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PAPER

Probing the stability of multicomponent self-assembled architectures based on cucurbit[8]uril in the gas phase†

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Aqueous supramolecular chemistry and highly controlled self-assembly of multi-component architectures are novel tools for investigating and answering questions with different biological implications. Among other self-assembly motifs the barrel-shaped host molecule cucurbit[8]uril (CB[8]) is of particular interest due to its capability of incorporating two guest molecules simultaneously in its hydrophobic cavity. This allows for its use as a supramolecular linking unit to conjugate two different entities such as polymers, peptides, and proteins as well as conjugation of various species to surfaces, colloids and nanoparticles. This study aims to improve our understanding of CB[8] ternary complex formation and stability. A series of CB[8] architectures of different size and chemistry have been analyzed in the gas phase to obtain information about their stability in the absence of solvent effects. While hydrophobic effects and solvation energies play a crucial role for host–guest affinities in solution, gas phase stabilities are determined by the guest's ability to form hydrogen bonding and electrostatic interactions. Increasing the size of the second guest resulted in an increase of gas phase stability, likely due to additional non-covalent interactions.

1. Introduction

Supramolecular chemistry provides unique opportunities and powerful strategies for investigating the relationship between structure and function from nano- to macroscopic scales. Moreover, it enables the design of entirely new architectures with desired macroscopic properties through control at the molecular level. Clever design and synthesis of self-assembled systems have led to convincing new concepts in drug delivery,[1] smart materials[2] and biomaterials[3] as well as a wide range of molecular switches[4,5] and machines.[6,7] Nature itself uses a host of supramolecular concepts to generate and support life, all of which function in an aqueous environment. Water as a solvent has attracted much attention in the scientific community[8,9] both for mimicking nature's concepts as well as for the development of entirely new systems for application in a biological context.

A number of recent approaches addressing questions with biological implications[10,11] are based on strong and specific non-covalent interactions between a family of barrel-shaped host molecules, cucurbit[*n*]urils (CB[*n*]s with n = 5–8,10), and suitably sized guest molecules in an aqueous environment.[12–14] CB[8] is unique amongst this class of macrocycles as it is capable of including two aromatic guests simultaneously into its hydrophobic cavity in a hetero-ternary complex,[15] a feature that has been exploited for the synthesis of supramolecular switches,[16,17] block copolymers,[18] bio-conjugates,[19–22] hydrogels[23] and functionalized surfaces.[24–28] Sequential binding of an electron-deficient species, such as methyl viologen (MV) or imidazolium derivatives,[29,30] and a wide variety of electron-rich aromatic compounds,[31] is typically monitored by an increase in visible absorbance on account of charge-transfer interactions between MV and the second guests.[32] This as well as other solution based methods such as ITC, fluorescence and NMR are limited to describing the average of an ensemble of complexes that are in rapid exchange with one another. The characterization of a single species within these mixtures remains challenging.

Gas phase experiments complement solution phase methods by producing a "kinetic snapshot" of the corresponding solution and enable exclusive analysis of a selected species of the mixture. Over the last few years electrospray mass spectrometry has become a powerful tool in the field of supramolecular chemistry[33] and has successfully been used in the identification of host–guest complexes with variable stability, including cyclodextrins,[34] calixarenes,[35] CBs[36–38] and other systems. However,

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†Electronic supplementary information (ESI) available: Synthesis of trimers **1** and **2**, synthesis of WG5 peptide, mass spectra of **1**, various ternary complexes (MV·CB[8] with Ant–NH_2, WG5 and Np–PEG), various 7-component complexes (**1**·CB[8]$_3$·DHN_3, **1**·CB[8]$_3$·Trp_3, **2**·CB[8]$_3$·DHN_3, **2**·CB[8]$_3$·Trp_3 and MS^n spectra of **1**·CB[8]$_3$·$WG5_3$). See DOI: 10.1039/c2ob06954g

only a few reports describe analysis and characterization of discrete multi-component assemblies,[39] with only one publication using CB[8] as the assembly motif.[40] In order to be able to use CB[8] to its full potential it is vital to gain fundamental insight into its molecular recognition properties. Detailed fragmentation studies (MS^n) presented herein aim to improve our understanding of the CB[8] ternary inclusion complex by analyzing architectures of varied size and chemistry in the absence of solvent effects. Extending the CB[8] ternary complex motif towards larger, well-defined architectures, such as the trimeric systems outlined below, allows for the stoichiometrically controlled assembly of peptides, proteins or other entities in a purposefully designed fashion and has potential in the area of bioconjugation.

2. Results and discussion

A variety of species have been used within this study to synthesize an array of supramolecular assemblies with CB[8] for subsequent gas phase analysis and fragmentation experiments (Fig. 1). Based on the well-characterized MV moiety, we designed and synthesized two trimeric variants of this highly affinitive first guest with different chemical properties and flexibility, trimers **1** and **2**. These MV-trimers self-assemble with three equivalents of CB[8] and three equivalents of a suitable second guest to form 7-component supramolecular architectures based on three inclusion complexes per entity. Besides small aromatic compounds, larger entities such as peptides,[41] proteins[42] and polymers[18] have been reported to from ternary complexes with MV·CB[8]. This study includes a variety of second guests, ranging from small aromatic compounds (dihydroxynaphthalene (DHN), anthracene amine (Ant–NH_2), fluorene amine (Flu–NH_2), L-tryptophan (Trp)) to larger entities. A WG5 model peptide and a series of five PEGylated second guests (1-hydroxynaphthalene (HN–PEG), anthracene (Ant–PEG), dibenzofuran (DBF–PEG), azobenzene (Azo–PEG) and fluorene (Flu–PEG) with PEG–OMe 1 kDa) were employed to establish and characterize this multi-component supramolecular platform. Both size and chemical composition of the second guest were found to influence the gas phase stability of the CB[8] ternary complex to a large extent.

The relationship between supramolecular binding forces in solution and in the gas phase is a topic of ongoing debate. It is well-understood that CB[*n*]'s affinity and selectivity towards suitable guest molecules in water is based on both hydrophobic interactions at the inner surface of the barrel and electrostatic and hydrogen-bonding interactions with the carbonyl groups at the rim.[12] Upon transfer from an aqueous environment into the gas phase these interactions change in their binding strength in opposite ways:[43] hydrophobic interactions become substantially weakened in the gas phase as their main contributing factor, the surrounding non-solvent, disappears. Both hydrogen bonding and electrostatic interactions in contrast increase in binding energy due to absence of competition with the solvent. In a recent study,[31] performed on a triple-quadrupole equipped with a nanoelectrospray source, we presented a way to correlate solution binding energies of a wide variety of CB[8] ternary inclusion complexes to the observed ion intensities in the gas phase. The instrumental setup and the mildness of ionization greatly influence how well (if at all) non-covalent complexes that are based on hydrophobic interactions in solution may be observed in the gas phase. The present study was performed on a LTQ Velos Orbitrap (Thermo Scientific Fisher) with infusion pump and HESI source (heated ESI). In contrast to the previous study a ternary complex based on hydrophobic interactions such as

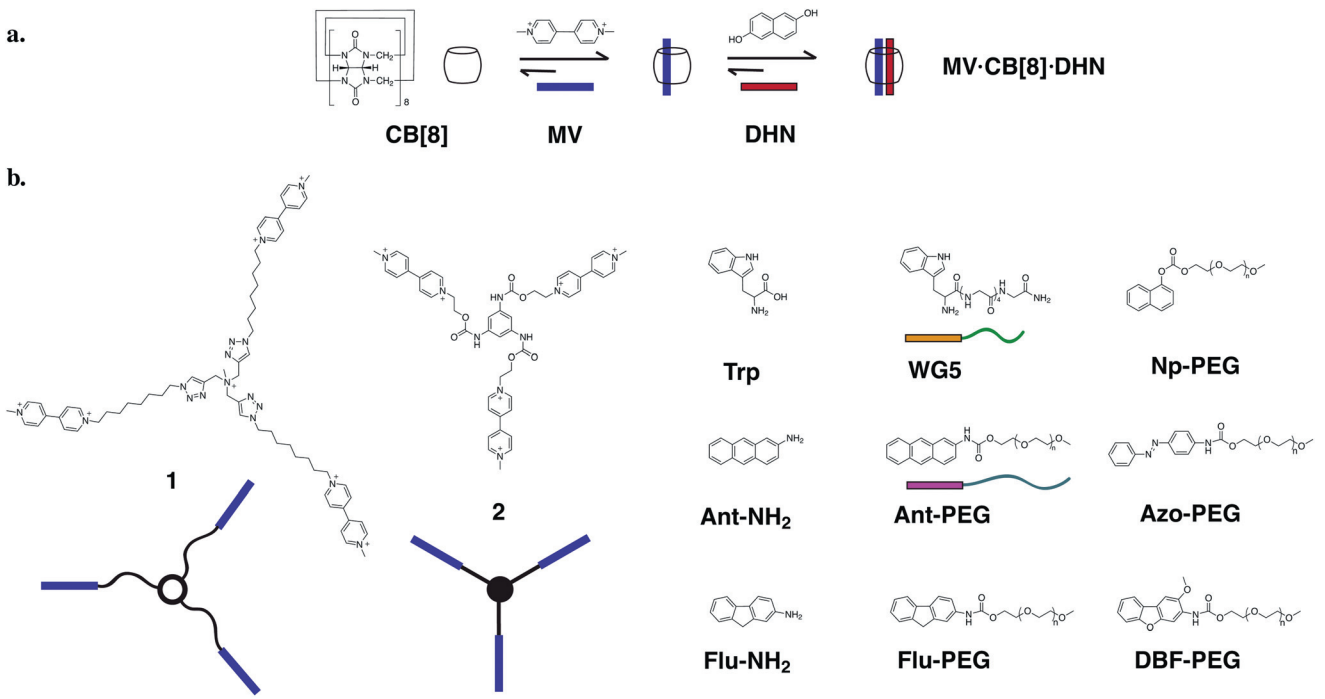

Fig. 1 a: Sequential binding of two guest molecules inside of CB[8]; b: structure of trimers **1** and **2**, and a variety of second guests.

MV·CB[8]·anthracene could not be observed regardless of the settings used. However, measurement of a sample containing Ant–NH_2 instead gave a very high signal for the ternary complex indicating the importance of interactions between the guest and the portal region of CB[8] in the gas phase. The strong signal for the complex with the correct stoichiometry and the absence of any aggregates of other compositions is a strong indication for highly specific interactions and inclusion complex formation rather than unspecific assembly. To further investigate this assumption, a mixture of an uncharged second guest DHN and CB[8] was measured under the same conditions as the ternary complex and revealed no assemblies. We are therefore confident that all observations of non-covalent assemblies in the gas phase are based on specific and strong interactions.

In an effort to gain a better understanding of the intrinsic properties of the CB[8] ternary inclusion complex a series of fragmentation experiments was performed. MS^n experiments were conducted at two different locations in the mass spectrometer: collision induced dissociation (CID) in the ion trap of the instrument served mainly to identify and confirm the various species. Higher energy collisionally activated dissociation (HCD) was performed in a multipole collision cell adjacent to the Orbitrap mass analyzer that enables triple quadrupole-like fragmentation. In the latter case parent ions were accelerated into the multipole where they remained for a set time to collide with a neutral gas, a process that allows for comparison of relative gas phase stabilities. Collision energies are given as % normalized collision energy (NCE). The supramolecular architectures under investigation vary largely in their masses and charges (m = 1700–7700, z = 2,6,7). It is well known that with increasing size of a molecule more energy can be dissipated in the form of vibrational energy before fragmentation occurs. Higher charge states of the same mass, however, fragment at lower collision energies due to charge repulsion effects. The NCE concept as implemented in recent Thermo instruments and software automatically compensates for this mass and charge dependency. Therefore, if significantly different NCEs are required for the dissociation of various CB[8] ternary complexes in the HCD cell, one can interpret these as differences in gas phase binding affinities of the second guest. It seemed most suitable to compare NCE values required for 50% loss of the parent ion intensity as illustrated in Fig. 2, hereafter referred to as E_{50}.

In all cases, both CID and HCD fragmentations of the ternary complex led to loss of the mass corresponding to the second guest. Comparing the E_{50} values for ternary MV·CB[8] complexes with different second guests shows a correlation between the molecular weight of the second guest and the gas phase stability of its ternary complex (Fig. 2). All "small" molecules, including DHN, Trp, Ant–NH_2 and Flu–NH_2 ranging from 160 to 204 amu required very little energy to be dissociated from the complex, typically 3–6% NCE. With increasing molecular weight higher energies were required, 17% NCE for the peptide (488 amu) and 22–24% NCE for the series of PEGylated second guests. Interestingly, no significant differences were observed among the five PEGylated guests, although their association constants in solution vary over two orders of magnitude (data not shown). Possible explanations for the increase of E_{50} with molecular weight of the second guest are stabilizing effects and additional interactions of both the penta-glycine chain and the PEG chains with the outside of the CB[8] barrel. Additional sets of experiments with the two trimeric systems further substantiated this hypothesis.

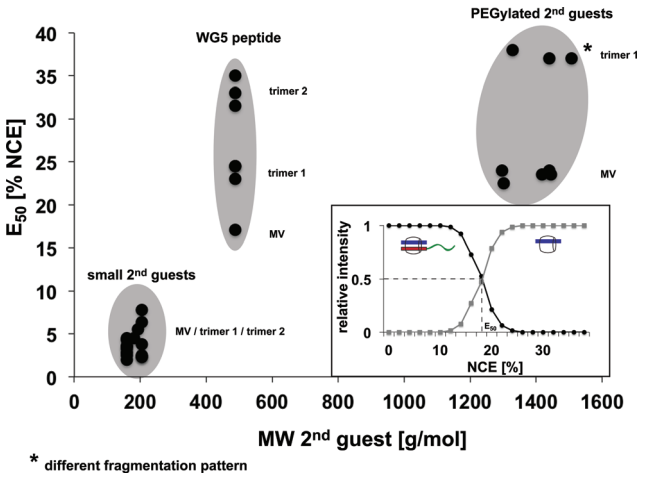

Fig. 2 HCD collision energies for 50% fragmentation of the parent ion; insert: determination of E_{50} for MV·CB[8]·WG5.

Trimers **1** and **2** were first studied on their own. While the larger and more flexible structure of trimer **1** allowed for detection of the naked 7+ ion in the gas phase (see Fig. S1†) the more compact and rigid trimer **2** fragments immediately upon transfer in the gas phase irrespective of the conditions. It has been reported[44] that viologen is intrinsically unstable as a doubly-positively charged ion in the gas phase on account of strong intramolecular charge repulsion effects and follows one of several mechanisms of stabilization: one electron reduction or proton loss leading to singly charged species, fragmentation into two singly-charged species or cluster formation with counter ions. Trimer **1** is likely able to stabilize itself through spatial rearrangement or one electron reduction, while the charge density of trimer **2** leads to destabilization and fragmentation. Upon addition of three equivalents of CB[8] formation of inclusion complexes in a 3 : 1 ratio was readily observed in the gas phase for both trimers. The inclusion complex of viologen inside of CB[8] shows remarkable stability in the gas phase, probably due to charge stabilizing effects of the host. In fact, dissociation was never observed in any of our fragmentation experiments.

Fig. 3 shows a typical spectrum obtained from an equimolar mixture of trimer **1**, CB[8] and WG5 as second guest. Similar spectra were found for both trimers with other "small" second guests and the peptide. A variety of different species is observed with **1**·$CB[8]_3$ usually being one of the most intense signals. Intensities drop with the number of second guests attached. In a control experiment with CB[7] which is capable of binding both MV and WG5, but not both of them simultaneously as a ternary complex, no assemblies beyond the **1**·$CB[7]_3$ complex were observed. This further supports the hypothesis that the observed species are based on highly specific interactions with CB[8] rather than unspecific aggregates.

Upon isolation of a 7-component complex and application of collision energy release of second guests was observed in a highly controlled fashion, giving further credibility to the

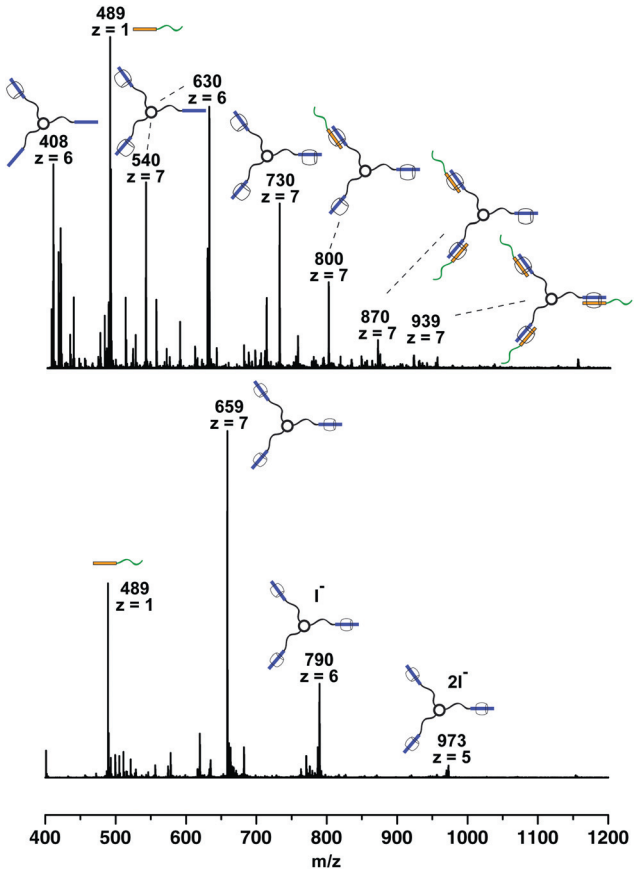

Fig. 3 Full spectra of 1·CB[8]$_3$·WG5$_3$ (top) and the control experiment with CB[7] instead of CB[8] (bottom) which cannot form 1 : 1 : 1 ternary complexes.

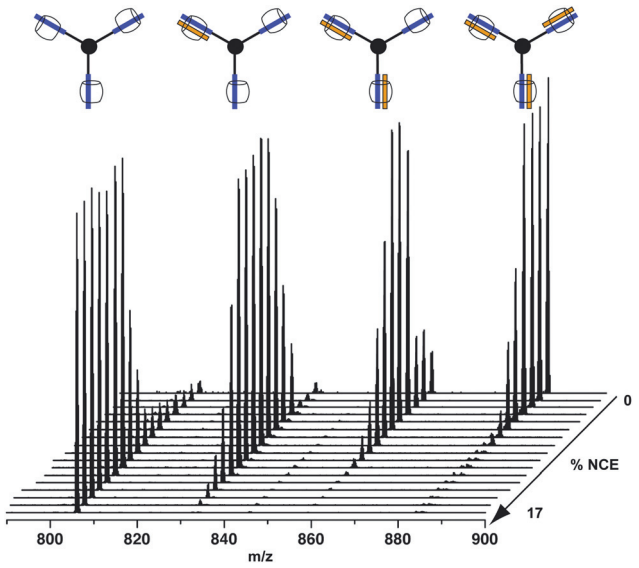

Fig. 4 Dissociation of **2**·CB[8]$_3$·DHN$_3$ in the HCD cell with increasing collision energy: **2**·CB[8]$_3$·DHN$_3$ *m/z* 886, **2**·CB[8]$_3$·DHN$_2$ *m/z* 860, **2**·CB[8]$_3$·DHN *m/z* 833, **2**·CB[8]$_3$ *m/z* 806, *z* = 6 for all ions.

envisioned structure. In a typical CID experiment the parent ion, *e.g.* **1**·CB[8]$_3$·WG5$_3$ (*m/z* 939, *z* = 7) loses the mass corresponding to one second guest, WG5 (488 amu) and results in a major signal for **1**·CB[8]$_3$·WG5$_2$ (*m/z* 870, *z* = 7). MS^3 of this species leads to **1**·CB[8]$_3$·WG5$_1$ (*m/z* 800, *z* = 7) and so on. After release of all three WG5 second guests the remaining species **1**·CB[8]$_3$ (*m/z* 730, *z* = 7) fragments into two main products with *m/z* 854 (*z* = 4) and *m/z* 555 (*z* = 3), corresponding to a covalent bond cleavage next to a triazole moiety resulting in a two-armed MV-species·CB[8]$_2$ (*m/z* 854) and a one-armed MV·CB[8] fragment (*m/z* 555), as illustrated in the ESI (Fig. S5†). This covalent bond cleavage happens preferentially over dissociation, which agrees with previous work,[45] suggesting that covalent bonds next to a non-covalent assembly of a doubly charged guest and a neutral host are significantly weakened in the gas phase. Further fragmentation of both viologen species with one or two CB[8] leads to the final product MV·CB[8].

Experiments in the HCD cell further confirmed the step-wise pattern of dissociation of all 7-component complexes. Fig. 4 illustrates how **2**·CB[8]$_3$·DHN$_3$ loses one DHN after another with increasing NCE. E_{50} values were measured for the three DHN guests independently by isolation of the corresponding 5-, 6- and 7-component complexes and subsequent MS^2 experiments. For DHN each dissociation step required 3.5% NCE, suggesting a non-cooperative binding mode. E_{50} values for the removal of second guests from either of the trimeric assemblies were similarly low for all "small" second guests. Values were in the same range for both trimers and for the ternary complexes with MV·CB[8] (see Fig. 2). These findings suggest that the stability of a CB[8] ternary complex in the gas phase, *i.e.* the energy required for its dissociation, is roughly equal for all small second guests under investigation. We conclude that differences in their solution based association constants are therefore largely influenced by differences in hydrophobic interactions and solvation energies.

As expected from the experiments with MV·CB[8]·WG5, HCD experiments with the 2 trimers and the WG5 peptide showed higher E_{50} values compared to the smaller second guests. Interestingly, E_{50} values of 24% NCE for trimer **1** and 31–35% NCE for trimer **2** were even higher than for the MV ternary complex. Upon transfer from the solution into the gas phase rearrangements of the 3-dimensional structure are expected to take place, most likely from an expanded structure in solution to a collapsed and more compact structure in the gas phase. We assume that elongated second guests such as WG5 or the PEGylated species can fold back onto CB[8] and even further onto the trimer itself and establish additional non-covalent interactions with suitable moieties. Clearly the carbamate structure of trimer **2** provides an environment for additional hydrogen bonding interactions with the C-terminal region of WG5. The alkyl chains of trimer **1** are less suited for this type of interaction.

Isolation of a 7-component complex containing PEGylated second guests proved difficult with the system used. A broad distribution of molecular weights for the second guest polymers leads to an even broader distribution of the corresponding *m/z* values for the complexes containing more than one second guest resulting in overall loss of relevant signals. Only **1**·CB[8]$_3$·HN–PEG$_1$, **1**·CB[8]$_3$·Ant–PEG$_1$ and **1**·CB[8]$_3$·Ant–PEG$_2$ could be isolated unambiguously and fragmented in the HCD cell (see Fig. 5). E_{50} values were around 37–38% and again higher than

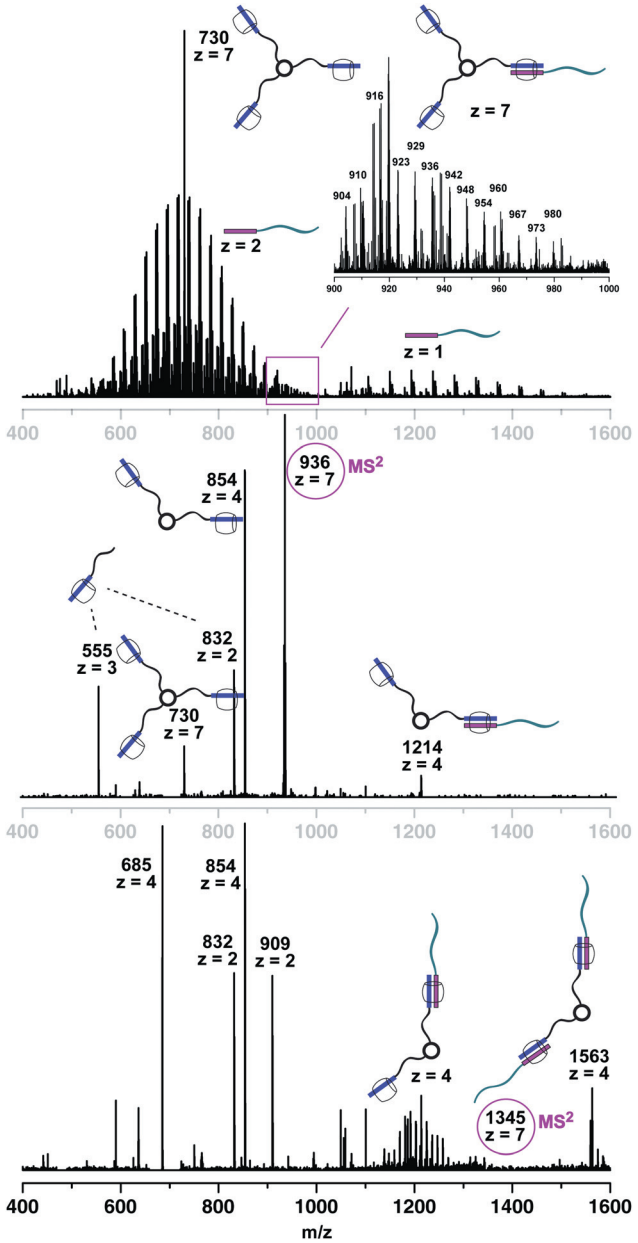

Fig. 5 (a) Full spectrum of **1**·CB[8]$_3$·Ant–PEG$_3$, inset showing the molecular weight distribution of **1**·CB[8]$_3$·Ant–PEG$_1$, (b) fragmentation spectrum of **1**·CB[8]$_3$·Ant–PEG$_1$ showing covalent bond cleavage as the main pathway, c) fragmentation spectrum of **1**·CB[8]$_3$·Ant–PEG$_2$, non-labelled peaks correspond to various fragments of **1**·CB[8]$_3$.

the corresponding values for smaller systems. These findings are in line with the envisioned collapsed gas phase structures of the supramolecular assemblies and additional interactions of the PEG chains with both the outer surface of CB[8] and elements of the trimeric viologen species. Further evidence comes from the fragmentation spectra of the PEG-containing species. In contrast to previous examples, loss of the mass corresponding to one second guest is now only a minor pathway (see Fig. 5b). The signal with highest intensity corresponds in all cases to the two-armed trimer fragment with two CB[8] hosts (*m*/*z* 854, *z* = 4). All other major signals can be assigned to the complex of the 2-armed MV species·CB[8]$_2$·HN–PEG$_1$ (*m*/*z* 1202, *z* = 4) and the Ant–PEG variant (*m*/*z* 1214, *z* = 4). Fragmentation of **1**·CB[8]$_3$·Ant–PEG$_2$ in fact resulted in a fairly high signal for the 2-armed MV species·CB[8]$_2$·Ant–PEG$_2$ (see Fig. 5c), suggesting that non-covalent interactions stay intact, while covalent bonds are being cleaved. As this behavior was not observed for smaller second guests secondary interactions of the PEG chains with CB[8] and the trimer are likely the cause of these results.

3. Conclusions

This study was aiming to improve our understanding of CB[8] ternary complexes by analyzing them in the absence of solvent effects. Both hydrophobic interactions and hydrogen bonding play an important role in the formation of CB[8] inclusion complexes in aqueous solution. However, only complexes containing second guests with the capability to form additional interactions such as hydrogen bonding could be observed in the gas phase with the instrumental setup used. Fragmentation studies in the HCD cell revealed increasing stability for ternary complexes with increasing size of the second guest. Back-folding of the chains appendant to the second guests onto the CB[8] host and trimeric first guest molecules are likely causing this effect by enhancing stability of the non-covalent assemblies *via* additional interactions. In conclusion, our findings suggest that hydrophobic interactions largely influence the affinity of second guests to the MV·CB[8] motif in aqueous solution, while their size and the ability to form additional interactions determines the gas phase stability of CB[8] ternary complexes. This enhanced understanding of the underlying molecular mechanisms of CB[8] ternary complex formation will greatly improve its use as a tool for the design and development of intriguing supramolecular architectures.

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