

## BRINGING SUPRAMOLECULAR CHEMISTRY TO LIFE

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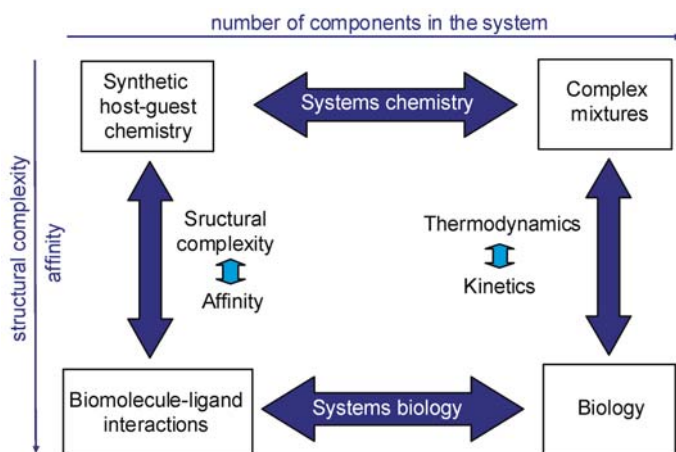
### ABSTRACT

The present impact of supramolecular chemistry in biology is not as large as it could be. The affinities of most water-soluble supramolecular receptors are many orders of magnitude lower than those of their biological counterparts, preventing their application in biological systems. We believe that the superiority of biological hosts is due to noncovalent interactions within their structures that enhance binding affinity. We have recently discovered that the synthetic receptors for anions that we have developed exhibit enhanced affinities as a result of similar intra-receptor interactions. This effect has as yet unexplored potential as a tool for pushing binding affinities of synthetic receptors into the desirable nanomolar affinity range. Another area where an expanding role of supramolecular chemistry is expected is that of complex systems. We have investigated the behaviour of dynamic combinatorial libraries of hosts in response to the introduction of guest molecules. These investigations have improved our understanding of thermodynamically controlled molecular networks, which is

relevant for the use of dynamic combinatorial libraries as a method to discover new receptors, but also provides a new entry into the emerging field of systems chemistry.

## INTRODUCTION

Nature has evolved to become enormously complex and this complexity is being investigated at various levels giving rise to a number of vibrant scientific disciplines. On a global level the interplay between different life forms is studied by ecologists while biologists tend to focus at the level of the individual organism. Biochemists investigate the role played by the individual molecules that are found within living organisms. Chemists have traditionally contributed by scrutinizing (synthetic) molecules in isolation. With the emergence of supramolecular chemistry also the noncovalent interactions between molecules have become fair game for chemists. However, the synthetic molecules developed in the course of these studies have generally been a lot smaller in size and simpler in structure than most biomolecules. Moreover, the study of complex mixtures has not been something chemists have traditionally been interested in. Chemists are trained to purify compounds and characterize substances in isolation and are therefore not naturally inclined to look at 'messy' complex mixtures. This has left a void between biology and (supramolecular) chemistry in terms of both structural complexity of the constituent molecules as well as complexity of the ensemble of molecules (Fig. 1). With regards to the latter: while systems biology (study of the interplay between the various molecules and mechanisms in a biological system) is a vibrant field, systems chemistry (the study of the behaviour of complex mixtures of man-made compounds) is still in its infancy.

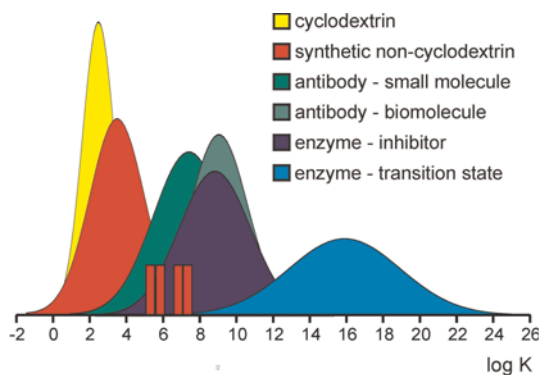


**Figure 1.** Bringing supramolecular chemistry to life will require coming to grips with complexity on the level of individual molecules as well as mixtures of molecules.

In an effort to begin filling this void we now present some of our results from our work on dynamic combinatorial chemistry [1–3]. In the course of these studies we have generated complex molecules as well as complex mixtures and have learned new things about both aspects of complexity.

## COMPLEX MOLECULES

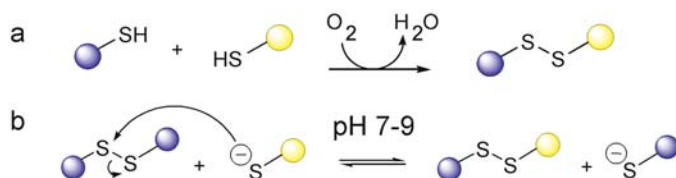
Biology has produced numerous molecules and molecular assemblies of stunning complexity and with a great variety of functions. Supramolecular chemists have made extensive efforts to produce molecules with similar functional properties, but have only rarely succeeded in rivalling nature's efficiency. This is clearly illustrated by the survey of Houk *et al.* in which binding affinities exhibited by biological hosts are compared with those of supramolecular systems [4]. On average the former outperform the latter by about six (!) orders of magnitude (Fig. 2).



**Figure 2.** Comparison between binding affinities of supramolecular receptors with their biological counterparts (data taken from ref.[4]). The affinities of the synthetic receptors we have developed using disulfide-based dynamic combinatorial chemistry (*cf.* Fig. 4) are shown as red bars.

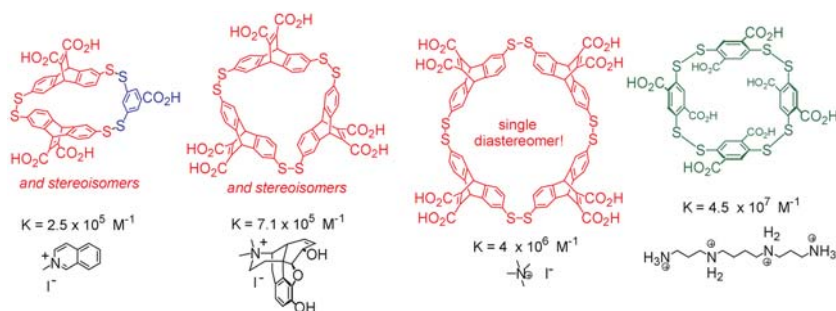
This raises the question whether we should be looking for new approaches to the development of synthetic receptors as alternatives to the traditional design approach. One such alternative is dynamic combinatorial chemistry [5–7]. This approach is based on the selection of the best receptors from large mixtures, reducing the need for a detailed design: instead of having to preconceive the structure of a complete synthetic receptor, only subunits need to be designed and synthesized. These are subsequently combined and linked together through a reversible chemical reaction to form a complex mixture of potential receptors that are all in equilibrium (a dynamic combinatorial library or DCL). Introducing a guest into such a mixture will result in the stabilization of the library members that bind the guest causing the equilibrium to shift in favour of, ideally (*vide infra*), the best binders. The compounds can be identified by comparing the library distributions before and after addition of the guest.

While several reversible covalent reactions can be used to generate DCLs, our work has focused on disulfide exchange [8]. In practise disulfide DCLs are made from thiols which are allowed to oxidize to form disulfides (Fig. 3a). During the irreversible oxidation process the mixture passes through an intermediate stage in which disulfides and thiols are present. Disulfide exchange occurs through nucleophilic attack of a disulfide by a thiolate anion, generating a new disulfide and liberating another thiolate anion (Fig. 3b). Exchange can be halted by addition of acid to protonate the thiolate or by allowing oxidation to go to completion [8].



**Figure 3.** Irreversible disulfide formation (a) and disulfide exchange (b) [8].

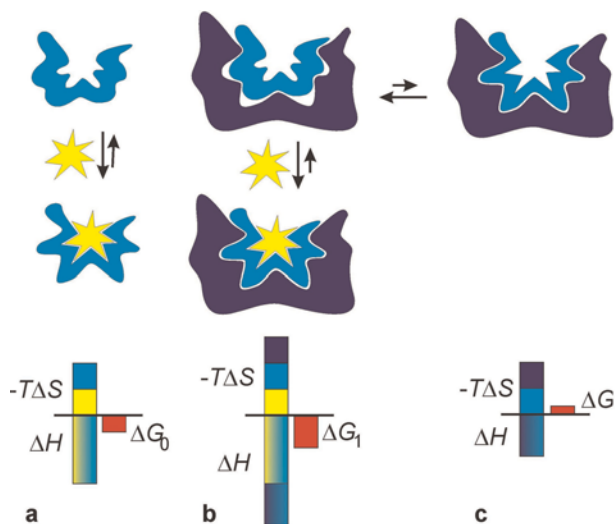
Some of the receptors that we have so far developed using this approach are shown in Fig. 4 [9–11] and include hosts for biologically relevant guest such as morphine alkaloids [9] and spermine [11]. The affinities exhibited by these receptors are shown by the red bars in Fig. 2 and are clearly at the higher end of the spectrum of synthetic receptors. Thus dynamic combinatorial chemistry appears successful in bringing us closer to the binding affinities of biomolecules. However, our dynamic combinatorial receptors are still roughly two orders of magnitude short of the biological affinities.



**Figure 4.** Synthetic receptors developed using disulfide-based dynamic combinatorial chemistry and their affinities for their guests (shown in black) in aqueous solution [9–11].

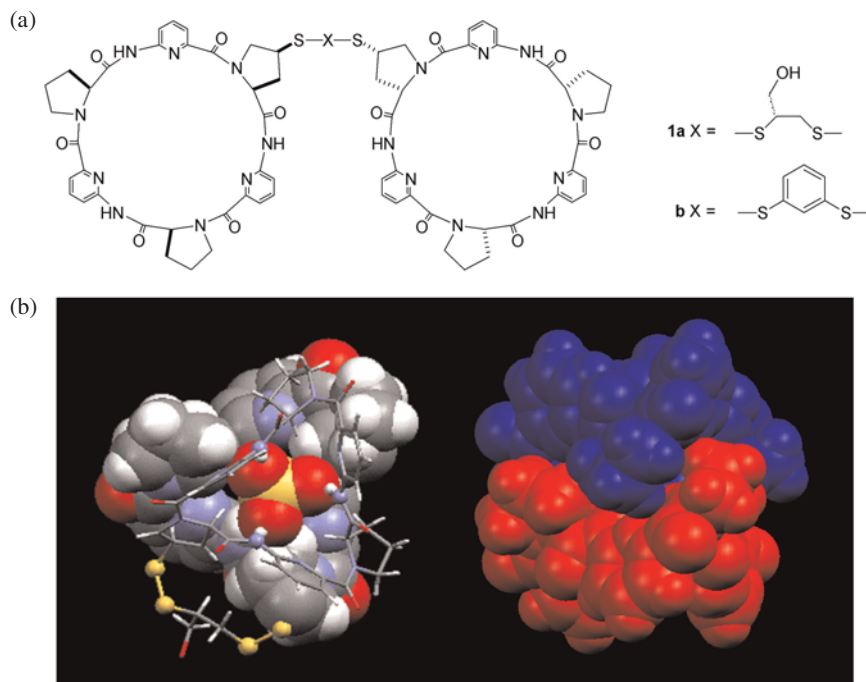
This raises the question whether there is anything special taking place in biological hosts that is not captured in the smaller synthetic counterparts? One important difference between biological receptors and synthetic ones is that the former are shaped through a complex set of noncovalent interactions within their structures, while the conformation of the latter is usually determined by the conformational preferences of covalent bonds. This raises the question of whether noncovalent interactions within biomolecules are important for their

function beyond merely providing some degree of structural integrity? A clue that this may well be the case comes from an analysis of one of the tightest noncovalent interactions known in biology: binding of biotin by streptavidin. Williams and coworkers have demonstrated that ligand binding in this system is felt throughout most parts of the protein resulting in a tightening of its structure [12]. For example, the melting temperature of streptavidin is increased by 37 °C upon binding of biotin [13, 14]. While tightening of protein structures upon binding of their ligands appears to be widespread, it is not immediately obvious whether this leads to an *increase* in ligand affinity or, conversely, whether structural tightening occurs *at the expense* of binding energy. We have recently reported a simple thermodynamic analysis that shows that the noncovalent interactions within the protein can indeed *reinforce* ligand binding [15]. Our analysis was based on comparing two binding scenarios: one in which binding is based on direct interactions between host and guest (Fig. 5a) and one which additional noncovalent interactions can be formed between different parts of the host (Fig. 5b). The interactions between host and guest can be split between the enthalpy associated with these and the entropy penalty related to bringing host and guest together and the entropy penalty of arranging the host in the optimum binding conformation. In case the conformational rearrangement of the host that is needed to form efficient interactions with the guest is (partly) the same as those needed to form efficient interactions within the host, then guest binding is more efficient when accompanied by the formation of intra-host interactions. Thus, structural complexity can indeed be beneficial for ligand binding.



**Figure 5.** Breakdown of the Gibbs energy of binding in the enthalpic and entropic contributions related to the guest (yellow), and the inner (light blue) and outer (dark blue) shells of the host. In (a) ligand binding is not accompanied by any change in intra-receptor interactions, while in (b) these interactions contribute to binding but they are not (fully) formed in the absence of the ligand (c). Reprinted with permission from ref. [15].

In our work on the dynamic combinatorial development of cyclic peptide based receptors for anions [3, 16] we have come across a system where intra-host interactions are occurring and we have been able to show that these interactions are indeed contributing to guest affinity. The X-ray crystal structure of the sulfate complex of receptor **1a** is shown in Fig. 6.



**Figure 6.** X-ray crystal structure of the complex of **1a** with  $(\text{NBU}_4)_2\text{SO}_4$ . (a) Representation in which one of the peptide rings is shown as space-filling and the other peptide ring and the disulfide spacer is shown as sticks; (b) space-filling representation showing the two peptide rings in different colours illustrating the close contacts between them. Counterions and solvent molecules are not shown for clarity [3].

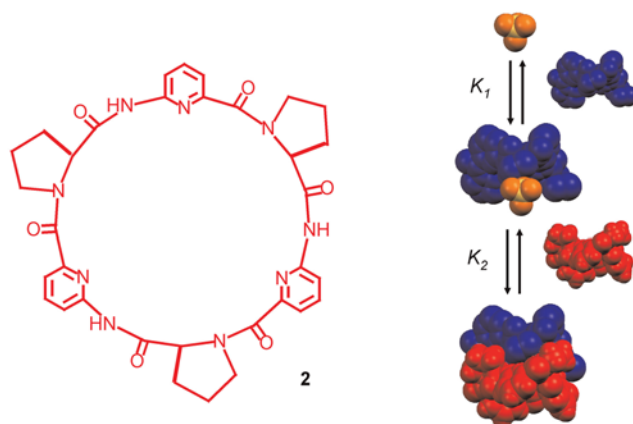
This system shows some remarkable similarities to proteins: it is a peptide that is folded into a particular structure and held together by disulfide bonds. It has a binding pocket that is shielded from the solvent and which contains hydrogen bonding sites for recognition of the guest (see Fig. 6a). Most importantly, in the conformation in which it binds the guest extensive noncovalent interactions within the receptor are possible. This is clearly illustrated in Fig. 6b, which shows the extensive interdigitation of the two peptide rings. As the parts of the peptide rings that come into close contact are mostly nonpolar, hydrophobic interactions play an important role in stabilizing the structure. This is evident from the solvent dependence of binding of sulfate and, in particular, iodide (Table 1) by analogous receptor **1b**.

**Table 1.** Binding constants and free energy of binding of iodide by receptor **1b** in different acetonitrile-water mixtures compared with the relative energy of desolvating iodide at 298 K. Data taken from ref [3].

Mol% water in acetonitrile	$K_{\text{iodide}}$ ( $M^{-1}$ )	$\Delta G^{\circ}_{\text{iodide}}$ (kJ/mol)	relative cost of desolvating iodide (kJ/mol)
49	$9.9 \times 10^4$	-28.5	0
80	$4.5 \times 10^4$	-26.5	+5.7

Whereas anion binding by synthetic receptors is normally extremely sensitive to the amount of water in the solvent mixtures, binding of iodide by receptor **1b** was remarkably solvent insensitive. Increasing the amount of water in acetonitrile from 49 to 80 mol% induced a decrease in the binding constant by a factor of only 2.2 (corresponding to 2.0 kJ/mol). The same change in solvent makes desolvating iodide (an intrinsic part of the binding process) 5.7 kJ/mol more difficult. We conclude that in our cyclic peptide this increased cost of desolvating the anion is partly offset by the increased stabilization of the iodide complex by hydrophobic intra-receptor interactions.

We have been able to quantitatively estimate the contribution of intra-receptor interactions in 1:1  $D_2O/CD_3OD$  from the analysis of the stepwise binding of sulfate by the monomer peptide **2** (Fig. 7) [17].



**Figure 7.** Stepwise binding of monomeric cyclopeptide **2** to sulfate to give a 2:1 sandwich complex [17].

The first binding step gives the 1:1 sulfate-peptide complex with a binding constant of  $360 M^{-1}$ . Statistically, the second binding event to give the 2:1 sandwich complex is expected to be 4 times weaker than the first. However, instead of the statistically expected binding constant of  $80 M^{-1}$  we found a binding constant of  $8760 M^{-1}$ . Thus the interactions between the two cyclic peptide rings which are only possible in the second binding event reinforce the stability of the complex by a factor 110 (12 kJ/mol). As these experiments

were performed in mixed aqueous solvent and given that the intra-receptor interactions are largely hydrophobic in nature, we expect that the reinforcement of binding in pure water should be even greater.

In conclusion, intra-receptor interactions can significantly boost the affinity of synthetic receptors. Thus there is an incentive to develop more complex receptors in which such interactions can take place. We have shown that dynamic combinatorial chemistry can be a useful tool in producing the required complex structures which are otherwise difficult to access. While we have to admit that the occurrence of intra-receptor interactions in our cyclic peptide system was serendipitous, the challenge is now to design a new generation of building blocks for dynamic combinatorial libraries that would allow us to further exploit intra-receptor interactions as a means of enhancing host-guest binding. This approach should allow us to close the affinity gap between supramolecular and biological hosts, which would create exciting opportunities to interfere with biological systems using synthetic receptors.

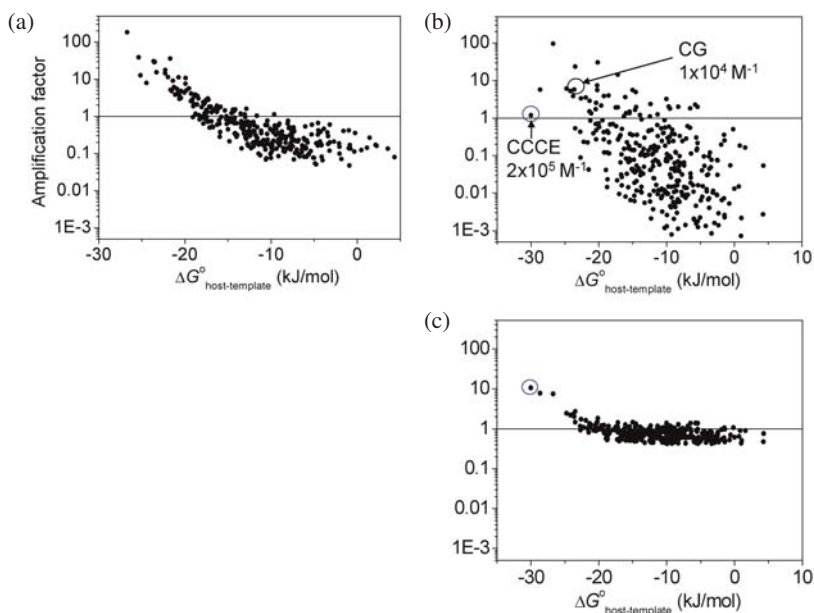
## COMPLEX MIXTURES

In the previous section we have used dynamic combinatorial chemistry as a tool for the development of new synthetic receptors. While the focus of these studies was on eventually obtaining a pure compound and studying its binding behaviour, the approach forced us to investigate the behaviour of complex equilibrium mixtures. It is becoming increasingly clear that the understanding of such mixtures is in fact an important goal on itself and highly relevant to the emerging field of systems chemistry (the study of the behaviour of complex mixtures of man-made compounds).

The ability of a dynamic combinatorial library to respond to the introduction of a template can be considered an emerging property of the system; it comes about through the interplay between the library members and cannot be ascribed to one single constituent of the system. However, the response of the library is clearly linked to the properties of the individual molecules in the mixture. In the context of our work on the development of synthetic receptors using dynamic combinatorial chemistry we have been particularly interested in how the amplification of library members relates to their binding affinity. Our expectation was that the amplification factors (the ratio of the concentration of a particular library member after addition of the template compared to that in the untemplated mixture) of the various hosts in the library would be correlated with the efficiency with which they bind the guest; i.e. the stronger the binder the better the amplification. However, pioneering theoretical work by Severin *et al.* on small model systems cast some doubt on the validity of this expectation [18, 19]. We therefore set out to investigate the behaviour of DCLs using an *in-silico* approach which would allow us to arrive at statistically significant results on complex mixture. We simulated DCLs based on an arbitrary number of 7 building blocks which were combined to form an equilibrium mixture of 28 dimers, 84 trimers and 210 tetramers in a statistical distribution using DCLSim software [2]. We then investigated how this mixture would respond to the introduction of a template. Of course, this response depends on how strong the various library members bind the

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template. To reflect the situation in a real DCL experiment where affinities of individual library members are unknown at the outset of the experiment, we chose to assign randomly a binding energy to every library member, based on a normal distribution. The result of a typical *in-silico* experiment is shown in Fig. 8a, in which the amplification factors of the library members are plotted as a function of their Gibbs energy of binding.

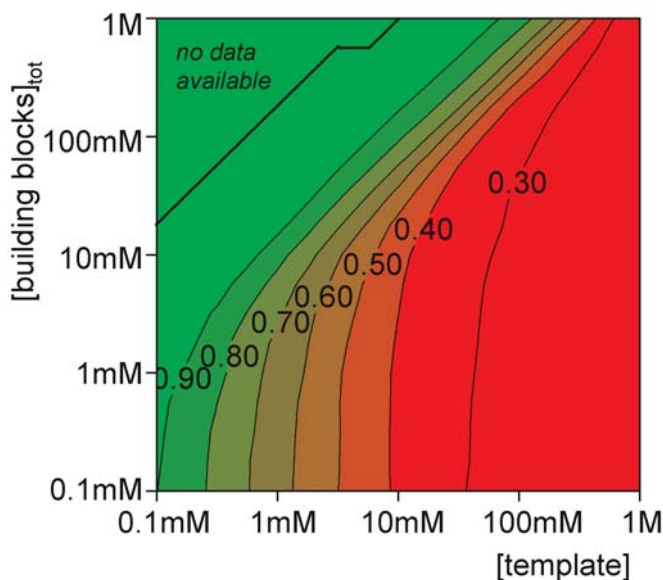


**Figure 8.** Correlation between amplification factor and the Gibbs energy of host-guest binding in simulated dynamic combinatorial libraries generated from 7 building blocks at a total concentration of 10 mM, using a template concentration of 10 mM (a and b) or 1 mM (c). The binding energies for the library members have been assigned randomly based on a normal distribution and are different for panels a and b, but the same for panels b and c [2].

While there is notable scatter of the data points the correlation between amplification factor and binding energy is satisfactory. However, using the very same 'experimental' conditions, but a different random assignment of binding affinities can give rise to dramatically different behaviour, as is apparent from Fig. 8b. In this example the correlation is weak and the best binder is not amplified at all. This behaviour is due to the fact that a single library member (CG) has ended up with a fairly high affinity for the template ( $K = 1 \times 10^4 \text{ M}^{-1}$ ). As CG is a dimer it has already a significant abundance in the library in the absence of the template (small oligomers are entropically favoured over higher oligomers). Under the specific 'experimental' conditions ( $[\text{template}] = [\text{building blocks}] = 10 \text{ mM}$ ) there is sufficient template around for all library members, in which case it is beneficial for the system to produce many copies of CG, at the expense of all other library members which contain building blocks C or G. Even the best binder in the system (CCCE;  $K = 2 \times 10^5 \text{ M}^{-1}$ ) is

unable to compete with CG, since for every molecule of CCCE the system can produce three molecules of CG and gain three times the binding energy of CG with the template versus only once the corresponding binding energy of CCCE.

When performing DCL experiments it is important to minimize the chance of encountering undesirable behaviour such as that exhibited by the library in Fig. 8b. The probability of encountering misbehaving libraries is dependent on the experimental conditions and we have investigated how the correlation between binding energy and amplification factor, quantified by the correlation coefficient  $R^2$ , depends on template and building block concentrations. The results are shown in Fig. 9 with good correlations in green and poor correlations in red. In general, using a modest template concentration of about one tenth of the total building block concentration appears to give satisfactory correlations. The reason for this is that forming large amounts of mediocre binders is no longer possible when the supply of template is limited, thus biasing the library towards the formation of the best binders. The effect the concentration of the template can have on the correlation between binding affinity and amplification factor is demonstrated by performing the simulation of the 'misbehaving' library of Fig. 8b at a 10-fold reduced template concentration, reassuringly restoring the correlation (see Fig. 8c).



**Figure 9.**  $R^2$  values for the correlation between the Gibbs energies of binding and the amplification factor as a function of the template and building block concentrations [2].

In the *in-silico* studies that are described above we used binding constants of individual host-guest pairs as the input for the simulations and were able to obtain library distributions as output. We are currently working on the reverse approach which should allow us to derive binding constants directly from the product distribution in complex equilibrium mixtures and the way these respond to varying the concentrations of the building blocks and template; that is, deriving information on individual molecules from the emerging properties of a complex molecular network, bypassing the need for time-consuming isolation procedures and binding studies. This would clearly be an important advance in the development of dynamic combinatorial libraries as a method for the development of new binders.

## CONCLUSIONS AND OUTLOOK

Man-made supramolecular systems have the potential to provide a unique perspective on the biological processes as they can, in theory, serve as controllable and manageable models of the latter. However, there is still a considerable gap between supramolecular chemistry and biology that prevents widespread application of supramolecular chemistry in biological contexts. This gap exists on the level of the complexity of individual molecules as well as on the complexity of systems comprised of mixtures of molecules. In our recent work we have started to bridge both of these gaps.

Firstly, we have produced elaborate synthetic receptors which have led to new insights into the interplay between structural complexity and binding affinity. Specifically, we demonstrated that intramolecular interactions within a synthetic receptor that do not directly involve the guest can contribute to guest binding. This effect has so far not been utilized in the design of synthetic receptors but has definite potential as a tool for pushing binding affinities of these compounds into the desirable nanomolar affinity range.

Secondly, our work on trying to understand the behaviour of dynamic combinatorial libraries has led us to investigate the multidimensional relationship between emerging properties (shifts in product distribution upon introducing a template molecule), the properties of individual library members and the experimental conditions. This has not only improved the effectiveness of the use of dynamic combinatorial chemistry but also led to a deeper understanding of the thermodynamics of complex equilibria. Extension of this work studying the interplay between irreversible reactions and complex equilibrium systems is currently underway. The emergence of connected chemical reactions that resemble metabolic pathways is conceivable. Such systems may well prove relevant to the way early life developed.

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