J|A|C|S COMMUNICATIONS

Published on Web 03/31/2009

Probing Cucurbituril Assemblies in Water with TEMPO-like Nitroxides: A Trinitroxide Supraradical with Spin−Spin Interactions

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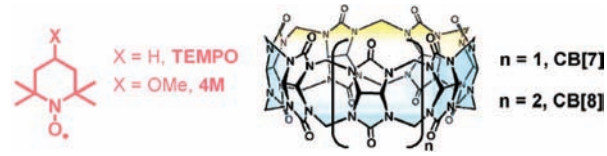
Cucurbit[*n*]urils (CB[*n*]) are a family of synthetic macrocycles with a pumpkin shape and a constricted cavity delineated by two identical carbonyl fringe portals.[1] The unique combination of the hydrophobic rigid cavity lying between the two oxygen-atom-rich rims makes this a very attractive situation for guest binding in water and the construction of advanced architectures as compared with cyclodextrins (CDs) or calixarenes. Studies of CB[*n*] expanded dramatically after the year 2000, when Kim and co-workers[2] reported the synthesis of the main members of the cucurbituril family (CB[5] to CB[8]). Since then, Kim's group,[3] the Isaacs group,[4] and others[5−7] have reported regularly on the synthesis of various analogues, thus expanding the scope of their molecular and supramolecular chemistry. However, their interactions in water remain poorly understood, and their limited water solubility is still an important drawback that restricts the extent of their potential use. Interestingly, at millimolar concentrations, several studies suggested the formation of aggregates,[8] the nature of which was not established. In two independent papers, Kim and co-workers showed that intermolecular CH···O interactions were responsible for (i) self-assembly of CB[7] in acidic solutions, leading to water gelation,[9] and (ii) formation of crystals of CB[6] in which the molecules self-associate by means of weak hydrogen bonds, rendering the crystal extremely stable.[10] In 2007, Lucarini and co-workers[11] used nitroxides as probes to investigate the binding properties of CB[7] in water. Here we report for the first time the X-ray crystal structure of a complex formed between a nitroxide (2,2,6,6-tetramethyl-4-methoxypiperidin-1-oxyl, **4M**) and CB[8] (Scheme 1). We show that the combination of nitroxide radicals and EPR spectroscopy can be used to directly observe the concentration-dependent aggregation of CB[7] and CB[8] in water. We also demonstrate that 2,2,6,6-tetramethylpiperidin-1-oxyl (**TEMPO**) included into CB[7] is very efficiently protected against reduction from ascorbate, a major in vivo reducing agent of nitroxides.

Orange prismatic crystals suitable for X-ray crystallography were grown from a solution of **4M** and CB[8] in water over a period of one month.[12] All of the other combinations of **TEMPO** or **4M** with CB[7] or CB[8] failed to produce any crystals. The asymmetric unit of **4M**@CB[8] contains six cucurbiturils, and the nitroxide inside each appeared to be disordered (Figure 1a). The host−guest couples are arranged in supramolecular equilateral triangles (Figure 1b) to form layers with water molecules in between. In each triangle, the distances between the oxygen atoms of the three nitroxides are in the range 8.4−9.0 Å, with an average value of 8.7 Å; this is expected to lead to three almost equivalent spin−spin interactions with large J values. In addition to the preexisting C−H···O interactions responsible for the cucurbituril assemblies, the aminoxyl (nitroxide) functions are involved in hydrogen bonds with neighboring CB[8] methine protons, which probably contribute to the triangular arrangement of the host−guest couples.[10] Additionally, the presence of the methoxy group of **4M** protruding from one rim of the cavity and pointing outside the CB[8] triangles is another important factor in the stabilization of this architecture. The TGA trace [Figure S1 in the Supporting Information (SI)] is in full agreement with the composition given by the single-crystal X-ray experiment. In the **4M**@CB[8] structure, the nitroxide is oriented parallel to the symmetry axis of CB[8]; this differs (by means of a 90° rotation) from the proposed **TEMPO**@CB[7] structure reported previously,[11] presumably as a result of the substitution of the methoxy in position 4 of the nitroxide and the larger macrocycle.

Scheme 1. **TEMPO**-Type Nitroxides Used to Study Cucurbit[*n*]uril (n = 7, 8) Assemblies in Water



When **4M**@CB[8] crystals were dissolved in pure water, we observed an X-band EPR signal (Figure S9 in the SI) composed of the superposition of the expected three-line spectrum from free **4M** and a seven-line spectrum. This additional spectrum exhibited a 1:3:6:7:6:3:1 seven-line pattern with a hyperfine coupling constant (a_N) of 5.13 G, corresponding to one-third of the value observed

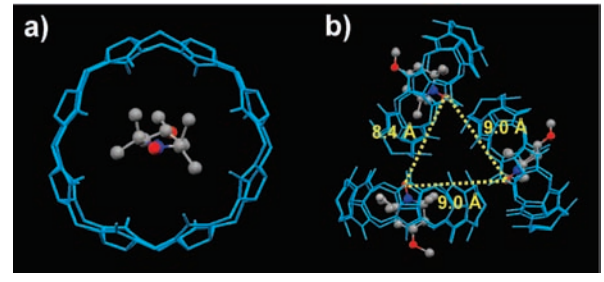


Figure 1. (a) X-ray structure of the **4M**@CB[8] inclusion complex. (b) Supramolecular triangle showing a three-spin-system interaction in the solid state (only one orientation of the guest inside the cavity is displayed, and hydrogen atoms have been omitted for clarity).

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10.1021/ja900306m CCC: $40.75

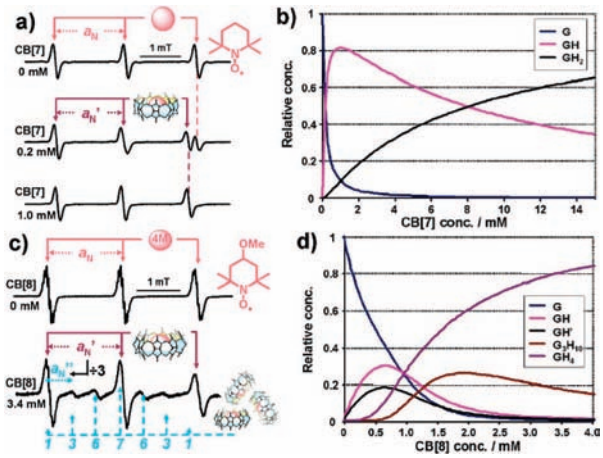

Figure 2. (a) EPR X-band spectra of a water solution of **TEMPO** (0.1 mM) in the presence of added amounts of CB[7]. (b) Concentration distribution of the guest (G) and guest−host$_n$ (GH$_n$) species present in solution for the **TEMPO**/CB[7] titration. (c) Influence of added amounts of CB[8] on the EPR spectrum of a water solution of **4M** (0.1 mM). (d) Concentration distribution of the different species for the **4M**/CB[8] titration.

for an included **TEMPO**-like nitroxide ($a_N \approx 15.5$ G). This spectrum is characteristic of a system composed of three identical nitroxide moieties coupled through spin exchange with J_{12}, J_{23}, $J_{13} \gg a_N$,[13] in agreement with the "trinitroxide" supraradical {**4M**@CB[8]}$_3$ shown in Figure 1. Even though the EPR spectrum pattern and the apparent coupling value $a_N' \approx a_N/3$ indicate that $J_{ij} \gg a_N$, the exchange couplings are expected to be lower than those reported for typical nitroxide diradicals and triradicals.[14] The intensity of the seven-line spectrum decreased with decreasing concentration of **4M**@CB[8]. These results indicate that the three **4M**@CB[8] units are still tightly associated in water within the triangular arrangement.

The paramagnetic signature of **TEMPO**-type nitroxides was investigated by EPR spectroscopy as a function of CB[7] and CB[8] concentration in water at pH 7. Alkaline hydroxide solutions (0.9−1.0 mM final concentrations) were used to adjust the solutions to neutral pH. The slow-exchange regime was observed by EPR with solutions of **TEMPO** and CB[7] in water. Additional signals due to the included guest were easily observed (Figure 2a).

The nitroxide was all-included at CB[7] concentrations above 1 mM, whereas further line broadening indicated the presence of larger complexes at higher concentrations (see the SI). As expected, the hyperfine coupling constant a_N, which is sensitive to the environment, was reduced for included nitroxides as a consequence of the hydrophobic cavity. Simulations of the EPR spectra using a 2D program[15] (with the magnetic field and CB[n] concentration as variable parameters) supported the presence of several species in equilibrium (see Table 1, the product distributions in Figure 2b,d, and the SI). EPR titrations were in line with 1:1 **TEMPO**/CB[7] complexation at low CB[7] concentrations and allowed quantitation of the nitroxide affinity to the macrocycle. A relatively large binding constant of 9750 M^{-1} was found, with the underlying interactions being almost exclusively hydrophobic.[11] At higher concentrations, additional guest−host$_n$ (GH$_n$) species had to be taken into account, and the best fit between the calculated and experimental spectra was obtained by also considering GH$_2$. The CB[7] dimer including the probe molecule was therefore found to be the major species at high CB[7] concentration (above 8 mM), whereas the cation/**TEMPO**/CB[7] ternary complex[11] was not detected, likely because of the tilted conformation of **TEMPO** in the cavity.

Table 1. EPR Parameters, Stability Constants of all **TEMPO**/CB[7] and **4M**/CB[8] Species in Equilibrium, and Half-Lives ($t_{1/2}$) of the Nitroxides in the Presence of 2 mM Sodium Ascorbate ([CB[7]] = 12.75 mM, pH Adjusted with NaOH, and [CB[8]] = 3.4 mM, pH Adjusted with LiOH)

TEMPO	G	GH	GH$_2$
g	2.00675	2.00726	2.00704
a_N (G)	17.29	16.04	16.36
log β	−	3.989	6.095
$t_{1/2}$ (min)	4	254 (apparent)	

4M	G	GH	GH′	GH$_4$	G$_3$H$_{10}$
g	2.00652	2.00628	2.00646	2.00641	2.00695
a_N (G)	17.04	16.87	17.04	17.03	5.13
log β	−	3.113	2.899	12.416	39.272
$t_{1/2}$ (min)	3	20 (apparent)			

CB[8] also showed interesting aggregation features when **4M** was used as a probe. At high CB[8] concentrations, broader additional lines appeared between the three lines expected for the free **4M** and GH$_n$ complexes (Figure 2c). Calculations showed that these lines belong to the seven-line spectrum corresponding to the trinitroxide supraradical already mentioned, which appears at high CB[8] concentrations. At low concentrations, a binding constant of 1297 M^{-1} was found for the formation of the 1:1 complex, and the presence of a ternary complex (GH′) having a smaller binding constant of 793 M^{-1} was clearly demonstrated from 2D EPR simulations. The axially aligned aminoxyl function of GH′ is available for interaction with an alkali cation coordinated at the carbonyl rim, increasing the a_N value with respect to that for the GH complex (Figures 1 and 2d).[11] This is in agreement with experiments performed using higher alkali salt concentrations. When the CB[8] concentration was increased, the best fit was obtained assuming the presence of larger aggregates of the GH$_4$ and G$_3$H$_{10}$ types. At concentrations above 8 and 1 mM respectively, aggregates of CB[7] and CB[8] become the major preponderant species in solution, as monitored by EPR (Figure 2b,d). The nontemplated CB[7] and templated CB[8] aggregation phenomena in solution are directly observed by EPR studies.

In the past decade, there has been an upsurge in interest in applications of nitroxides in biological systems, for instance as contrast agents for NMR[16] and EPR[17] imaging or as probes for redox[18] or pH status.[19] Unfortunately, nitroxides are readily reduced to diamagnetic hydroxylamines in biological samples, hampering further developments for such applications. Increasing the steric hindrance around the nitroxide function has been shown to be an interesting approach for slowing the kinetics of reduction, but this approach still needs further development for biological applications.[20] On the other hand, Rassat and co-workers[21] showed that supramolecular encapsulation of nitroxides by cyclodextrins could be an interesting strategy for improving their resistance to ascorbate reduction. However, a covalent system appeared more promising because of the weak binding capacity of cyclodextrins toward **TEMPO**-like nitroxides (K_a for β-CD/**TEMPO** = 607 M^{-1}). In this vein, Lucarini and co-workers[22] have recently synthesized a [1]rotaxane-type nitroxide-appended β-cyclodextrin with improved resistance to glutathione reduction. The appealing high stability of the **TEMPO**@CB[7] complex prompted us to investigate the effect of CB[7] on the nitroxide reduction process by sodium ascorbate (Figure 3).

A strikingly high resistance was observed, with a half-life ($t_{1/2}$) of ∼254 min (i.e., a 60-fold increase), in the presence of 2 mM ascorbate anion with CB[7], in agreement with the slow kinetics of decomplexation. For comparison, using β-CD as the host led to

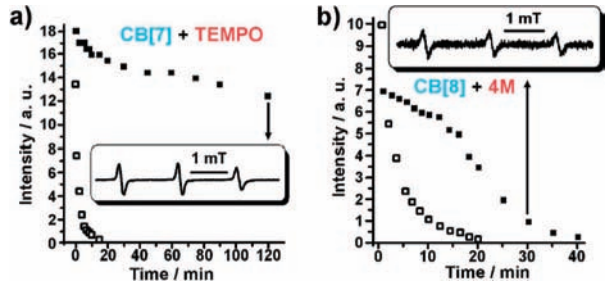

Figure 3. Supramolecular protection of the **TEMPO** and **4M** nitroxides inside CB[7] and CB[8], respectively, against reduction by sodium ascorbate in water. (a) Concentration of **TEMPO** (+12.75 mM CB[7]) as a function of time after addition of 20 equiv of sodium ascorbate (2 mM), as deduced from the intensity of the first line in the EPR spectrum. Inset: EPR spectrum recorded 2 h after the addition of the reductant. (b) Concentration of **4M** (+3.8 mM CB[8]) as a function of time after addition of 20 equiv of sodium ascorbate (2 mM). The □ data were recorded in the absence of CB[*n*].

an increase by a factor of less than 2. CB[8] also showed significant activity toward **4M** protection, albeit to a lesser extent, presumably because of a weaker binding constant and the axial orientation of the nitroxide inside the cavity.

In summary, we have shown that the EPR study of small paramagnetic probes interacting inside CB[*n*] cavities is a powerful tool for directly observing cucurbituril aggregates in aqueous solutions. EPR spectroscopy also provides critical and unique information regarding the guest orientation within the CB[*n*] cavity and possible interactions with coordinated cations. The macrocycle concentrations are observed to be of prime importance in CB[*n*] self-assemblies. Moreover, we have shown that nitroxide encapsulation by CB[*n*] could widen the use of nitroxides at biologically relevant concentrations for in vivo applications. We envision that this result could benefit other research disciplines, such as NMR and EPR imaging, particularly for applications that need long in vivo lifetimes of nitroxides.

Acknowledgment. Région PACA, Radical Vision, CNRS, Université de Provence, and the National Research Council of Canada are acknowledged for financial support.

Supporting Information Available: Crystallographic data (CIF) and TGA and DSC traces for crystals of the **4M**@CB[8] inclusion compound and additional EPR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA900306M