Ag^+ Labeling: A Convenient New Tool for the Characterization of Hydrogen-Bonded Supramolecular Assemblies by MALDI-TOF Mass Spectrometry**

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Abstract: Herein we describe our results on the characterization of a wide variety of different hydrogen-bonded assemblies by means of a novel matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique with Ag^+ labeling. The labeling technique with Ag^+ ions is extremely mild and provides a nondestructive way to generate charged assemblies that can be detected by mass spectrometry. Up to now more than 25 different single ($\mathbf{1}_3 \cdot \mathbf{2}_3$), double ($\mathbf{3}_3 \cdot \mathbf{2}_6$), and tetrarosettes ($\mathbf{4}_3 \cdot \mathbf{2}_{12}$) have been successfully characterized by the use of this method. The success of the method entirely depends on the presence of a suitable binding site for the Ag^+ ion. A variety of functionalities has been identified that provide strong binding sites for Ag^+, either acting in a cooperative way (π-arene and π-alkene donor functionalities) or individually (cyano and crown ether functionalities). The method works well for assemblies with molecular weights between 2000 and 8000 Da, and most likely far beyond this limit.

Keywords: hydrogen bonds · ion labeling · mass spectrometry · noncovalent interactions · supramolecular chemistry

Introduction

The synthesis of large multicomponent noncovalent assemblies has developed into an area of enormous interest for a wide variety of scientific and technological disciplines which ranges from materials science to molecular electronics.[1, 2] However, their characterization is far from straightforward and provides the chemists in the field with a formidable challenge.[3–5] Most studies rely on solution ^{1}H NMR spectroscopy data in combination with either vapor pressure osmometry (VPO), gel permeation chromatography (GPC),[1, 6] or light-scattering data.[7] Occasionally, a single-crystal X-ray structure analysis has been reported.[8] Until recently, mass spectrometry, the only method that provides quantitative data on the molecular composition was undoubtedly the missing link in the standard set of characterization tools for the supramolecular chemist.

The critical issue in the mass spectrometric detection of noncovalent molecular assemblies is the nondestructive generation of stable corresponding gas-phase ions.[9] A variety of soft ionization methods is currently available, such as field desorption (FD),[10] fast atom bombardment (FAB),[11] electrospray ionization (ESI),[12] and matrix-assisted laser desorption ionization (MALDI).[13] Of these methods FAB has been applied successfully in studies of noncovalent complexes of organic[14] and bioorganic[15] compounds with β-cyclodextrin. Most noncovalent complexes which involve large biomolecules have been studied with ESI[16–23] or MALDI techniques.[24–27] These ionization methods also allow an easy characterization of metal-coordinated assemblies that contain weakly coordinating counteranions.[4c, 28] However, for hydrogen-bonded assemblies, these methods are generally not successful because of the absence of suitable ionization sites in the molecules. Occasionally, acidic additives, such as trifluoroacetic acid (TFA) or acidic matrices, such as 2,5-

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[**] MALDI-TOF = matrix-assisted laser desorptoion ionization time-of-flight.

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dihydroxybenzoic acid (DHB), are used to promote ionization by protonation of basic sites; however, these tricks are usually not compatible with the formation of hydrogen bonds and lead to destruction of the assemblies.

In recent years a variety of ion-labeling techniques for the detection of hydrogen-bonded assemblies by means of ESI-MS has been developed. Lehn[29] and others[30] used K^+ labeling for the detection of hydrogen-bonded assemblies $\mathbf{1}_3 \cdot (DEB)_3$ (DEB = 5,5-diethylbarbituaric acid), that used benzo[18]crown-6 functionalized melamine **1a** which provides a strong binding site for K^+ (Figure 1 left). Although the detection of the corresponding assemblies as mono- and divalent potassium complexes was successful, severe fragmentation of the labeled assemblies was observed. A different ESI-MS labeling method that uses Cl^- ions was reported by Smith and Whitesides et al.[31] for the detection of $Hub(M)_3 \cdot (DEB)_3$ assemblies (Figure 1 right). This method has been successful only for assemblies that contain Hub, most likely as a result of cooperative binding of Cl^- to the three NH moieties in the spacer. Other labeling methods involve the oxidation of ferrocene moieties in the assemblies with molecular oxygen (O-ESMS),[32] and the use of quaternary ammonium guest molecules encapsulated as guest molecules in spherical hollow assemblies.[33]

Although these methods work very well for selected examples, a general MS characterization method that allows the widest possible structural variation within the assembly is still lacking. In a preliminary communication we reported a novel Ag^+ labeling technique for the MALDI-TOF mass spectrometric characterization of multicomponent hydrogen-bonded assemblies.[34, 35] The method is based on the remarkably high affinity of Ag^+ for a variety of aromatic and aliphatic π-donor systems, cyano groups,[36] crown ethers, or a combination thereof, and provides a *nondestructive* way to generate positively charged, hydrogen-bonded assemblies that can be easily detected by MALDI-TOF mass spectrometry. In this paper we describe the complete results of a detailed study on the characterization of hydrogen-bonded assemblies that consist of six, nine, and fifteen components by MALDI-TOF MS by means of Ag^+ labeling. We report 25 examples of hydrogen-bonded assemblies that were successfully characterized with this novel technique. In general, the MALDI-TOF MS data correlate very well with ^1H NMR spectroscopic data on the stability of these assemblies in solution.[8a, 37]

The interaction of Ag^+ with organic π-donor ligands: The interaction of metal cations with the π electrons of organic molecules, such as arenes, alkenes, and alkynes, has been studied in detail. Gas-phase binding enthalpies as high as 38, 28, 19, and 16 kcal mol^{-1} have been measured for Li^+, Na^+, K^+, and Rb^+ to benzene, respectively; however, the binding enthalpies are largely compensated by equally high solvation and ion-pair formation enthalpies.[38, 39] Dougherty and Ma established the relevance of cation–π interactions to biological recognition phenomena through interactions of especially NR_4^+ ions with the aromatic side chains of the amino acids Phe, Tyr, and Trp, and also showed that a binding site that consists of aromatic rings can favorably compete with the high solvation energies of cations in water.[39]

The existence of stable complexes between Ag^+ and π-electron donors, such as alkenes[40–42] and benzene,[43] has been known for decades.[44] Binding is the result of electron donation from the bonding π orbital to the empty 5s orbital on Ag^+ and backdonation from a filled d orbital on Ag^+ to the antibonding π orbital.[45, 46] A drastic increase in the stability of Ag^+ π complexes was observed for several cyclophanes as a result of cooperative binding of the Ag^+ ion by the aromatic rings.[47–49] Several papers describe the interaction of Ag^+ ions with the aromatic π faces at the upper rim of calix[4]arenes.[50] Highly stable sandwich-type complexes are formed in which

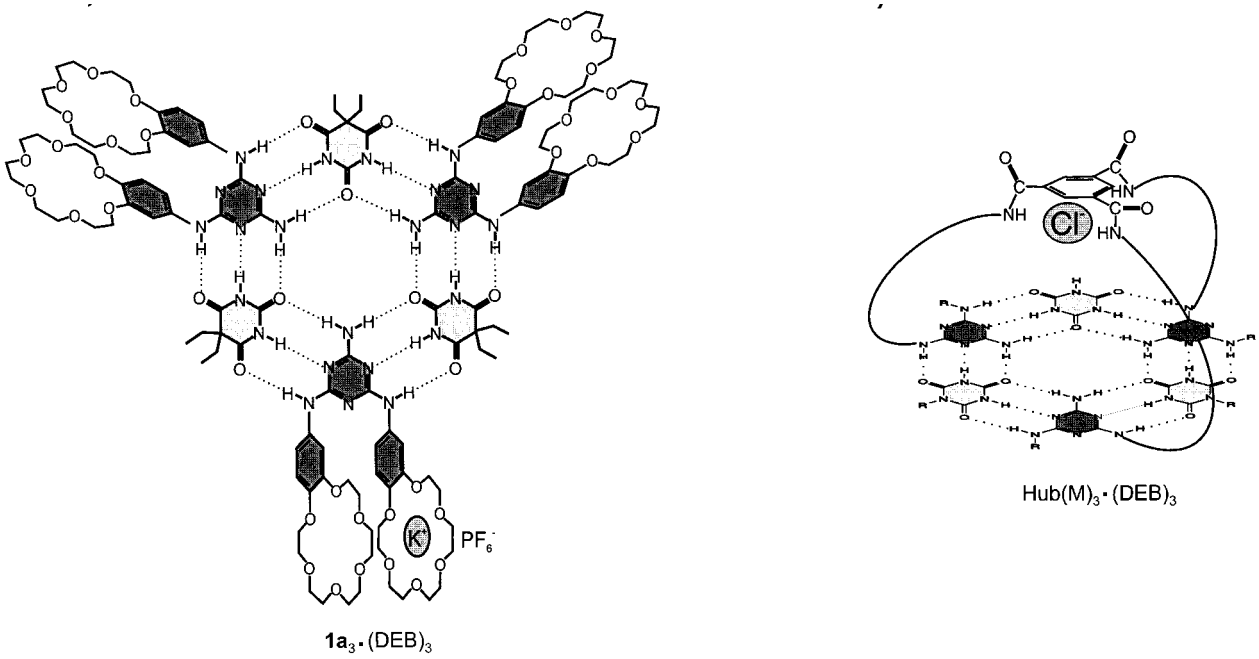

Figure 1. Schematic representations of two hydrogen-bonded assemblies $\mathbf{1a}_3 \cdot (DEB)_3$ and $Hub(M)_3 \cdot (DEB)_3$ that were characterized by ESI-MS.

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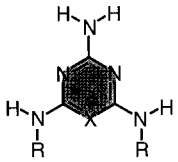
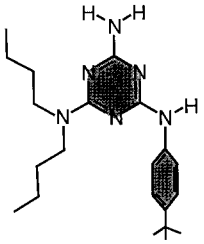
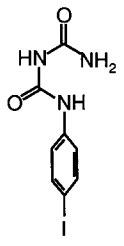


1b R = *p*-C_6H_4-$C(CH_3)_3$, X = N
c R = *p*-C_6H_4-$C(CH_3)_3$, X = CH
d R = C_6H_5, X = N
e R = *p*-C_6H_4-$O(CH_2)_{11}CH_3$, X = N
f R = $(CH_2)_3CH_3$, X = N



1g



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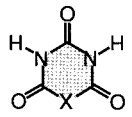
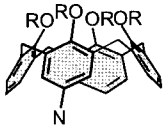
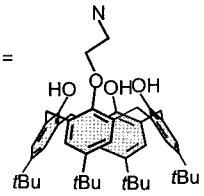


2a X = $C(CH_2CH_3)_2$
b X = $NCH_2CH_2C(CH_3)_3$
c X = $NCH_2CH_2C(C_6H_5)_3$
d X =

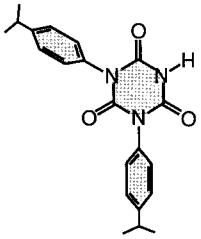


R = $CH_2(CH_2)_{10}CH_3$

2e X =



f X = $C(CH_2CH_3)(C_6H_5)$
g X = $C(CH_2CH{=}CH_2)_2$
h X = N-*p*-C_6H_4I
i X = N-*p*-C_6H_4CN
j X = N-(*R*)-α-$CH(C_6H_5)CH_3$



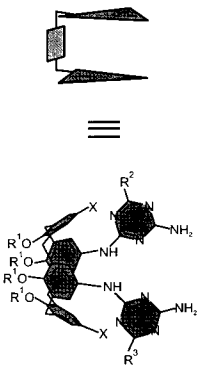
2k

the two distal aromatic rings bind the Ag^+ ions in a highly cooperative way. Shinkai et al. determined that the stability of the Ag^+ complexes of a variety of alkylated calix[4]arenes is $\approx 10^5\,M^{-1}$ in MeOH.[50a] In a similar manner to Ag^+, Cu^+ also has a large affinity for π-donor ligands, such as alkenes and aromatic rings.[51, 52] However, the complexes are extremely unstable upon exposure to air, and the Cu^+ ions quickly disproportionate to give Cu^{2+} and Cu^0.[51]

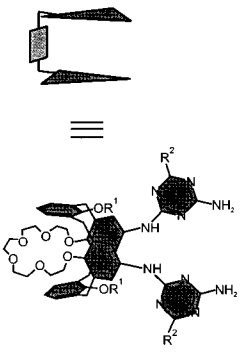
Apart from binding to π-electron donor ligands, the extremely soft Ag^+ ion binds very strongly to a variety of other soft donor ligands, such as nitriles, thiols, and amines.[53]

Results and Discussion

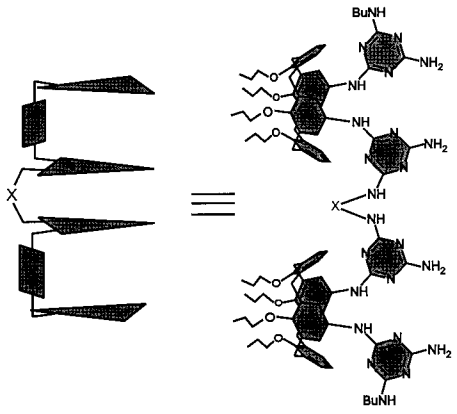
General: Samples prepared by stirring the hydrogen-bonded assemblies $\mathbf{1}_3\cdot\mathbf{2}_3$, $\mathbf{3}_3\cdot\mathbf{2}_6$, or $\mathbf{4}_3\cdot\mathbf{2}_{12}$ (Figure 2) with 1.5–2.0 equivalents of $AgCF_3COO$ for 24 hours in chloroform (for details see the Experimental Section) clearly show signals in the



3a $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(CH_2)_3CH_3$, X = H
b $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(CH_2)_3CH_3$, $X = NO_2$
c $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NHCH_2C_6H_5$, X = H
d $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(R)$-α-$CH(C_6H_5)CH_3$, X = H
e $R^1 = CH_2CH_2CH_3$, $R^2 = NH(R)$-α-$CH(C_6H_5)CH_3$,
$R^3 = NH(S)$-α-$CH(C_6H_5)CH_3$, X = H
f $R^1 = CH_3$, $R^2 = R^3 = NH(CH_2)_3CH_3$, X = H
h $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NHCH_2CH{=}CH_2$, X = H
i $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(CH_2)_4CH{=}CH_2$, X = H
j $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(CH_2)_6CH{=}CH_2$, X = H
k $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(CH_2)_8CH{=}CH_2$, X = H
l $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(CH_2)_3CH_3$, $X = C{\equiv}CSi(CH_3)_3$
m $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(CH_2)_3CH_3$, $X = C{\equiv}CH$
n $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = NH(CH_2)_3CH_3$, $X \equiv CN$
o $R^1 = CH_2CH_2CH_3$, $R^2 = R^3 = Cl$, X = H



3g $R^1 = CH_2CH_2CH_3$
$R^2 = NH(CH_2)_3CH_3$



4a X = CH_2(*m*-C_6H_4)CH_2
b X = $(CH_2)_6$

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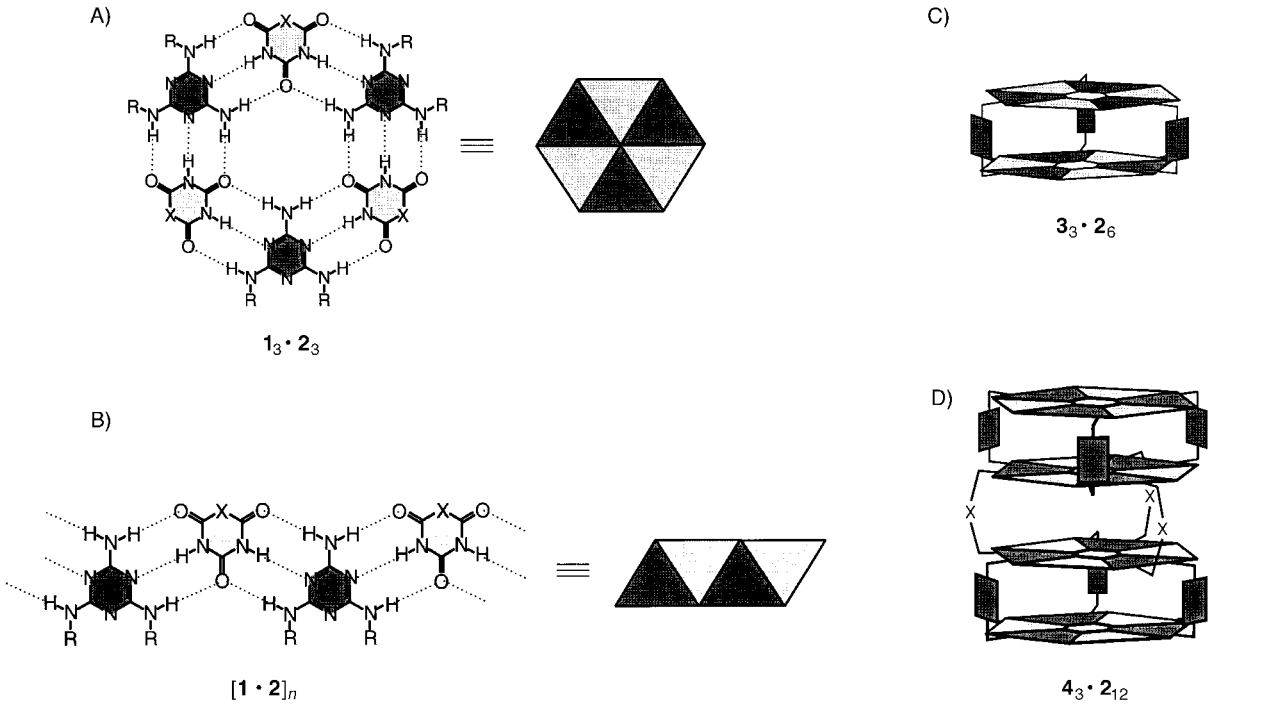

Figure 2. Chemical structure and schematic representations of A) single-rosette assemblies $\mathbf{1}_3 \cdot \mathbf{2}_3$ (6 components), B) linear tapelike assemblies $[\mathbf{1} \cdot \mathbf{2}]_n$, C) double-rosette assemblies $\mathbf{3}_3 \cdot \mathbf{2}_6$ (9 components), and D) tetrarosette assemblies $\mathbf{4}_3 \cdot \mathbf{2}_{12}$ (15 components).

MALDI-TOF spectra for the corresponding Ag^+ complexes only when the assembly is stable in solution and contains a binding site for Ag^+ (see Tables 1–3). The assemblies contain many carbon atoms and a relatively large number of nitrogen atoms. Consequently, the intensities of the peaks attributed to ions that contain natural isotopes are high. In addition, the peaks overlap because of the limited resolution of the MALDI-TOF instrument used (3900 FWHM). The masses of the Ag^+ complexes of the assemblies therefore have been calculated from the chemical atomic weights of carbon, hydrogen, nitrogen, oxygen, and silver. They agree very well with the observed masses of the Ag^+ complexes, taken as the maximum of the signal formed by the overlap of the unresolved natural isotope peaks (Tables 1–3). As a further check of the correct mass assignment, the appearance of the observed mass signal was compared to that obtained from the calculated isotope pattern obtained with the limited resolution of the instrument. As a typical example, the observed and calculated spectrum for the assembly $\mathbf{3c}_3 \cdot \mathbf{2a}_6$ ($R^2 = R^3 = CH_2Ph$) are given in Figure 3, which exemplifies the very good agreement between the shapes of the observed and calculated mass signals (on the average < 300 ppm mass difference).

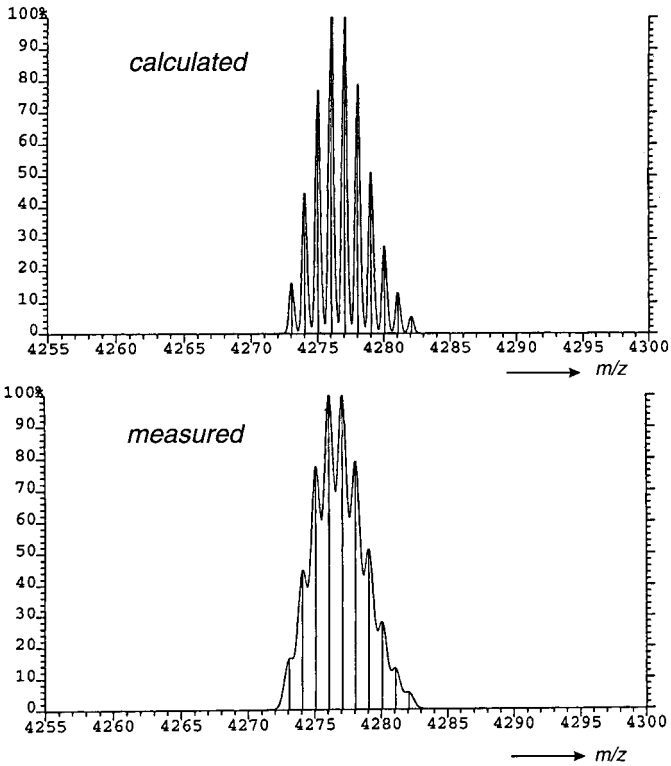

Figure 3. Experimental and calculated MALDI-TOF mass spectrum (+ isotope distribution) of the Ag^+ complex of assembly $\mathbf{3c}_3 \cdot \mathbf{2a}_6$.

Single-rosette assemblies $\mathbf{1}_3 \cdot \mathbf{2}_3$ (6 components, 18 hydrogen bonds)

The formation of multiply hydrogen-bonded networks that consist of disubstituted melamines or pyrimidines **1** (DAD) and barbiturates or isocyanurates **2** (ADA) have been extensively studied by several groups.[54] Two different assemblies can be formed depending on the relative orientation of the individual components, that is the cyclic hexameric *rosette* assembly $\mathbf{1}_3 \cdot \mathbf{2}_3$,[55] or *tapelike* assemblies $[\mathbf{1} \cdot \mathbf{2}]_n$, which can be subdivided into linear[56] and crinkled tapes (Figure 2).[57] It has been suggested by Whitesides and co-workers that melamines with bulky substituents, such as **1b** ($R = t$BuPh), exclusively form the cyclic rosette assembly $\mathbf{1}_3 \cdot \mathbf{2}_3$ as a direct

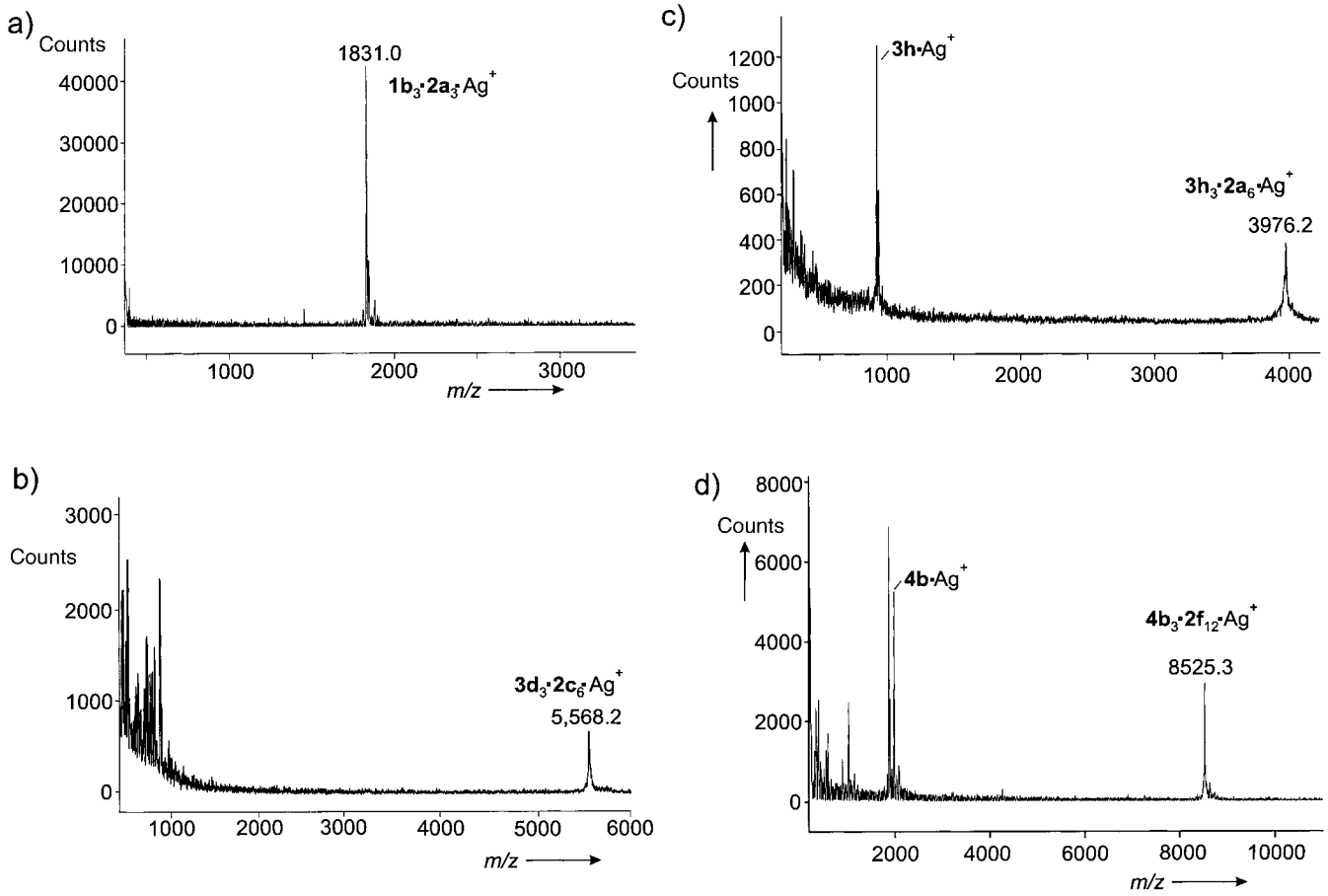

Figure 4. MALDI-TOF mass spectra of the monovalent Ag^+ complexes of a) single-rosette assembly $\mathbf{1b}_3 \cdot \mathbf{2a}_3$, b) double-rosette assembly $\mathbf{3d}_3 \cdot \mathbf{2c}_6$, c) double-rosette assembly $\mathbf{3h}_3 \cdot \mathbf{2a}_6$, and d) tetrarosette assembly $\mathbf{4b}_3 \cdot \mathbf{2f}_{12}$.

result of severe peripheral crowding in the corresponding tapelike structures $[\mathbf{1} \cdot \mathbf{2}]_n$. In contrast to this, less bulky melamines, such as **1d** (R = Ph), preferentially form linear (or crinkled) tapes. The cyclic rosettes are fully assembled in chloroform only at concentrations of >10 mM and are held together by a total of 18 hydrogen bonds. Mass spectrometrical characterization of these types of hydrogen-bonded assemblies with MALDI-TOF MS has not been successful so far.[31a] Our results show that MALDI-TOF MS with Ag^+ labeling provides an easy and straightforward technique to characterize a variety of single-rosette assemblies $\mathbf{1}_3 \cdot \mathbf{2}_3$.

For example, both melamine **1b** (R = *t*BuPh) and pyrimidine **1c** (R = *t*BuPh) give intense signals at *m*/*z* 1831.0 (calcd for $C_{93}H_{126}N_{24}O_9 \cdot Ag^+ = 1832.1$, Figure 4a) and at *m*/*z* 1828.9 (calcd for $C_{96}H_{129}N_{21}O_9 \cdot Ag^+ = 1829.1$) for the monovalent Ag^+ complexes of the corresponding assemblies $\mathbf{1b}_3 \cdot \mathbf{2a}_3$ and $\mathbf{1c}_3 \cdot \mathbf{2a}_3$ (Table 1, entries 1 and 6). Signals corresponding to the doubly or triply charged assemblies $\mathbf{1b}_3 \cdot \mathbf{2a}_3 \cdot (Ag^+)_2$ (calcd *m*/*z* 970.0) or $\mathbf{1b}_3 \cdot \mathbf{2a}_3 \cdot (Ag^+)_3$ (calcd *m*/*z* 682.6) or fragmented assemblies were not observed. The cyclic rosettes bind Ag^+ ions by simultaneous coordination to the two aromatic rings of the *tert*-butylphenyl groups of component **1b** or **1c** (Figure 5). Evidence for this comes from the fact that only components **1b–e**, that bear phenyl substituents, give signals for the Ag^+ complexes of free **1** (*m*/*z* 497, $[\mathbf{1b} \cdot Ag]^+$; *m*/*z* 496, $[\mathbf{1c} \cdot Ag]^+$) in the MALDI-TOF spectrum, while for instance, the butyl-substituted melamine **1f** does not. ^{1}H NMR complexation experiments with free **1b** support this view. Addition of 10 equivalents of $AgClO_4$ to a 2 mM solution

Table 1. MALDI-TOF MS data for six-component assemblies $\mathbf{1}_3 \cdot \mathbf{2}_3$ (18 hydrogen bonds) after treatment with 1.5–2.0 equivalents $AgCF_3COO$ for 24 h at RT.

Entry	Assembly	Molecular composition	Stability in chloroform[a]	Calcd mass [Da] of Ag^+ complex[b]	Observed mass [Da]
1	$\mathbf{1b}_3 \cdot \mathbf{2a}_3$	$C_{93}H_{126}N_{24}O_9$	+	1832.1	1831.0
2	$\mathbf{1b}_3 \cdot \mathbf{2b}_3$	$C_{96}H_{135}N_{27}O_9$	+[c]	1919.2	1919.1
3	$\mathbf{1b}_3 \cdot \mathbf{2c}_3$	$C_{141}H_{153}N_{27}O_9$	+	2477.8	–[d]
4	$\mathbf{1b}_3 \cdot \mathbf{2d}_3$	$C_{306}H_{453}N_{27}O_{21}$	+	4954.0	4947.9
5	$\mathbf{1b}_3 \cdot \mathbf{2e}_3$	$C_{216}H_{273}N_{27}O_{21}$	+	3691.6	3692.0
6	$\mathbf{1c}_3 \cdot \mathbf{2a}_3$	$C_{96}H_{129}N_{21}O_9$	+	1829.1	1828.9
7	$\mathbf{1c}_3 \cdot \mathbf{2b}_3$	$C_{99}H_{138}N_{24}O_9$	+	1916.2	1915.0
8	$\mathbf{1c}_3 \cdot \mathbf{2c}_3$	$C_{144}H_{156}N_{24}O_9$	+	2474.9	2476.9
9	$\mathbf{1d}_3 \cdot \mathbf{2a}_3$	$C_{69}H_{78}N_{24}O_9$	–	1495.4	–[d]
10	$\mathbf{1e}_3 \cdot \mathbf{2a}_3$	$C_{141}H_{222}N_{24}O_{15}$	–	2601.4	–[d]
11	$\mathbf{1f}_3 \cdot \mathbf{2a}_3$	$C_{57}H_{102}N_{24}O_9$	–	1375.5	–[d]

[a] Notation "+" means that assembly is stable at concentrations $>10^{-2}$ M and "–" means that assembly is not stable in solution. [b] The calculated isotopic patterns are in good agreement with the experimentally observed molecular mass signals. [c] The ^{1}H NMR spectrum shows a complex pattern for the isocyanurate NH protons ($\delta = 14-16$), which suggests that the cyclic hexameric assemblies is not the only species present. [d] The Ag^+ complex was not observed in the MALDI TOF mass spectrum.

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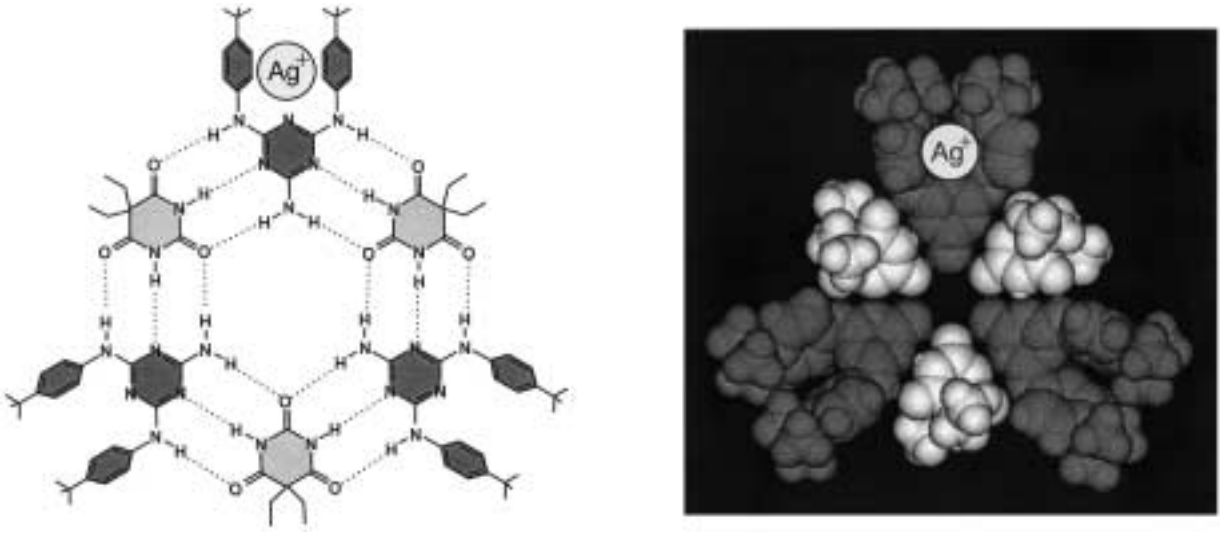

Figure 5. Proposed structure (left) and space-filling model (right) of the Ag^+ complex of assembly $\mathbf{1b}_3 \cdot \mathbf{2a}_3$.

of **1b** in $CDCl_3/CD_3OD$ (1:1, v/v) causes significant shifts for the *tert*-butyl ($\Delta\delta = 0.03$ upfield) and aromatic ($\Delta\delta = 0.085$ downfield) proton signals. Formation of the cyclic hexameric assembly preorganizes the phenyl substituents of **1** to form a sandwich-type π complex with Ag^+ (Figure 5). Additional coordination of Ag^+ to the triazine ring nitrogen, which is absent in **1c**, does not seem to play a significant role, judging from the close similarity between the spectra of $\mathbf{1b}_3 \cdot \mathbf{2a}_3$ and $\mathbf{1c}_3 \cdot \mathbf{2a}_3$. Coordination of Ag^+ to the nitrogen or the carbonyl oxygen atoms of **2a** is unlikely to account for the high stability of the $\mathbf{1}_3 \cdot \mathbf{2a}_3 \cdot Ag^+$ complexes, in view of the low affinity of free **2a** for Ag^+ (very weak signal at *m*/*z* 291 for $\mathbf{2a} \cdot Ag^+$).

Single-rosette assemblies of melamine **1b** with isocyanurates **2b–e** (Table 1, entries 2–5) also give strong signals in the MALDI-TOF spectra for the monovalent Ag^+ complexes, except for triphenyl isocyanurate **2c** (Table 1, entry 3). However, the MALDI-TOF spectrum of **2c** with pyrimidine **1c** (Table 1, entry 8) does show an intense signal at *m*/*z* 2476.9 (calcd for $\mathbf{1c}_3 \cdot \mathbf{2c}_3 \cdot Ag^+ = 2474.9$) for the Ag^+ complex. The reason for this discrepancy is not clear because the ^1H NMR spectra of these assemblies are not significantly different.

Also assemblies with a much higher molecular weight, such as the calix[4]arene-based assemblies $\mathbf{1b}_3 \cdot \mathbf{2d}_3$ and $\mathbf{1b}_3 \cdot \mathbf{2e}_3$, give clean signals at *m*/*z* 4947.9 and 3692.0 (calcd for $\mathbf{1b}_3 \cdot \mathbf{2d}_3 \cdot Ag^+ = 4954.0$ and for $\mathbf{1b}_3 \cdot \mathbf{2e}_3 \cdot Ag^+ = 3691.6$). For assembly $\mathbf{1b}_3 \cdot \mathbf{2e}_3$, the observed and calculated masses match very well (100 ppm mass difference), while for assembly $\mathbf{1b}_3 \cdot \mathbf{2d}_3$ there is a significant difference between both values (1200 ppm mass difference). The reason for this relatively large deviation is still unclear. Despite the fact that these assemblies have a total of six strong binding sites for Ag^+ (also the calix[4]arenes bind Ag^+ very strongly),[50] the monovalent Ag^+ complexes are the only significant signals in the spectrum between 2000 and 8000 Da.

For the melamines **1d** (R = phenyl), **1e** (R = 4-dodecyloxyphenyl), and **1f** (R = *n*-butyl), stable Ag^+ complexes of the corresponding rosette assemblies $\mathbf{1}_3 \cdot \mathbf{2a}_3$ were not observed. These results support earlier observations by Whitesides and co-workers that cyclic rosette assemblies $\mathbf{1}_3 \cdot \mathbf{2}_3$ without bulky substituents at the periphery are thermodynamically unstable and preferentially form *linear* tapelike structures $[\mathbf{1} \cdot \mathbf{2}]_n$ under the conditions used (Figure 2B).[55] However, no signals for the Ag^+ complexes of tapelike structures (or fragments) were observed when equimolar mixtures of **1d–f** and **2a** were measured under standard Ag^+ labeling conditions. Model experiments with compounds **1g** and **2k**, which form a well-defined hydrogen-bonded assembly by the formation of three hydrogen bonds, also do not show a signal for the Ag^+ complex of the assembly, which suggests that the stability of the complex is too low to be observed under the conditions of the MALDI-TOF measurements.

Influence of the metal cation (Cs^+, Cu^{2+}, Zn^{2+}) and of the counteranion (OTs^-, $CF_3SO_3^-$, BF_4^-, PF_6^-): In order to establish the unique role of the soft Ag^+ ion, several ion-labeling experiments with assembly $\mathbf{1b}_3 \cdot \mathbf{2a}_3$ were carried out in which Ag^+ was replaced by other cations. However, none of the cations tested ($M = Cs^+, Cu^{2+}, Zn^{2+}$) gave similar results to those of Ag^+. Only with Cs^+ was a small signal ($S/N \approx 3-4$) for the ion-labeled hydrogen-bonded assembly observed (*m*/*z* 1857.1, 60%, calcd for $\mathbf{1b}_3 \cdot \mathbf{2a}_3 \cdot {}^{133}Cs^+ = 1857.9$). However, in this case severe decomposition of the complex was also observed (additional signals at 1823.8 (45%), 1804.4 (100%), 1788.7 (70%), 1747.1 (20%)). With Cu^{2+} and Zn^{2+}, the corresponding ion-labeled hydrogen-bonded assemblies were not observed. The low-mass region of these spectra only showed the formation of metal ion complexes with component **1b** (*m*/*z* 452.7, 60%, $[\mathbf{1b} \cdot Cu - H]^+$, calcd 452.6; *m*/*z* 842.7, 20%, $[\mathbf{1b}_2 \cdot Cu - H]^+$; calcd 843.4), (*m*/*z* 460.9, 40%, $[\mathbf{1b} \cdot Zn - H]^+$, calcd 453.6). These complexes are most probably formed by coordination of the metal ion to the triazine ring nitrogen atoms, thus destroying the hydrogen-bonded assemblies.

Additionally, several experiments were performed in order to determine the role of the counteranion used. In addition to CF_3COO^- (standard conditions), four other anions were tested: OTs^-, $CF_3SO_3^-$, BF_4^-, and PF_6^-. For both OTs^- ($S/N \approx 50$) and $CF_3SO_3^-$ ($S/N \approx 20$) the spectra show very similar

signals to those of CF_3COO^- (S/N ≈ 50). The samples are stable for at least one month as the intensity of the signals does not decrease over this period of time. However, with BF_4^- and PF_6^- as counteranions, the results are significantly worse. Initially, intense signals for the Ag^+-labeled assemblies are observed for both anions (S/N ≈ 20), while in the case of BF_4^- an additional signal at *m/z* 1652 (50%) is observed that most likely corresponds to the loss of one molecule of **2a**. Similar decomposition patterns have never been observed in any of the labeling experiments with CF_3COO^-. Moreover, the intensity of the signals with BF_4^- and PF_6^- as counteranions severely diminishes over time. After one month the Ag^+-labeled assemblies cannot be detected anymore, which is most probably the consequence of the relatively low hydrolytic stability of both anions.

Double-rosette assemblies $\mathbf{3}_3\cdot\mathbf{2}_6$ (9 components, 36 hydrogen bonds)

Hydrogen-bonded double-rosette assemblies $\mathbf{3}_3\cdot\mathbf{2}_6$ (see Figure 2) which comprise three molecules of calix[4]arene dimelamines **3** and six molecules of barbiturates or isocyanurates **2**, have been an active topic of research in our group.[8a, 37, 58] These assemblies exclusively exist as cyclic double rosettes according to ^{1}H NMR spectroscopy, while the corresponding tapelike structures are totally absent. The extremely high thermodynamic stability of these assemblies is the result of the presence of 36 cooperative hydrogen bonds, which makes these structures stable, even at 10^{-4} M in chloroform.[8a] All attempts to characterize these assemblies by mass spectrometry without making use of labeling techniques have so far been unsuccessful. Also the $PPh_4^+Cl^-$-labeling ESMS technique introduced by Smith, Whitesides and co-workers did not give positive results for these assemblies in our hands, which is in agreement with the negative results reported by Whitesides and co-workers for related assemblies.[31] Therefore, we have investigated the feasibility of our Ag^+-labeling technique for the MALDI-TOF MS characterization of double-rosette assemblies $\mathbf{3}_3\cdot\mathbf{2}_6$.

In order to promote the formation of strong Ag^+ complexes of the hydrogen-bonded assemblies $\mathbf{3}_3\cdot\mathbf{2}_6$, additional functionalities with a strong affinity for Ag^+ ions need to be introduced into the individual components. When such functionalities are present, the corresponding assemblies show intense signals for the corresponding Ag^+ complexes in the MALDI-TOF mass spectrum. We have found four different types of functionalities that support the formation of strong Ag^+ complexes of hydrogen-bonded assemblies $\mathbf{3}_3\cdot\mathbf{2}_6$, namely aromatic groups (A), olefinic groups (B), cyano groups (C), and crown ethers (D). Below we describe a selection of 16 assemblies (see Table 2) that bear these functionalities either on the dimelamine component **3** or on the barbiturate/isocyanurate component **2**, which were successfully characterized by MALDI-TOF MS with Ag^+ labeling.

Assemblies functionalized with aromatic groups: The presence of aromatic functionalities in either dimelamine **3** or barbiturate/isocyanurate **2** generally leads to the formation of stable Ag^+ complexes upon treatment with $AgCF_3COO$ that can be observed in the MALDI-TOF spectrum. For example, treatment of the benzyl-substituted assembly $\mathbf{3c}_3\cdot\mathbf{2a}_6$ ($R^2 = R^3 = CH_2Ph$) and the chiral assembly $\mathbf{3d}_3\cdot\mathbf{2a}_6$ ($R^2 = R^3 = (R)$-CHPhMe)[58] with $AgCF_3COO$ produced intense signals at *m/z* 4278.3 (calcd for $\mathbf{3c}_3\cdot\mathbf{2a}_6\cdot Ag^+ = 4276.9$) and 4358.3 (calcd for $\mathbf{3d}_3\cdot\mathbf{2a}_6\cdot Ag^+ = 4361.1$), respectively, in the corresponding MALDI-TOF spectra (Table 2, entries 21 and 22). In view of the inability of assemblies $\mathbf{3a}_3\cdot\mathbf{2a}_6$ and $\mathbf{3b}_3\cdot\mathbf{2a}_6$ ($R^2 = R^3 = (CH_2)_3CH_3$) to form stable Ag^+ complexes (vide infra), it is evident that the benzyl groups are involved in the Ag^+ complexation. It is quite unlikely that one benzyl group alone binds Ag^+, because such complexes are not expected to have sufficient stability in the presence of excess 2,5-dihydroxybenzoic acid (used as the matrix), which can form Ag^+ complexes of comparable stability. Molecular models clearly show that the benzyl group (oriented perpendicularly to the plane of the assembly) together with the calix[4]arene aromatic ring carrying the melamine, provides a binding site in which Ag^+ is bound cooperatively (Figure 6 left). Supporting evidence for this comes from the fact that 2D ROESY connectivities measured for assembly $\mathbf{3d}_3\cdot\mathbf{2a}_6$ reveal a very similar orientation of the 1-phenylethyl substituents in this assembly.[58] The possibility that two benzyl groups of neighboring calix[4]arene fragments combine to provide a binding site for Ag^+ can be excluded, because the distance between two benzyl groups is > 7 Å.

Table 2. MALDI-TOF MS data for nine-component assemblies $\mathbf{3}_3\cdot\mathbf{2}_6$ (36 hydrogen bonds) after treatment with 1.5–2.0 equivalents of $AgCF_3COO$ for 24 h at RT.

Entry	Assembly	Molecular Composition	Stability in chloroform[a]	Calcd mass [Da] of Ag^+ complex[b]	Observed mass [Da]
12	$\mathbf{3a}_3\cdot\mathbf{2a}_6$	$C_{210}H_{288}N_{48}O_{30}$	++	4072.8	–[c]
13	$\mathbf{3a}_3\cdot\mathbf{2b}_6$	$C_{216}H_{306}N_{54}O_{30}$	++	4247.0	–[c]
14	$\mathbf{3a}_3\cdot\mathbf{2f}_6$	$C_{234}H_{288}N_{48}O_{30}$	++	4361.1	4360.0
15	$\mathbf{3a}_3\cdot\mathbf{2g}_6$	$C_{222}H_{288}N_{48}O_{30}$	++	4216.9	–[c]
16	$\mathbf{3a}_3\cdot\mathbf{2h}_6$	$C_{222}H_{252}N_{60}O_{30}$	++	4348.7	4348.1
17	$\mathbf{3b}_3\cdot\mathbf{2a}_6$	$C_{210}H_{282}N_{54}O_{42}$	++	4342.8	–[c]
18	$\mathbf{3b}_3\cdot\mathbf{2b}_6$	$C_{216}H_{300}N_{60}O_{42}$	++	4517.0	4516.0
19	$\mathbf{3b}_3\cdot\mathbf{2h}_6$	$C_{222}H_{246}N_{66}O_{42}$	++	4618.7	4620.4
20	$\mathbf{3b}_3\cdot\mathbf{2j}_6$	$C_{228}H_{282}N_{60}O_{42}$	++	4643.0	4643.4
21	$\mathbf{3c}_3\cdot\mathbf{2a}_6$	$C_{228}H_{276}N_{48}O_{30}$	++	4276.9	4278.3
22	$\mathbf{3d}_3\cdot\mathbf{2a}_6$	$C_{234}H_{288}N_{48}O_{30}$	++	4361.1	4358.3
23	$\mathbf{3d}_3\cdot\mathbf{2b}_6$	$C_{240}H_{306}N_{54}O_{30}$	++	4535.3	4536.4
24	$\mathbf{3d}_3\cdot\mathbf{2c}_6$	$C_{324}H_{330}N_{54}O_{30}$	++	5568.4	5568.2
25	$\mathbf{3e}_3\cdot\mathbf{2a}_6$	$C_{234}H_{288}N_{48}O_{30}$	–	4361.1	4362.3
26	$\mathbf{3e}_3\cdot\mathbf{2j}_6$	$C_{252}H_{288}N_{54}O_{30}$	++	4661.3	4658.2
27	$\mathbf{3f}_3\cdot\mathbf{2a}_6$	$C_{186}H_{240}N_{48}O_{30}$	++	3736.2	3745.8
28	$\mathbf{3g}_3\cdot\mathbf{2a}_6$	$C_{222}H_{306}N_{48}O_{42}$	++	4427.1	4429.3
29	$\mathbf{3h}_3\cdot\mathbf{2a}_6$	$C_{204}H_{264}N_{48}O_{30}$	++	3976.5	3976.2
30	$\mathbf{3i}_3\cdot\mathbf{2a}_6$	$C_{222}H_{300}N_{48}O_{30}$	++	4229.0	–[c]
31	$\mathbf{3j}_3\cdot\mathbf{2a}_6$	$C_{234}H_{324}N_{48}O_{30}$	++	4397.4	4398.0
32	$\mathbf{3k}_3\cdot\mathbf{2a}_6$	$C_{246}H_{348}N_{48}O_{30}$	++	4565.7	–[c]
33	$\mathbf{3l}_3\cdot\mathbf{2a}_6$	$C_{240}H_{336}N_{48}O_{30}Si_6$	++	4650.0	–[c]
34	$\mathbf{3m}_3\cdot\mathbf{2a}_6$	$C_{222}H_{288}N_{48}O_{30}$	++	4216.9	–[c]
35	$\mathbf{3n}_3\cdot\mathbf{2a}_6$	$C_{216}H_{282}N_{54}O_{30}$	++	4222.8	4220.0
36	$\mathbf{3o}_3\cdot\mathbf{2a}_6$	$C_{222}H_{288}N_{48}O_{30}$	++	4216.9	–[c]

[a] Notation "++" means that assembly is stable at 10^{-3} M concentration, and "–" means that formation of the assembly is not observed in solution. [b] The calculated isotopic patterns are in good agreement with the experimentally observed molecular mass signals. [c] The Ag^+ complex was not observed in the MALDI TOF mass spectrum.

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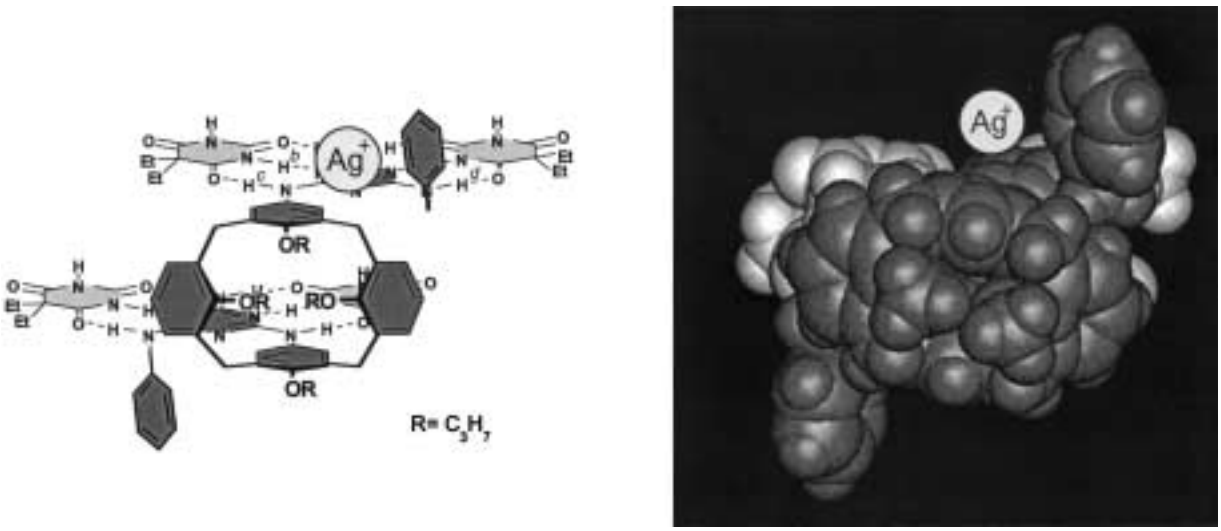

Figure 6. Proposed structure (left) and space-filling model (right) of the Ag^+ complex of assembly $\mathbf{3c}_3 \cdot \mathbf{2a}_6$ (part of the structure is omitted for clarity).

Functionalization of barbiturates or isocyanurates **2** with aromatic groups provides an alternative way to incorporate Ag^+ binding sites in the corresponding assembly. For example, treatment of the chiral assembly $\mathbf{3d}_3 \cdot \mathbf{2c}_6$ ($R^2 = R^3 = (R)$-CHPhMe) with $AgCF_3COO$ gives rise to intense signals at *m*/*z* 5568.2 (calcd for $\mathbf{3d}_3 \cdot \mathbf{2c}_6 \cdot Ag^+ = 5568.4$) in the corresponding MALDI-TOF spectra (Table 2, entry 24, Figure 4b). In this case, the three phenyl substituents in **2c** can bind the Ag^+ in a cooperative way. In certain cases, binding of Ag^+ takes place as a result of cooperativity between the melamine and the barbiturate or isocyanurate component. For example, the treatment of assembly $\mathbf{3a}_3 \cdot \mathbf{2f}_6$ with $AgCF_3COO$ shows an intense signal at *m*/*z* 4360 (calcd for $\mathbf{3a}_3 \cdot \mathbf{2f}_6 \cdot Ag^+ = 4361.1$), despite the fact that **2f** does not have an appreciable affinity for Ag^+ by itself. It is most likely that the phenyl group in **2f** also binds Ag^+ in cooperation with one of the aromatic rings of calix[4]arene **3a**, in a similar manner to that observed for assembly $\mathbf{3c}_3 \cdot \mathbf{2a}_6$. The alternative option that two phenyl groups of different components of **2f** combine together to provide a suitable binding site for Ag^+ is very unlikely for steric reasons; this has been supported by ^1H NMR studies with assembly $\mathbf{3a}_3 \cdot \mathbf{2f}_6$.[59] For reasons of generality, the use of barbituric acid **2f** has a small disadvantage because many different isomeric assemblies can be formed (different *up* and *down* orientations of the phenyl groups). This leads to desymmetrization of the ^1H NMR spectrum, the interpretation of which, therefore, becomes very complicated.

Functionalization of isocyanurates with aromatic groups, as in chiral isocyanurate **2j**, also promotes the formation of stable Ag^+ complexes of the corresponding assemblies $\mathbf{3}_3 \cdot \mathbf{2j}_6$. The assemblies $\mathbf{3b}_3 \cdot \mathbf{2j}_6$ and $\mathbf{3e}_3 \cdot \mathbf{2j}_6$ (Table 2, entries 20 and 26) showed intense signals in the MALDI-TOF spectrum at *m*/*z* 4643.4 (calcd for $\mathbf{3b}_3 \cdot \mathbf{2j}_6 \cdot Ag^+ = 4643.0$) and at *m*/*z* 4658.2 (calcd for $\mathbf{3e}_3 \cdot \mathbf{2j}_6 \cdot Ag^+ = 4661.3$), respectively, after treatment with $AgCF_3COO$ (S/N ≈ 25). For these assemblies it cannot be determined exactly how Ag^+ binding takes place; however, molecular models clearly show that the aromatic functionality in **2j** can easily adopt an orientation consistent with the formation of a sandwich-type Ag^+ complex.

Alkenyl- and alkynyl-functionalized assemblies: Similar to arenes, a variety of alkenes and alkynes are known to form strong complexes with Ag^+ ions.[40–42] We have found two examples in which functionalization of dimelamine **3** with alkene functionalities resulted in the formation of strong Ag^+ complexes that can be observed by MALDI-TOF MS. In general, the intensity of the signals is lower (S/N < 8) than for the assemblies with aromatic functionalities (S/N ≈ 12). Moreover, our results show that the affinity of assemblies functionalized with alkene or alkyne functionalities is strongly dependent on the position of the alkene or alkyne functionality within the individual components. For example, the allyl-substituted assembly $\mathbf{3h}_3 \cdot \mathbf{2a}_6$ ($R^2 = R^3 = CH_2CH{=}CH_2$) showed a signal at *m*/*z* 3976.2 (calcd for $\mathbf{3h}_3 \cdot \mathbf{2a}_6 \cdot Ag^+ = 3976.5$) after treatment with $AgCF_3COO$. This assembly most probably binds Ag^+ ions in a very similar way to that described for the benzyl-substituted assembly $\mathbf{3c}_3 \cdot \mathbf{2a}_6$, namely by means of cooperative binding both with the alkene and one of the calix[4]arene aromatic rings. In sharp contrast to this, we found that of the assemblies with longer alkenyl side chains (Table 2, entries 30–32), only assembly $\mathbf{3j}_3 \cdot \mathbf{2a}_6$ ($R^2 = R^3 = (CH_2)_6CH{=}CH_2$) with 7-octenyl side chains showed a significant signal at *m*/*z* 4398.0 (calcd for $\mathbf{3j}_3 \cdot \mathbf{2a}_6 \cdot Ag^+ = 4397.4$) after treatment with $AgCF_3COO$. These results clearly indicate that the ability of arene and alkene units to bind Ag^+ in a cooperative way quickly decreases with increasing chain length of the spacer that connects the two binding partners. However, it remains unclear as to why assembly $\mathbf{3j}_3 \cdot \mathbf{2a}_6$ is indeed able to bind Ag^+.

Supporting evidence that cooperativity is important in the binding of Ag^+ was given by the fact that neither assembly $\mathbf{3l}_3 \cdot \mathbf{2a}_6$ ($R^2 = R^3 = C{\equiv}CSi(CH_3)_3$) nor assembly $\mathbf{3m}_3 \cdot \mathbf{2a}_6$ ($R^2 = R^3 = C{\equiv}CH$) showed detectable signals for the corresponding Ag^+ complexes in the MALDI-TOF spectrum, despite the fact that both assemblies are thermodynamically stable in solution.[8a] Clearly, the isolated acetylene moieties in these assemblies cannot bind Ag^+ in a cooperative way (too remote from aromatic ring or a second acetylene unit), which indicates that Ag^+ complexes of single acetylenes (and also

single arenes and alkenes, vide supra) are insufficiently stable to be observed by MALDI-TOF mass spectrometry.

The presence of two alkene units in close proximity to each other does not guarantee the cooperative formation of strong Ag^+ complexes. For example, assemblies that contain 5,5-diallylbarbituric acid **2g** do not form stable Ag^+ complexes under MALDI-TOF MS conditions (Table 2, entry 15). The free barbiturate **2g** itself also does not bind Ag^+ significantly, despite the fact that it contains two alkene moieties that are close enough to be able to bind Ag^+ in a cooperative manner. These results strongly emphasize the importance of the relative orientation of the functionalities that form the binding site for Ag^+. Furthermore, these results clearly show that it remains difficult to predict, a priori, whether a particular assembly can be detected by MALDI-TOF by means of the Ag^+-labeling method as long as combinations of functionalities, such as arenes and alkenes, are required.

Cyano-functionalized assemblies: So far we only have discussed the labeling experiments for which cooperativity between two functionalities is required for Ag^+ binding, since binding of the individual functionalities to Ag^+ is too weak to be detected. The disadvantage of these labeling experiments is that one cannot predict the outcome of a labeling experiment. In this respect the introduction of cyano groups is of interest, because they are known to have an extremely high affinity for Ag^+.[36] For example, a distinct signal for the monovalent Ag^+ complex of assembly $\mathbf{3n}_3 \cdot \mathbf{2a}_6$ was observed at *m*/*z* 4220.0 (calcd for $\mathbf{3n}_3 \cdot \mathbf{2a}_6 \cdot Ag^+ = 4222.9$) after treatment with $AgCF_3COO$ (Table 2, entry 35). Any form of cooperativity between two cyano centers can be excluded, because the cyano centers are separated by at least 7 Å within the assembly. These results seem to suggest that $[RCN-Ag]^+$ complexes are significantly stronger than $[arene-Ag]^+$ complexes. Similarly, double-rosette assemblies $\mathbf{3a}_3 \cdot \mathbf{2i}_6$ and $\mathbf{3b}_3 \cdot \mathbf{2i}_6$ with *N*-(4-cyanophenyl)isocyanurate **2i**, form very stable Ag^+ complexes. Both assemblies showed intense signals at *m*/*z* 4348.1 (calcd for $\mathbf{3a}_3 \cdot \mathbf{2i}_6 \cdot Ag^+ = 4348.7$) and at *m*/*z* 4620.4 (calcd for $\mathbf{3b}_3 \cdot \mathbf{2i}_6 \cdot Ag^+ = 4618.7$), respectively, after treatment with $AgCF_3COO$. In the meantime, we have investigated more than 10 other examples (described elsewhere)[60] in which **2i** has been successfully used to characterize the corresponding assembly $\mathbf{3}_3 \cdot \mathbf{2i}_6$, while the corresponding assemblies with **2a** could not be detected by MALDI-TOF MS.

Crown ether-functionalized assemblies: Crown ethers are known to bind a variety of alkali metal cations in apolar solvents; this is the basis of the ion-labeling method described by Lehn.[29] In addition to this capability, crown ethers also have a reasonable affinity for a variety of other metal ions, such as Ag^+.[53] Therefore, assembly $\mathbf{3g}_3 \cdot \mathbf{2a}_6$,[61] which comprises the crown ether functionalized dimelamine **3g** in which the calix[4]arene is chemically fixed in the 1,3-alternate conformation, should be able to bind Ag^+ very strongly by means of complexation inside the crown-6 ring, assisted by interaction with the π electrons of the parallel aromatic rings of the calix[4]arene. Indeed, a sample prepared by treatment of assembly $\mathbf{3g}_3 \cdot \mathbf{2a}_6$ with $AgCF_3COO$ showed a very intense signal at *m*/*z* 4429.3 (calcd for $C_{222}H_{306}N_{48}O_{42} \cdot Ag^+$: 4427.0) in the MALDI-TOF spectrum corresponding to the monovalent Ag^+ complex (Table 2, entry 28). This result clearly shows that in addition to π-electron-based binding sites, crown ether-based binding sites can also serve as Ag^+-labeling sites.

Miscellaneous assemblies: In most of the assemblies discussed so far, cooperativity of two arene and/or alkene functionalities played a crucial role in the binding of Ag^+. An exception to this was assembly $\mathbf{3b}_3 \cdot \mathbf{2b}_6$ (Table 2, entry 18) which showed a strong signal at *m*/*z* 4516.0 (calcd for $C_{216}H_{300}N_{60}O_{42} \cdot Ag^+$: 4517.0) in the MALDI-TOF MS spectrum after treatment with $AgCF_3COO$. In this particular case, the binding of Ag^+ seems to occur by cooperative interaction of the nitro functionality in dimelamine **3b** and (most probably) the C(O)N(Ar)C(O) functionality in the isocyanurate **2b**, since none of the assemblies $\mathbf{3a}_3 \cdot \mathbf{2b}_6$ (Table 2, entry 13) or $\mathbf{3b}_3 \cdot \mathbf{2a}_6$ (Table 2, entry 17) comprising only one of the individual components has an appreciable affinity for Ag^+. This surprising result nicely illustrates that any combination of functional groups with low individual binding affinities for Ag^+ can, in principle, form stable Ag^+ complexes when acting in a cooperative manner.

Nonfunctionalized double-rosette assemblies: Calix[4]arene-based assemblies without additional Ag^+-binding functionalities, such $\mathbf{3a}_3 \cdot \mathbf{2a}_6$ and $\mathbf{3b}_3 \cdot \mathbf{2a}_6$ (Table 2, entries 12 and 17), do not give significant signals in the MALDI-TOF mass spectra after treatment with $AgCF_3COO$. This is quite remarkable because the extremely large affinity of upper-rim tetraalkylated calix[4]arenes for Ag^+ ions ($K_{ass} \approx 10^5\,M^{-1}$ in 1:1 $CDCl_3/CD_3OD$) has been described in several papers.[50] Moreover, we observed intense signals for the corresponding monovalent Ag^+ complexes of calix[4]arene **3a** and **3b** in the MALDI-TOF mass spectrum. Normally, calix[4]arenes bind Ag^+ at the upper rim by means of simultaneous interaction of two distal (1,3) aromatic rings with the soft metal ion. Apparently, the calix[4]arenes within these assemblies have totally lost their affinity for Ag^+ ions upon formation of the hydrogen-bonded assembly, probably as a result of the extreme conformational change. Atomic distances obtained from the X-ray crystal structure analysis of assembly $\mathbf{3b}_3 \cdot \mathbf{2a}_6$ support this view and show that the melamine-substituted aromatic ring carbons are 4.05 Å apart,[8a] which is 0.75 Å less than the distance measured in the X-ray crystal structure of a related $calix[4]arene-Ag^+$ complex.[50] Formation of the hydrogen-bonded assembly thus leaves too little space in between the parallel aromatic rings of **3a** for the complexation of Ag^+. Complexation of Ag^+ can still occur at the outside of assemblies $\mathbf{3a}_3 \cdot \mathbf{2a}_6$, but in this way the binding is not cooperative.

When the conformation of the calix[4]arene units in assemblies $\mathbf{3}_3 \cdot \mathbf{2a}_6$ is changed from cone to 1,3-alternate, as in **3g** (vide supra), the corresponding assemblies have the possibility to bind Ag^+ to the outside of the assembly by means of a cooperative interaction with the aromatic rings of the calix[4]arene that do not carry the melamine units. However, from the experiments with assembly $\mathbf{3g}_3 \cdot \mathbf{2a}_6$ it cannot be concluded with certainty whether the aromatic

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rings in the calix[4]arene participate in binding. ^{1}H NMR spectroscopic studies with assembly $\mathbf{3f}_3\cdot\mathbf{2a}_6$, which is present in solution as a mixture of the cyclic rosette and other (undefined) assemblies, indicate that the flexible calix[4]arene units **3f** adopt the 1,3-alternate conformation at room temperature. MALDI-TOF MS measurements of this assembly did indeed show a small but distinct signal at *m*/*z* 3745.8 (calcd for $C_{186}H_{240}N_{48}O_{30}\cdot Ag^+$: 3736.1) which corresponds to a stable monovalent Ag^+ complex. This data somehow suggests that double-rosette assemblies $\mathbf{3}_3\cdot\mathbf{2a}_6$, in which the calix[4]arene units adopt a 1,3-alternate conformation, can also be detected by means of MALDI-TOF MS after Ag^+ labeling.

Tetrarosette assemblies $\mathbf{4}_3\cdot\mathbf{2}_{12}$ (15 components, 72 hydrogen bonds)

Finally, the MALDI-TOF characterization of tetrarosette assemblies $\mathbf{4}_3\cdot\mathbf{2}_{12}$ (Figure 2D) is discussed. These assemblies are formed from three molecules of calix[4]arene tetramelamines **4** and 12 molecules of barbiturates **2** by the formation of 72 cooperative hydrogen bonds. They are thermodynamically stable at millimolar concentrations.[62]

The initial objective of the MALDI-TOF MS experiments with assemblies $\mathbf{4}_3\cdot\mathbf{2}_{12}$ was to determine if the Ag^+-labeling method has certain limits with respect to the molecular weight of the assemblies. In line with previous experiments, we used barbiturate **2f** to introduce the required Ag^+ binding sites in the assembly. After treatment with $AgCF_3COO$, the assemblies $\mathbf{4a}_3\cdot\mathbf{2f}_{12}$ and $\mathbf{4b}_3\cdot\mathbf{2f}_{12}$ gave clean MALDI-TOF spectra and only showed distinct signals for the corresponding monovalent Ag^+ complexes at *m*/*z* 8585.2 (calcd for $\mathbf{4a}_3\cdot\mathbf{2f}_{12}\cdot Ag^+ = 8584.0$) and at *m*/*z* 8525.3 (calcd for $\mathbf{4b}_3\cdot\mathbf{2f}_{12}\cdot Ag^+ = 8524.1$) (Figure 4d), respectively, (Table 3, entries 38 and 40). The observed masses are in perfect agreement with the calculated data (both 140 ppm mass difference) and clearly show that hydrogen-bonded assemblies with molecular masses in the order of 10000 Da can be conveniently characterized by means of the Ag^+ labeling technique, provided that appropriate Ag^+-binding sites are incorporated. The latter cannot be emphasized too often, because this item really determines the success of the method. A clear illustration of this was provided by the negative results obtained from experiments with assemblies $\mathbf{4a}_3\cdot\mathbf{2a}_{12}$ and $\mathbf{4b}_3\cdot\mathbf{2a}_{12}$, which apparently do not form stable Ag^+ complexes.

Table 3. MALDI-TOF MS data for fifteen-component assemblies $\mathbf{4}_3\cdot\mathbf{2}_{12}$ (72 hydrogen bonds) after treatment with 1.5–2.0 equivalents of $AgCF_3COO$ for 24 h at RT.

Entry	Assembly	Molecular Compositon	Stability in chloroform[a]	Calcd Mass [Da] of Ag^+ complex[b]	Observed mass [Da]
37	$\mathbf{4a}_3\cdot\mathbf{2a}_{12}$	$C_{420}H_{546}N_{96}O_{60}$	++	8007.5	–[c]
38	$\mathbf{4a}_3\cdot\mathbf{2f}_{12}$	$C_{468}H_{546}N_{96}O_{60}$	++	8584.0	8585.2
39	$\mathbf{4b}_3\cdot\mathbf{2a}_{12}$	$C_{414}H_{558}N_{96}O_{60}$	++	7947.5	–[c]
40	$\mathbf{4b}_3\cdot\mathbf{2f}_{12}$	$C_{462}H_{558}N_{96}O_{60}$	++	8524.1	8525.3

[a] Notation "++" means that assembly is stable at 10^{-3} M concentration, and "–" means that formation of the assembly is not observed in solution. [b] The calculated isotopic patterns are in good agreement with the experimentally observed molecular mass signals. [c] The Ag^+ complex was not observed in the MALDI TOF mass spectrum.

Although this result was expected for the latter assembly (no binding sites present), it was quite surprising for assembly $\mathbf{4a}_3\cdot\mathbf{2a}_{12}$. This assembly contains a *m*-xylyl linker which connects the two double-rosette structures. Based on the results for the benzyl-substituted assembly $\mathbf{3c}_3\cdot\mathbf{2a}_6$, from which we learned that the Ag^+ binding site is provided by a benzyl substituent in cooperation with one of the calix[4]arene aromatic rings, it was expected that the tetrarosette assembly $\mathbf{4a}_3\cdot\mathbf{2a}_{12}$ should be able to bind Ag^+ in a very similar manner. This surprising result again shows that a subtle change in the relative orientation of the functionalities that make up the Ag^+ binding site can drastically reduce the affinity for Ag^+ and is therefore the critical parameter in determining whether a particular assembly can form strong Ag^+ complexes.

Conclusions

The results in this paper show that MALDI-TOF MS in combination with Ag^+ labeling is a convenient new tool for the mass spectrometric characterization of hydrogen-bonded assemblies. The method gives excellent results both for assemblies with moderate stability (single rosettes) and for high stability (double and tetrarosettes) in the MW range between 2000 and 8000 Da. The absence of any signal which corresponds to fragmented assemblies in the spectra illustrates the unprecedented mildness of the technique. The method requires the presence of a strong binding site for the Ag^+ ion in order to charge the noncovalent assembly in a nondestructive way. We have identified a variety of different binding sites, namely arene or alkene π-donor functionalities that can bind the Ag^+ ion in a cooperative way cyano functionalities or crown-ether moieties that bind the Ag^+ ion alone. Therefore, we feel that MALDI-TOF MS in combination with Ag^+ labeling provides a method of general interest for the MS characterization of noncovalent hydrogen-bonded assemblies or host–guest complexes. Our results clearly show that the absence of a suitable binding site for the labeling ion and not the thermodynamic stability of the hydrogen-bonded assembly is most often the limiting factor for the characterization of such assemblies.

Experimental Section

General: All experiments were carried out under Ar. THF was distilled from Na/benzophenone ketyl, hexane (referring to petroleum ether fraction with b.p. 60–80 °C), CH_2Cl_2 and EtOAc from K_2CO_3. All chemicals were of reagent grade and used without further purification. NMR spectra were recorded on a Bruker AC250 (^{1}H NMR 250 MHz) or a Varian Unity 300 (^{1}H NMR 300 MHz) spectrometer in [D_1]chloroform at room temperature, unless stated otherwise. Residual solvent protons were used as internal standard and chemical shifts are given relative to tetramethylsilane (TMS). EI and FAB spectra were measured on a Finnigan MAT 90 spectrometer; the FAB spectra were obtained in a matrix of *m*-nitrobenzyl alcohol (NBA). Identification of the Ag^+ complexes of the hydrogen-bonded assemblies was performed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry[13] on a PerSeptive Biosystems Voyager-DE-RP MALDI-TOF mass spectrometer (PerSeptive Biosystems, Inc. Framingham, MA, USA) equipped with delayed extraction.[63] A UV nitrogen laser ($\lambda = 337$ nm) which

produced 3 ns pulses was used and the mass spectra were obtained both in the linear and reflectron mode. Mass assignments were performed with nonmanipulated spectra (no smoothing or centering, etc.) for an optimal correlation between observed and calculated masses. Melting points were determined with a Reichert melting point apparatus and are uncorrected. Flash chromatography was performed on silica gel (SiO_2, E. Merck, 0,040–0.063 mm, 230–240 mesh). The presence of solvents in the analytical samples was confirmed by ^{1}H NMR spectroscopy. Barbiturates **2a**, **2f**, and **2g** were purchased from Aldrich. Melamines **1b** and **1d** and isocyanurates **2b** and **2c** were prepared according to literature procedures.[63] The synthesis of isocyanurates **2d** and **2e**,[65] and dimelamines **3a–c** and **3l–o**[8] has been described previously. The synthesis of melamine **1e**, isocyanurates **2j**[58] and **2k**, and dimelamines **3d** and **3e**,[58] **3f** and **3g**,[61] **3i–k**, and **4a** and **4b** will be described elsewhere.

Sample preparation for Ag^+-labeling experiments: Samples were prepared as follows. A 5–10 mM solution of the appropriate hydrogen-bonded assembly in $CHCl_3$ was stirred for 24 h at room temperature with 1.5 equiv (per mol assembly) of solid $AgCF_3COO$ (ACROS, 98% purity). Subsequently, 10 μL of this solution was mixed with 30 μL of a solution of 2,5-dihydroxybenzoic acid (DHB; 0.5 mg L^{-1}) and/or hydroxy-benzylidene malonitrile (HBM; 0.5 mg L^{-1}) in $CHCl_3$. 1 μL of the resulting solution was loaded onto a gold-sample plate, whereupon the solvent was removed in warm air and the sample transferred to the vacuum of the mass spectrometer for analysis. Solutions containing the undiluted Ag^+ complexes can be stored (protected from light) for several days without detectable decomposition. Storage over periods of more than a week occasionally led to extensive Ag^+/H^+ exchange.

The amount of added $AgCF_3COO$ is crucial to the success of the labeling experiment. ^{1}H NMR spectroscopy experiments with assemblies $\mathbf{3}_3\cdot\mathbf{2a}_6$ have shown that the addition of more than 4.0 molar equivalents of $AgCF_3COO$ (per assembly) led to irreversible destruction of the assemblies. However, when less than 2.0 molar equivalents of $AgCF_3COO$ (per assembly) was used, the assemblies remained intact and were not significantly affected.

2-Amino-4,6-bis{4-(1,1-dimethylethyl)phenylamino}pyrimidine (1c): To a solution of 2-amino-4,6-dichloropyrimidine (1.0 g, 6.1 mmol) in ethanol (20 mL) was added 4-*tert*-butylaniline (3.9 mL, 24.3 mmol) and the resulting solution was heated at reflux for 18 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography to yield **1c** as a colorless solid (1.7 g, 70%). M.p. 290–291 °C; ^{1}H NMR (250 MHz): $\delta = 7.32$ (m, 4H; ArH), 7.17 (m, 4H; ArH), 6.42 (br s, 2H; NH), 5.62 (s, 1H; CH), 4.65 (br s, 2H; NH_2), 1.30 (s, 18H; CH_3); ^{13}C NMR (62.5 MHz, [D$_6$]DMSO): $\delta = 162.7$ (Cq), 161.6 (Cq), 143.2 (Cq), 138.6 (Cq), 125.1 (CH), 119.5 (CH), 77.2 (CH), 33.8 (Cq), 31.3 (CH_3); MS (FAB) *m*/*z*: 390 (100) $[M+H]^+$; $C_{24}H_{31}N_5$ (389.5): calcd C 74.0, H 8.0, N 18.0; found: C 73.8, H 8.2, N 17.8.

***N*-(4-Iodophenyl)-biuret (5)**: To a solution of 4-iodoaniline (0.9 g, 4.1 mmol) in DMF (20 mL) were added H_2O (10 mL) and nitrobiuret (0.6 g, 4.1 mmol). The resulting mixture was heated at 95 °C for 1 h, then a second portion of nitrobiuret (0.6 g, 4.1 mmol) was added. After heating for another 1 h, a third portion of nitrobiuret (0.6 g, 4.1 mmol) was added and the mixture was finally stirred at 95 °C for a further 1 h, and then cooled to room temperature. Addition of H_2O (15 mL) resulted in the precipitation of crude **5**, which was triturated with CH_3CN to give pure **5** (0.87 g, 70%). ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 10.1$ (s, 1H; ArNH), 8.9 (s, 1H; NH), 7.6 (d, 2H; ^2J(H,H) = 7.8 Hz, ArH), 7.3 (d, 2H; ^2J(H,H) = 7.8 Hz, ArH), 7.0–6.8 (br s, 2H; NH_2); MS (FAB) *m*/*z*: 304.9 (100) ($[M]^+$, calcd: 304.9).

1-(4-Iodophenyl)-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (2h): To a solution of **5** (0.25 g, 0.82 mmol) and carbonyldiimidazole (CDI; 0.64 mL, 3.9 mmol) in THF (8 mL) was added a solution of potassium *tert*-butoxide in THF (1 M, 5.9 mL). The resulting solution was stirred for 8 h at room temperature, then the solvent was removed under reduced pressure. The residue was partitioned between EtOAc (100 mL) and aqueous HCl (1 M, 100 mL) and the organic layer was subsequently washed with brine (2 × 100 mL), dried on $NaSO_4$, and evaporated to dryness. The resulting pale brown solid was triturated with EtOAc to give pure **2h** (0.2 g, 77%). ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 11.6$ (s, 1H; NH), 7.8 (d, 2H; ^2J(H,H) = 7.8 Hz, ArH), 7.1 (d, 2H; ^2J(H,H) = 7.8 Hz, ArH); MS (FAB): *m*/*z*: 331.7 (100) ($[M+H]^+$, calcd 330.9).

1-(4-Cyanophenyl)-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (2i): To a heated solution (150–155 °C) of CuCN (0.3 mL, 3.4 mmol) in DMF (20 mL) was added a solution of **2h** (0.56 g, 1.7 mmol) in DMF (15 mL). The resulting solution was heated for another 5 h at 150 °C, and then the solvent was removed under reduced pressure. The residue was partitioned between EtOAc (100 mL) and aqueous $FeCl_3$ solution (50 mL). The organic layer was successively washed with aqueous HCl (1 M, 100 mL), $NaHSO_4$ (2 × 100 mL), and brine (2 × 100 mL), and then dried on Na_2SO_4. Removal of the solvent gave a pale brown solid, which was triturated with EtOAc to give pure **2i** (0.2 g, 51%). ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 11.7$ (s, 1H; NH), 8.0 (d, 2H; ^2J(H,H) = 7.8 Hz, ArH), 7.5 (d, 2H; ^2J(H,H) = 7.8 Hz, ArH); ^{13}C NMR (62.5 MHz, [D$_6$]DMSO): $\delta = 149.3$, 148.4 (C=O), 138.6 (Cq), 132.8, 130.5 (CH), 117.7 (CN), 111.2 (Cq); MS (FAB): *m*/*z* 229.9 (100) $[M+H]^+$; $C_{10}H_6N_4O_3$ (230.2): calcd:C 52.18, N 24.34, H 2.63; found: C 52.15, N 24.36, H 2.62.

5,17-*N*,*N*′-Bis(4-amino-6-(2-propenylamino)-1,3,5-triazin-2-yl)-diamino-25,26,27,28-tetrapropoxycalix[4]arene (3h): A solution of bis(chlorotriazine) **3o** (150 mg, 0.17 mmol) and allylamine (10 mL) was refluxed for 15 h. Addition of H_2O (10 mL) gave dimelamine **3h** as a white precipitate. Recrystallization from $CHCl_3$ afforded pure **3h** in 89% yield. ^{1}H NMR (300 MHz): $\delta = 7.1–6.0$ (m, 14H; ArH + NH), 5.9–5.8 (m, 2H; CH), 5.1–4.7 (m, 8H; $CH_2 + NH_2$), 4.42 and 3.10 (ABq, ^2J(H,H) = 13.3 Hz, 8H; CH_2), 4.0–3.9 (m, 8H; CH_2), 3.8–3.6 (t, ^3J(H,H) = 8.0 Hz, 4H; CH_2), 2.0–1.8 (m, 8H; CH_2), 1.06 (t, ^3J(H,H) = 7.5 Hz, 6H; CH_3), 0.90 (t, ^3J(H,H) = 7.3 Hz, 6H; CH_3); MS (FAB): *m*/*z*: 921.4 (100) $[M+H]^+$; $C_{52}H_{64}N_{12}O_4$ (920.5) + 0.86 $CHCl_3$: calcd:C 62.01, N 16.39, H 6.29; found: C 62.10, N 16.19, H 6.29.

Preparation of assemblies $\mathbf{1}_3\cdot\mathbf{2}_3$: The assemblies were prepared by dissolving the melamine and isocyanurate components in THF, followed by the removal of this solvent under reduced pressure and drying under high-vacuum. Subsequently, the resulting solid was redissolved in an appropriate solvent.

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