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Peptide Complexes

Assessment of Cooperativity in Ternary Peptide-Cucurbit[8]uril Complexes

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Abstract: Evaluating cooperativity for cucurbit[8]uril (CB[8])-mediated ternary complexation is required for understanding and advancing designs of such ternary self-assembled systems. A key issue is to dissect the contributions of the binding steps of the first and second guest molecules to the overall ternary complex formation energy. This is addressed by performing concentration-dependent titrations between CB[8] and guests by means of concentration-dependent calorimetric and ^{1}H-NMR titrations. The sensitivity of the fitting of the cumulative heat of complexation of the calorimetric titrations is evaluated in terms of fitting error and enthalpy–entropy compensation and, together with the NMR spectroscopic analysis of the separate species, non-cooperative binding is conceived to be the most probable binding scenario. The binding behavior of CB[8] homoternary complexes is similar to CB[8] heteroternary complexes, with an enthalpy-driven tight fit of the guests in the CB[8] cavity overcoming the entropic penalty. Also for these types of complexes, a non-cooperative binding is the most probable.

Specific molecular recognition properties between ligands (guests) and receptors (hosts) allow non-covalent synthesis of artificial receptor–ligand complexes to occur.[1–4] Cucurbit[*n*]urils (CB[*n*]) form a new class of macrocyclic hosts that show remarkable molecular recognition properties in water.[5] The highest affinities between CB[*n*]s and their guests occur when high energy solvation water molecules are released from the cavity, which generates an enthalpic gain upon complexation.[3] CB[8] is the first homologue large enough to promote binding of two equivalents of guest forming a ternary complex.[6,7] For example, a heteroternary complex forms through the well-defined sequential binding of two different guests inside the CB[8] cavity and this can drive the self-assembly of copolymers,[8] hydrogels,[9] particles,[10–11] and monolayers.[12] Also homoternary complexes can be used for such purposes, in particular as demonstrated for the binding of *N*-terminal aromatic amino acidic residues such as tryptophan (Trp) or phenylalanine (Phe) to CB[8].[13] This type of CB[8]-peptide complex extends the application of CB[8] assemblies into the biological arena.[14–18]

A ternary complex offers the opportunity for tuning the assembly properties by cooperativity. Cooperativity describes the relationship between the affinities of binding of the first and second equivalent of guest by the host.[19] In comparison to the affinity of the first guest molecule, the binding of the second guest can either be favored, unfavored, or unaffected (i.e., positive, negative, or non-cooperative, respectively). The principle of cooperative interactions is common in living systems and modulates the function of a receptor by the concentration of the ligands. For example, the binding of oxygen to the four pockets of hemoglobin is a positive cooperative process resulting in an increase of the binding affinity of hemoglobin for the substrate oxygen upon each molecule of oxygen bound.[20] Proper design of the stability and dynamics of self-assembled systems based on ternary interactions requires a thorough understanding of the, possibly cooperative, binding behavior of the ternary complex interaction motif. In a systematic study of the sequence-specific recognition of peptides by CB[8], the homoternary complex between $PheGly_2$ and CB[8] was proposed as a synthetic, positively cooperative receptor–ligand interaction.[13] An overall ternary binding constant K_{ter} of $1.5\times10^{11}\,M^{-2}$ was reported for the complex CB[8]·$(PheGly_2)_2$.[13] The positively cooperative nature of this complex was suggested on the basis of ^{1}H-NMR experiments, but the extent of cooperativity was not quantified.[13] Here, we assess the degree of cooperativity for ternary complexes of CB[8] and two peptides both with an *N*-terminal phenylalanine, followed by either two ($PheGly_2$) or six glycine ($PheGly_6$) residues. Isothermal titration calorimetry (ITC) and ^{1}H-NMR titrations were used to study the dependence of the affinity of CB[8] on the concentration of the guest. A key issue is to dissect the contributions of the bindings of the first and second guest molecules to the overall ternary complex formation. This is addressed by performing concentration-dependent titrations, an evaluation of the error sensitivity in the ITC experiments, and by a spectroscopic analysis of the separate species by ^{1}H NMR spectroscopy.

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Supporting information, including the synthesis, concentration assessment, experimental methods for ITC and ^{1}H NMR, description of fitting model, and summary of fitting results, for this article can be found under http://dx.doi.org/10.1002/chem.201605284.

Figure 1 shows the first and the second binding events between the host CB[8] (H) and the peptide guest (G), leading to the formation of the 1:1 complex HG and the homoternary 1:2

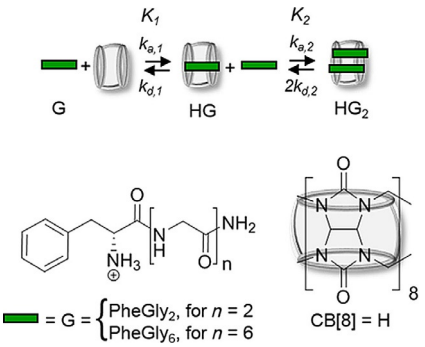

Figure 1. Equilibria of complexation of CB[8] (host, H) and peptide $PheGly_n$ (guest, G).

complex HG_2, respectively. The first equilibrium binding constant K_1 arises from the interaction of a single guest G with the host H. For the second binding step, the dissociation of a guest is associated with a pre-factor 2 ($2*k_{d,2}$) to account for the presence of two identical guest molecules in the cavity. Overall, the degree of cooperativity, defined by the ratio K_1/K_2, governs which of the three scenarios, positive, negative, and non-cooperativity, applies, depending on whether K_2 is larger than, smaller than, or equal to $^1/_2K_1$, respectively.

An important aspect for the assessment of the degree of cooperativity is to work in an as wide as possible range of concentrations of H and G to make use of the different concentration dependencies of the binding constants for the formation of HG and HG_2. For a given overall binding constant K_{ter}, different degrees of cooperativity are expected to give different species distributions. This means that the distributions of the concentrations of H, HG, and HG_2, while keeping the initial concentrations of host and guest constant, correspond to unique scenarios of K_1/K_2. To be able to accurately determine the ratio K_1/K_2, different distributions of H, HG, and HG_2 can be measured starting from different initial concentrations of host and guest. A proper working range of concentrations was determined to be between 1 and 50 μM (see the Supporting Information for details). ITC studies were performed to determine the ratio between K_1 and K_2 for the ternary complexes of CB[8] with the peptides $PheGly_2$ and $PheGly_6$. The simultaneous fitting of the ITC data sets measured at three different host concentrations provided a restricted range of physically acceptable K_1/K_2 ratios. Specifically, consistent with the optimal range of concentrations, CB[8] was loaded in the cell at concentrations between 10 and 50 μM and titrated with a solution of the peptide guest. The enthalpograms obtained for each host–guest complex are given in Figure 2a, e. A mathematical model was used to fit the experimental heats with a least-squares minimization routine (see the Supporting Information for details). Briefly, the heat of complex formation was expressed as a function of the species concentrations, and the thermodynamic parameters K_1, K_2, ΔH^0_1, and ΔH^0_2 were used as fit parameters. Heats of dilution for each set of initial concentration were also included in the model, and calculated values were confirmed by reference experiments. The best fits provided the optimal four parameters ΔH^0_1, ΔH^0_2, K_1 and K_2, and thus the optimal K_1/K_2 ratio for each peptide guest. K_1/K_2 values of around 2 were found for both peptides ($K_1/K_2 = 2.1 \pm 0.8$ for $PheGly_2$ and 1.8 ± 0.4 for $PheGly_6$, Table 1), which agrees with a non-cooperative binding scenario.

To evaluate how sensitive the fit error is to variations of the K_1/K_2 ratio, the least-squares error was calculated for different degrees of cooperativity. Thus, the parameters ΔH^0_1, ΔH^0_2, and K_2 (correlated to K_1) were optimized for chosen values of K_1/K_2. Figure 2 shows the dependence of the fit error (Figure 2b, f) on the ratio K_1/K_2, and the correlated enthalpies (Figure 2c, g) and entropies (Figure 2d, h). Figure S4 in the Supporting Information shows the changes in fit of the ITC titrations at very high and very low K_1/K_2. The trends in fit show, in short, that: (a) a much higher K_1/K_2 should be visible by a plateau of Q at low $[G_{tot}]/[H_{tot}]$ combined with a clear inflection at $[G_{tot}]/[H_{tot}] =$

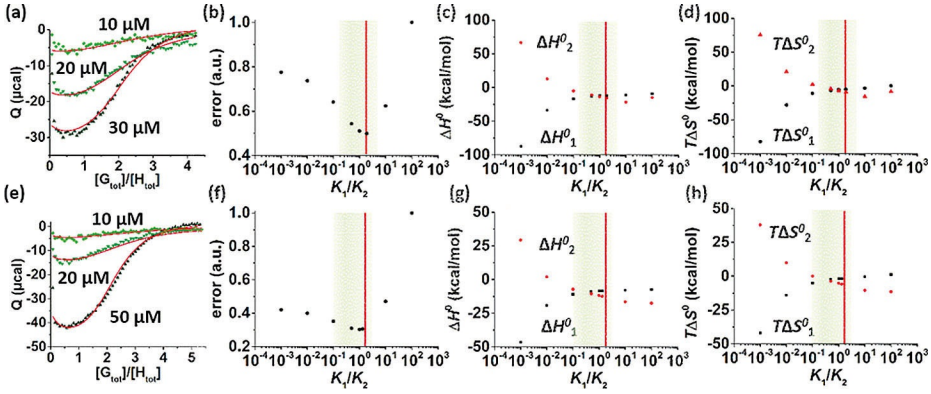

Figure 2. ITC data (markers) of binding CB[8] (H, three initial concentrations) with $PheGly_2$ (G) (a) and $PheGly_6$ (G) (e) in PBS (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4). ITC data (see also Figures S1–S6 in the Supporting Information) were simultaneously fitted (solid lines) to a model with K_1, K_2, ΔH^0_1, and ΔH^0_2 as fit parameters. Representative plots of the normalized least-squares fit error, ΔH^0 and $-T\Delta S^0$ calculated at fixed values of the K_1/K_2 ratio for $PheGly_2$ (b–d) and $PheGly_6$ (f–h). Red vertical lines indicate the non-cooperative case ($K_1/K_2 = 2$), green areas represent the acceptable ranges of K_1/K_2 within 20% of the minimum fit error and of enthalpy–entropy compensation.

Table 1. Thermodynamic binding constants for complexes of CB[8][a] and $PheGly_n$.

	ITC $PheGly_2$[b]	ITC $PheGly_2$[c]	ITC $PheGly_6$[b]	^{1}H NMR $PheGly_2$[d]	^{1}H NMR $PheGly_6$[d]
K_1/K_2	2.1 (0.8)	–	1.8 (0.4)	0.5	1.2
K_1 [M^{-1}]	2.2 (1.1)×10^5	–	8.7 (0.6)×10^4	3.8×10^5	9.2×10^4
K_2 [M^{-1}]	1.0 (0.2)×10^5	–	5.1 (1.3)×10^4	7.8×10^5	7.7×10^4
K_{ter} [M^{-2}][e]	2.3 (1.4)×10^{10}	1.5 (0.2)×10^{11}	4.4 (1.1)×10^9	3.0×10^{11}	7.1×10^9
ΔH^0_1 [kcal mol^{-1}]	−11.6 (0.3)	−29.6 (0.2)	−8.3 (0.2)	–	–
ΔH^0_2 [kcal mol^{-1}]	−13.7 (1.7)		−14.7 (2.5)	–	–
ΔG^0_1 [kcal mol^{-1}]	−7.2 (0.3)	−15.4 (0.1)	−6.7 (0.1)	−7.6	−6.8
ΔG^0_2 [kcal mol^{-1}]	−6.8 (0.1)		−6.4 (0.2)	−8.0	−6.7
$T\Delta S^0_1$ [kcal mol^{-1}][f]	−4.3 (0.5)	−14.2 (0.3)	−1.5 (0.2)	–	–
$T\Delta S^0_2$ [kcal mol^{-1}][f]	−6.9 (2.2)		−8.3 (3.3)	–	–

Standard deviations are given in parentheses. [a] Concentration of CB[8] was spectrophotometrically determined.[24] [b] See Figure 2 and text for details. Data obtained at 25 °C in PBS (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4). [c] Data as reported[13] for the overall ternary complex HG_2. Data based on three ITC experiments titrating 2 mM of $PheGly_2$ into 0.1 mM CB[8] in 10 mM sodium phosphate, pH 7.0 at 27 °C. [d] See Figure 3 and text for details. Data obtained at 25 °C in D_2O [e] Product of K_1 and K_2 gives K_{ter}. [f] Difference ΔG and ΔH gives $T\Delta S^0$.

1, and (b) a much lower K_1/K_2 should lead to a rather shallow slope at around $[G_{tot}]/[H_{tot}] = 1$ (in Figure S4a, most visible in the two higher concentrations, and in Figure S4b, at the two lower concentrations), which clearly conflict with the observed data. An evaluation of all thermodynamic parameters presented in Figure 2 allowed for the determination of a range of possible degrees of cooperativity (indicated in green in Figure 2). Values of the fit error within 20% from the minimum error were defined as acceptable. This 20% cut-off value was selected based on the variability of the minimum error observed in triplicate calorimetric experiments. Therefore, the upper boundary of the range of acceptable degrees of cooperativity was set at values of K_1/K_2 equal to 6 for $PheGly_2$ and to 3.5 for $PheGly_6$. For higher values of K_1/K_2 (strongly negative cooperativity), the fit errors became quickly unacceptably high (Figure 2b, f). Regarding the thermodynamic parameters, such high K_1/K_2 ratios gave more exothermic enthalpies and less favorable entropies for the second step (Figure 2).

The lower limit of the range was determined considering that, even though the fit errors did not rise as quickly as at the upper limit, the binding enthalpies and entropies for the first and second binding events diverged more and more for values of K_1/K_2 lower than 0.5. Specifically, an inversion of the signs and order of ΔH^0_1 and ΔH^0_2, as well as of $T\Delta S^0_1$ and $T\Delta S^0_2$, was observed for values of K_1/K_2 below 0.2 for $PheGly_2$ and below 0.1 for $PheGly_6$ (Figure 2). Under these conditions, the second binding event became less enthalpically favored (and more entropically favored) than the first step. Both steps would thus be associated with large enthalpy–entropy compensation effects and opposite driving forces, that is, strongly enthalpy-driven for the first step and strongly entropy-driven for the second. In particular, the unfavorable positive enthalpy contribution (Figure 2c, g) and the highly favorably entropy (Figure 2d, h) for the second step are not realistic considering that CB[8] complexation is known to be enthalpically driven and entropically unfavorable.[21–23] Overall, the considerations made in terms of fit error and of enthalpy–entropy compensation determined a range of acceptable K_1/K_2 ratios between 0.2 and 6 for $PheGly_2$ and between 0.1 and 3.5 for $PheGly_6$, which are highlighted in green in Figure 2. For both peptides, these ranges indicate either a non-cooperative or a weakly, negative or positive cooperative system.

For both $PheGly_2$ and $PheGly_6$, the second binding event has a larger enthalpic gain than the first, as well as a larger entropy loss (Table 1). This indicates a tighter fit for the second guest in the CB[8] cavity, which is logical as it involves interaction with an already partially filled cavity. It is also in agreement with studies performed by Biedermann and co-workers[22] that show, in the case of heteroternary complexes, a more favorable enthalpy for the second aromatic guest correlates with a less favorable entropy contribution. Similar to what was shown for the heteroternary complexes, this can be expected also in the case of the homoternary complexes studied here; the first guest reduces the cavity volume of CB[8] in such a way that the potential energy of the residual cavity water molecules is increased, thus leading to a stronger enthalpic response upon release of these water molecules upon the binding of the second guest. In constrast, the tightly packed ternary complex reduces the degrees of freedom of both guests and therefore brings an additional unfavorable entropy contribution.[22]

Another observation from our calorimetric results is that when comparing the thermodynamic data for the two peptides, a stronger binding affinity was found for $PheGly_2$ with respect to $PheGly_6$, arising from differences for both the first and second guest binding steps. In particular, the first $PheGly_6$ seems to have a weaker interaction with the host (less favorable ΔH^0_1).

Moreover, our results reveal a slightly weaker overall binding than the one reported in the literature[13] for the overall ternary complexation of the peptide $PheGly_2$ with CB[8] (see K_{ter} in Table 1), which can be explained by a higher concentration of cations competing with the guest for the binding to the host in our buffer.[25] The crystal structure of the complex[13] shows that the shorter $PheGly_2$ can assume a circular conformation to maximize its dipole–dipole interactions of the amidic protons with the carbonyl on the CB[8] rims. This cannot be achieved for a longer chain in the case of $PheGly_6$, which may explain

the observed difference in affinity. Unfortunately, the X-ray structure of the complex CB[8]·$(PheGly_6)_2$ is not available to confirm this hypothesis. Our observations are in agreement with calorimetric experiments on heteroternary complexes of CB[8], paraquat and $TrpGly_2$ or $TrpGly_5$ that have shown a tighter binding for the short peptide compared to the long one.[22] Taken together, the calorimetric data indicate that the most realistic scenario is the non-cooperative binding of the peptides.

However, further narrowing the range of possible K_1/K_2 values could not be achieved by ITC alone, due to both the restricted operative concentration range (see above) and the convolution of the heat effects arising from the first and the second binding events. To overcome the latter limitation, ^{1}H-NMR was used to provide direct spectroscopic insight into the (relative) concentrations of all participating species separately. This technique has a relatively low sensitivity, so fairly high concentrations are preferred; however, to prevent precipitation of CB[8], experiments were performed at 50 μM, which contrasts an earlier study that used CB[8] at a concentration that exceeded the solubility limit.[13] A titration experiment was performed at a constant total CB[8] concentration (in D_2O) of 50 μM, while titrating from 0.5–4 equivalents of the peptides (Figure 3a, d and see full spectra in Figure S3 of the Supporting Information). The three species G, HG, and HG_2 were distinguished based on the signals of the aryl protons of the guests.[13] Upon the first complexation, the upfield shifts of the phenyl protons of the Phe residue verified the shielding of the surrounding CB[8] host molecule. With the second complexation, the interaction among the two guests in the cavity of the CB[8] caused an additional upfield shift.[26] Under non-saturation conditions for CB[8], the HG complex is well visible at low concentrations for both peptides, thus excluding a strongly positive cooperative system, in contrast to what has been described in an earlier study.[13] By monitoring the signals of the aromatic protons (Figure 3a, d), the distributions of all species G, HG, and HG_2 were determined for each titration step (Figure 3b, e). These distributions were fitted to a model expressing the calculated distributions of species as a function of the fitting parameters K_2 and K_1 (see the Supporting Information for details). The calculated data are shown as lines in Figure 3b, e for the peptides $PheGly_2$ and $PheGly_6$, respectively. Table 1 summarizes the values found for the optimized parameters K_1, K_2, the corresponding free energies ΔG^0_1, ΔG^0_2 (see also Figure S7), and the overall binding constant K_{ter}. Higher overall binding affinities (K_{ter} in Table 1) were found as expected because the cations in the PBS solutions used for ITC can compete with the guest for the interaction with the host, thus destabilizing the complex,[25] whereas these salt effects are absent in the solvent (D_2O) used for the ^{1}H-NMR experiments. In agreement with ITC, CB[8] binds more strongly with the shorter peptide $PheGly_2$ (3.0×10^{11} M^{-2}) than the longer $PheGly_6$ (7.1×10^{9} M^{-2}, Table 1). The optimal fits gave $K_1/K_2 = 0.5$ and 1.2, for $PheGly_2$ and $PheGly_6$, respectively, indicative of non-cooperative or slightly positive cooperative binding.

To assess the sensitivity of the degree of cooperativity, the graphs in Figure 3c, f were obtained by optimizing K_2 (and the correlated K_1) at chosen values of the ratio K_1/K_2. The values of the least-squares error for each K_1/K_2 ratio are reported for each peptide (Figure 3c, f). A cut-off value of 20% from the minimum fit error was arbitrarily chosen to find the acceptable range of degree of cooperativity. The values of K_1/K_2 are in a range between 0.2 and 1 for the shorter peptide $PheGly_2$, and between 0.6 and 10 for the longer $PheGly_6$. Notably, the minima by ^{1}H NMR are within the range of K_1/K_2 obtained by calorimetry, indicating a non-cooperative system. Taken togeth-

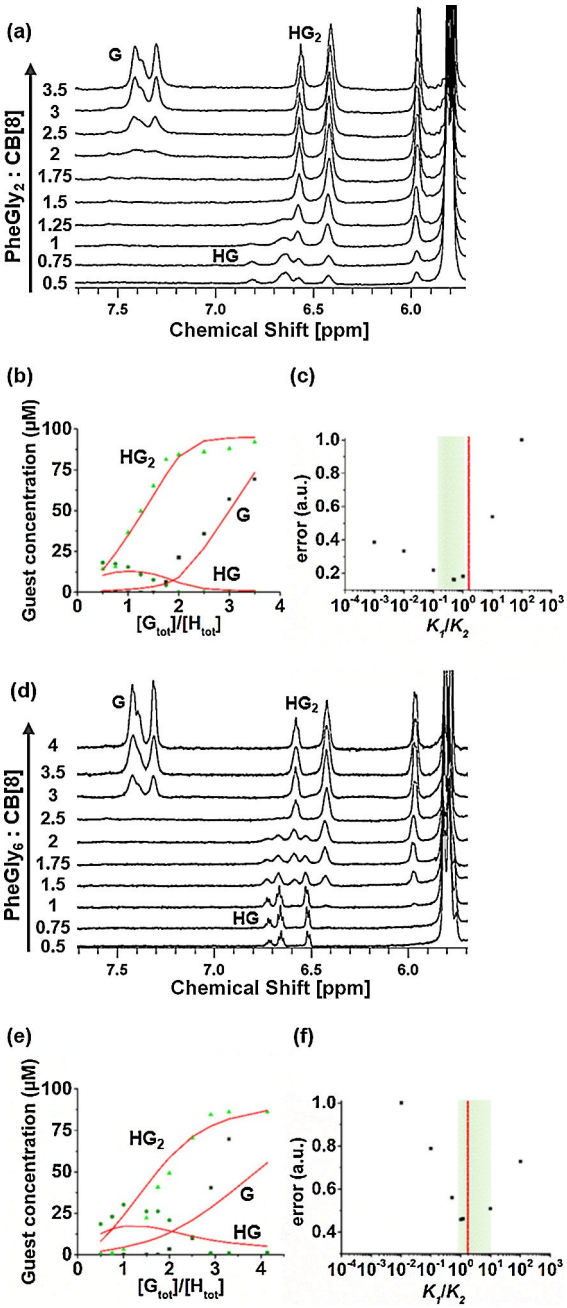

Figure 3. ^{1}H-NMR titrations of CB[8] (50 μM) with $PheGly_2$ (a) and $PheGly_6$ (d) in D_2O at 25 °C. Experimental [G] in G, HG, and HG_2 (data points) are simultaneously fitted (see also Figure S7) to a model varying K_1 and K_2 (solid lines) for (b) $PheGly_2$ and (e) $PheGly_6$. Plots of the normalized fit error calculated at fixed values of the ratio K_1/K_2 for (c) $PheGly_2$ and (f) $PheGly_6$. Red vertical lines in c and f indicate the non-cooperative value of $K_1/K_2 = 2$. Green areas indicate the acceptable ranges of K_1/K_2 within 20% of the minimum error.

er, these results confirm a most probable scenario in which the ternary complexation between the peptides and CB[8] is non-cooperative.

It should be noted that these ternary CB[8]-peptide complexes cannot be compared directly to, for example, the cooperativity observed in hemoglobin, because in the former case, the first guest does not occupy one of two identical, well-spaced binding sites, but resides somewhere in the same cavity to which also the second one binds in the next step. As a result, the second guest experiences interactions with the first guest directly, as witnessed by the correlation between enthalpy and entropy.

In conclusion, combining the pieces of evidence from calorimetric and ^{1}H-NMR titrations shown in this work, the most probable scenario to describe the homoternary complexation of phenylalanine-based peptides by CB[8] is a non-cooperative mode of interaction. This is independent of the tail length of the peptides studied in this work. Remarkably, whereas the second guest experiences a stronger interaction with the host after the first complexation step, there appears to be a counterbalancing entropic contribution that leads to an overall non-cooperative behavior in affinity. This contrasts the normal non-cooperative behavior of well-separated binding sites, in which case the binding enthalpies of all steps are equal, and entropy differences arise solely from differences in statistical pre-factors. The binding behavior of the homoternary peptide complexes resembles that observed for heteroternary complexes. The PheGly binding motif offers the synthetic flexibility and biocompatibility of peptides, and can have an active role in natural functional structures as well, such as in nuclear membrane pores.[27] The insights in the complexation between peptides and CB[8] allow for a rational design of more complex self-assembled systems built on this powerful interaction motif.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: complexation · cooperativity · cucurbit[*n*]uril · self-assembly · titration

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