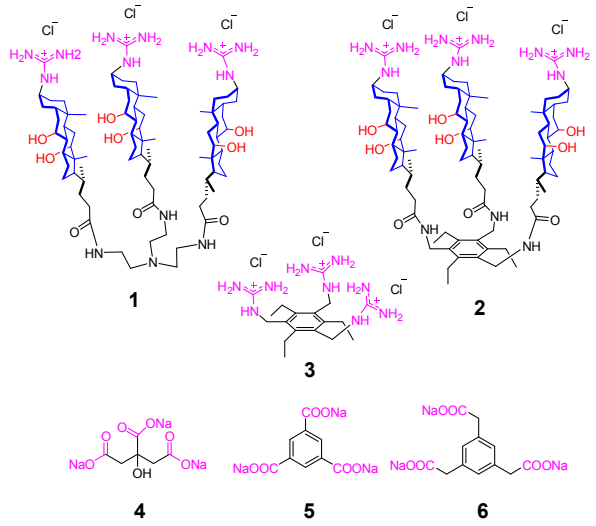
Molecular recognition is at the heart of biology and vital to countless processes including ligand–receptor interactions, gene expression, transport, and catalysis.[1, 2] Over the last decades, chemists have made remarkable progress in constructing receptors functional in organic solvents, doing so in aqueous solution, however, remains challenging.[3, 4] The difficulty in the latter partly derives from the nature of the noncovalent forces used in the binding: whereas polar interactions such as hydrogen bonds are directional and highly programmable, they tend to be ineffective in protic solvents due to competition from solvent. Conversely, although hydrophobic interactions can be strong in water, their nondirectionality makes it difficult to achieve high selectivity in binding.

We recently reported a method to create guest-complementary hydrophobic binding pockets within water-soluble nanoparticles through molecular imprinting of surfactant micelles.[5-8] Strong and selective binding was achieved for a variety of water-soluble molecules including bile salt derivatives,[5] aromatic carboxylates and sulfonates,[6, 7] and nonsteroidal anti-inflammatory drugs (NSAIDs).[8] To be successfully imprinted within the micelles, however, the guest needs to possess significant hydrophobicity.

Citric acid is a natural preservative found in citrus fruits. It is also an important intermediate in the citric acid cycle. To create receptors for such guest molecules with little or no hydrophobicity, we have to deal with the challenge in utilizing inherently weak polar binding forces in water. One possible solution to the problem is multivalency.[10, 11] If multiple binding groups in a concave receptor can be oriented to interact with the (polar) guest, strong binding should be achievable even if the individual interaction are weak. Anslyn and co-workers, indeed, in a classic paper described such a tripodal receptor that bound citrate in D_2O with an impressive binding constant (K_a) of 6.9×10^3 M^{-1}.[12] Many citrate receptors have been reported using similar strategies, sometimes using metal–ligand complexation for higher binding affinity.[13-22]

Nature has a different strategy to deal with weak binding forces. Streptavidin binds biotin with a K_a of $10^{13.4}$ M^{-1}. Having two highly polar functional groups (i.e., carboxylic acid and urea) and a rather small size (M.W. = 244.3), its tight binding cannot be fully explained by enthalpy gain or displaced water molecules in the binding pocket. Upon binding with biotin, the melting point of the protein increases by 37 °C and numerous backbone amide proteins become resistant to H/D exchange.[23] These results suggest that the guest-binding has turned on previously disengaged intrareceptor interactions, which contribute to the binding equilibrium even though they are not at the binding interface. With these hidden "binding interactions" delocalized throughout the protein, nature is able to achieve high binding affinity even when direct host–guest binding forces are of limited strength. Similar synthetic cooperatively enhanced receptors (CERs),[24] although still few and far between, began to emerge in the literature in recent years.[25-30]

To bind citrate in water, we designed CERs **1** and **2**. The C3-symmetrical receptors have three facially amphiphilic cholates functionalized with guanidinium groups on the top. The positively charged guanidinium groups are on the β face of the cholate, opposite to the hydroxyl groups. Their electrostatic repulsion is engineered to hinder close contact of the cholate groups, making it difficult for the intrahost hydrophobic interactions to fully engage. When citrate, a trianonic guest, binds the receptor, the repulsion among the guanidinium groups is eliminated while the citrate carboxylate groups, being close to one another, promote the intermolecular aggregation of the cholates. The guest-triggered hydrophobic interactions are expected to contribute to the binding, even though the guest itself has negligible hydrophobicity.



The major difference between **1** and **2** is the scaffold on which the cholates were assembled: one was built on a flexible scaffold and the other on the preorganized 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene.[31, 32] To compare our CERs with conventional receptors, we also prepared **3**, based on the same hexasubstituted benzene, without the cholate groups responsible for the hypothesized intramolecular hydrophobic enhancement.

Table 1. Binding data for receptors **1**–**3** obtained by ITC.[a]

Entry	Complex	K_a (10^3 M^{-1})	K_{rel}[b]	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
1	**1•4**	10.4 ± 1.1	1	-5.5 ± 0.04	10.4 ± 0.3	15.9
2	**1•5**	7.6 ± 0.9	0.73	-5.3 ± 0.05	-4.5 ± 0.5	0.8
3	**1•6**	3.2 ± 0.2	0.31	-4.8 ± 0.06	-9.7 ± 1.0	-4.9
4	**2•4**	77.9 ± 4.5	1	-6.7 ± 0.04	2.5 ± 0.1	9.2
5	**2•5**	2.1 ± 0.7	0.03	-4.5 ± 0.3	-14.0 ± 0.2	-9.5
6	**2•6**	-[b]	-[b]	-[b]	-[b]	-[b]
7	**3•4**	16.3 ± 2.7	1	-5.7 ± 0.1	-1.6 ± 0.4	4.1
8	**3•5**	3.9 ± 0.8	0.24	-4.8 ± 0.5	-5.8 ± 2.4	-0.9
9	**3•6**	-[b]	-[b]	-[b]	-[b]	-[b]

[a] The titrations were performed in duplicates in Millipore water and the errors in K_a between the runs were generally < 20%. The number of binding site (N) determined by ITC averaged ~0.4 for **4** and ~0.8 for **5** and **6** for all three receptors. The lower-than-unity N in the citrate–receptor complexes could be caused by the presence of small amounts of higher order complexation, as in Anslyn's tripodal guanidinium receptor which bound citrate mainly in the 1:1 stoichiometry but formed small amounts of higher order complexes.[9] In our hands, ESI-MS confirmed the 1:1 complex between **4** and the preorganized receptor **3** (Figure S4 ESI†).Since the diffusion coefficient of **2** (our strongest and most selective citrate receptor) changed very little upon binding citrate (vide infra), the higher order binding processes must be minor. [b] Binding was not detectable by ITC.

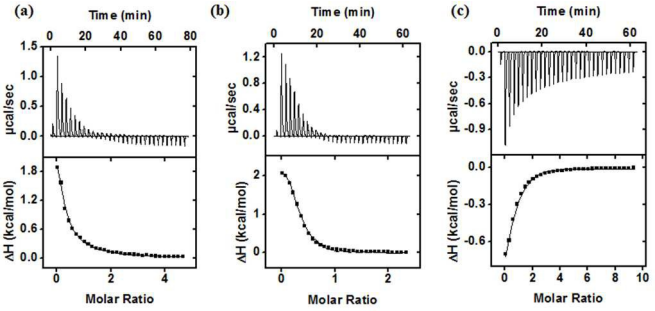


Figure 1. ITC titration curves obtained at 298 K for the binding of citrate **4** by (a) **1**, (b) **2**, and (c) **3**. The data correspond to entries 1, 4, and 7, respectively, in Table 1.

Table 1 shows the binding data for the three receptors. Selected ITC titrations curves are shown in Figure 1 and more in the Supplementary Information (Figures S1–S3, ESI†). We chose to study two additional anionic guests (**5** and **6**) in addition to citrate (**4**). All the guests possess three carboxylates, with the distance between the binding groups more or less increasing from **4** to **5** to **6**. Our hypothesis was that, as the carboxylate groups in the guest are separated by a larger distance, their ion-pairing interactions with the host would keep the cholates apart, preventing their effective intramolecular hydrophobic contact. Consequently, the cooperative enhancement designed in the citrate binding will either diminish or disappear in **5** and **6**.

The binding data support our hypothesis. Receptor **1** bound citrate **4** with a very significant K_a of 10.4×10^3 M^{-1} in water (Table 1, entry 1). Although the binding was weaker than that of the preorganized control receptor **3** ($K_a = 16.3 \times 10^3$ M^{-1}, entry 7), it is encouraging to see that a highly flexible receptor could bind citrate with such an affinity. The preorganized cholate receptor was clearly the best among the three to afford a K_a of 77.9×10^3 M^{-1} or a binding free energy of $-\Delta G$ = 6.7 kcal/mol (entry 4). Its ^{1}H NMR spectrum in D_2O showed well-resolved peaks at submillimolar concentrations (Figure S5 ESI†). Because the ITC was performed with the concentration of the receptor at 0.1–0.2 mM, host aggregation was not expected to be a problem under our experimental conditions.

We also studied the most stable host–guest complex (**2•4**) by two additional NMR techniques. 2D diffusion-ordered NMR spectroscopy (DOSY) experiments showed that **2** and **4** (both at 1.5 mM) had a diffusion coefficient of 2.43 and 5.57×10^{-10} m^2s^{-1} in D_2O, respectively (Figures S6 and S7, ESI†). The slower movement of the former was expected from its larger size. The homogeneous distribution of the diffusion peaks rules out any significant host aggregation at 1.5 mM. Most importantly, citrate in a 1:1 mixture of **2** and **4** had a diffusion coefficient of 2.16×10^{-10} m^2 s^{-1} in D_2O (Figure S8, ESI†), slightly slower than that of **2** and thus fully in agreement with the host–guest complexation.

The complexation was further supported by 2D NOESY experiments, which showed close contact between **2** and **4**, as well as cholate–cholate contact that resulted from the citrate-triggered intramolecular aggregation of the cholates (for details, see Figures S9 and S10, ESI†).

An interesting difference between the cholate receptors and the control receptor was in the driving force for the binding. Table 1 indicates that the binding of citrate by either **1** or **2** was endothermic with a positive ΔH, but was exothermic by **3** (compare entries 1, 4, and 7). The endo- and exothermic difference is seen clearly in the ITC titration curves (Figure 1). Note that all three bindings have significant entropic contributions, with $T\Delta S$ being 15.9, 9.2, and 4.1 kcal/mol, respectively, for receptors **1**, **2**, and **3**.

For ionic binding between citrate and the preorganized receptor **3**, the entropic contribution normally is considered to come from the release of water molecules that solvate the ionic groups prior to the binding.[9, 33-35] The two cholate receptors are considerably more flexible than **3**. Because binding reduces freedom of the cholate hosts substantially, the entropic driving force normally is expected to decrease but increased instead. In fact, receptor **1**, the most flexible among the three, had the largest entropic driving force (15.9 kcal/mol). Since the enthalpy of binding citrate was positive/unfavorable for the two cholate receptors, the only reason the complex could form at all was the increased entropy.

The unusually large entropic driving force for **1** and **2** are consistent with our hypothesized hydrophobically enhanced binding.[36] When citrate ion-pairs with the three guanidinium groups, hydrophobic contact among the cholates is anticipated to improve as the electrostatic repulsion among the guanidinium groups is eliminated. Since (tighter) contact among the cholates would release water molecules formerly associated with the cholate β faces, strong entropic driving forces in the citrate binding of **1** and **2** are predicted by our binding model. The flexible tether in **1** probably makes it easier for the cholates to interact with one another, thus creating the strongest hydrophobic/entropic driving force. The large entropic driving force in receptor **1** was compensated by a significant enthalpic penalty, making its overall binding weaker than that of **2**.

Receptor **2** was not only the strongest but also the most selective among the three citrate receptors. Table 1 lists the relative binding constants (K_{rel}) of guests **5** and **6** to that of citrate. According to our data, the flexible cholate receptor **1** had the poorest selectivity, with K_{rel} being 0.73 and 0.31 for **5** and **6**, respectively. The preorganized cholate receptor **2** afforded a K_{rel} of 0.03 for **5** and showed nondetectable binding for **6**. Although the control receptor **3** also exhibited no binding for **6**, it bound **5** with a K_{rel} of 0.24, thus less discriminating than cholate receptor **2**. Stronger citrate-binding receptors have been prepared by Schmuck and Schwegmann using the principle of preorganization and multivalency, but the selectivity was lower.[37]

Our data so far strongly supports the intimate contact among the cholates being responsible for the unusual stability of complex **2•4** in water. Our hypothesized binding model also suggests that poor contact among the cholates should be the cause of the lower stability of **2•5**. If these predictions are true, a hydrophobic "gap" should exist among the cholates of **2•5**, which is lacking in the former. To confirm these features, we titrated receptor **2** with **4** and **5**, respectively, *in the presence of 1.0 μM pyrene* in water. Pyrene has five vibronic bands in emission. The first band (I_1) near 372 nm becomes more intense in more polar environment and the third (I_3) near 384 is rather insensitive to the environmental polarity. As a result, the I_3/I_1 ratio becomes larger as the probe enters a nonpolar

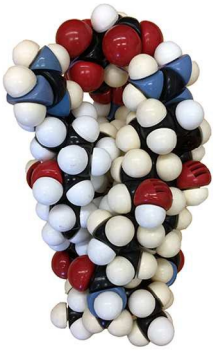


Figure 2. CPK model of complex **2•4**, showing the hydrogen-bonded citrate on the top and tightly packed cholates groups.

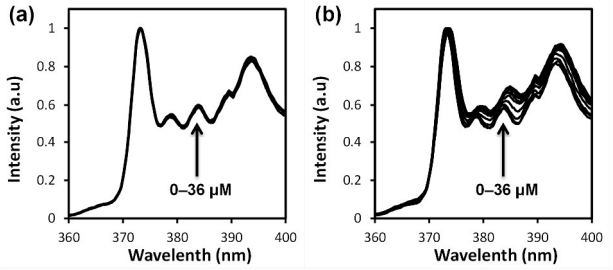


Figure 3. Emission spectra of pyrene normalized to vibronic band I_1 in different concentrations of (a) citrate **4** and (b) benzene-1,3,5-tricarboxlate **5** in the presence of receptor **2** in Millipore water. [Pyrene] = 1.0 μM; [**2**] = 20 μM.

microenvironment.[38] If indeed a hydrophobic gap is created when **5** binds **2**, pyrene, being hydrophobic, should insert itself into the gap, provided that the gap is large enough to accommodate the probe. With the cholates tightly associated with one another in **2•4** (CPK model shown in Figure 2), pyrene is expected to remain in the aqueous phase, displaying a nearly constant and rather low I_3/I_1 value.

Figure 3a shows the normalized emission spectra of pyrene as 0–36 μM of citrate **4** was added to 20 μM of receptor **2**. As predicted, the emission of pyrene stayed unchanged, suggesting that pyrene remained in water throughout the titration. Calculated from the binding constant and the concentrations, the percentage of **2** being complexed with citrate ranged from 0 to 67% during the titration. The nearly constant I_3/I_1 indicates that pyrene under our experimental conditions bound neither the free receptor nor the **2•4** complex.

When **2** was titrated with benzene-1,3,5-tricarboxlate **5**, the results were completely different (Figure 3b). The I_3 band intensified continuously relative to I_1, shown also by Figure 4, in which the I_3/I_1 ratio was plotted against the concentration of the guest. The gradual increase of I_3/I_1 suggests that a hydrophobic gap indeed was created in complex **2•5** that could accommodate pyrene. The three carboxylate groups in **5** are separated by a phenyl spacer. Formation of three amidinium–carboxylate salt bridges, therefore, is anticipated to keep the cholates apart—this is how the hydrophobic gap is formed in the complex. The net result is that, when a CER binds a mismatched guest, the intrareceptor interactions that have enhanced the binding of a well-matched guest turn against the poorly-fitted guest because the guest-binding creates unfavorable intrareceptor

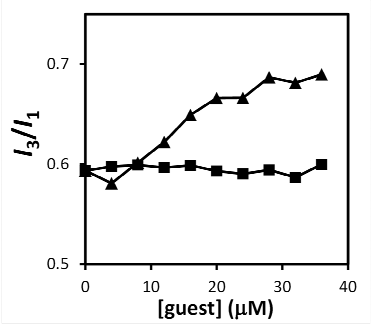


Figure 4. Pyrene I_3/I_1 ratio as a function of the concentration of citrate **4** (■) and benzene-1,3,5-tricarboxylate **5** (▲) in Millipore water. [Pyrene] = 1.0 μM. [**2**] = 20 μM.

interactions. Put it in a different way, the guest-triggered intrareceptor interactions are a double-edged sword: they reward the "fittest" guest by contributing to their binding and penalize the "misfitted" ones by taking away what can be obtained through direct host–guest binding interactions.

Cooperative enhancement is beneficial to selectivity only if the CER is properly preorganized, as demonstrated by receptor **2** in our study. For nonpreorganized CER **1**, the flexible scaffold gives the cholates too much freedom to adjust themselves, both in the free receptor and after binding the guest. The result is very poor selectivity of binding, as shown by the binding data.

One of the most interesting properties of the CERs is that what controls the binding—both in terms of affinity and selectivity—could be completely away from the binding interface. This feature is the key difference between a CER and traditional preorganized hosts whose binding action mainly happens at the host–guest binding interface. The most significant finding of this work is that the intrareceptor interactions can be rationally engineered to favor one guest over others to *magnify both the affinity and selectivity*. As supramolecular chemistry continues to evolve, this strategy should be very useful in the design of biomimetic receptors, even when direct binding forces are weak due to either environmental or structural reasons.

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Notes and references

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† Electronic Supplementary Information (ESI) available: syntheses and characterization of materials, experimental procedures for the binding studies, additional data and Figures. See DOI: 10.1039/c000000x/

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