Supramolecular and Biomacromolecular Enhancement of Metal-Free Magnetic Resonance Imaging Contrast Agents

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Many contrast agents for magnetic resonance imaging are based on gadolinium, however side effects limit their use in some patients. Organic radical contrast agents (ORCAs) are potential alternatives, but are reduced rapidly in physiological conditions and have low relaxivities as single molecule contrast agents. Herein, we use a supramolecular strategy where cucurbit[8]uril binds with nanomolar affinities to ORCAs and protects them against biological reductants to create a stable radical in vivo. We further overcame the weak contrast by conjugating this complex on the surface of a self-assembled biomacromolecule derived from the tobacco mosaic virus.

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Contrast thanks to cucurbituril

The contrast agent died :(

2 h
Supramolecular and Biomacromolecular Enhancement of Metal-Free Magnetic Resonance Imaging Contrast Agents

Hamilton Lee,1 Jenica L. Lumata,2 Michael A. Luzuriaga,3 Candace E. Benjamin,2 Olivia R. Brohlin,3 Christopher R. Parish,2 Steven O. Nielsen,2 Lloyd L. Lumata2 and Jeremiah J. Gassensmith2,4 *

Many contrast agents for magnetic resonance imaging are based on gadolinium, however side effects limit their use in some patients. Organic radical contrast agents (ORCAs) are potential alternatives, but are reduced rapidly in physiological conditions and have low relaxivities as single molecule contrast agents. Herein, we use a supramolecular strategy where cucurbit[8]uril binds with nanomolar affinities to ORCAs and protects them against biological reductants to create a stable radical in vivo. We further over came the weak contrast by conjugating this complex on the surface of a self-assembled biomacromolecule derived from the tobacco mosaic virus.

Of the many spatially resolved biomedical imaging techniques available, magnetic resonance imaging (MRI) is of particular importance in modern medicine due to its non-invasive nature, potential for high spatial resolution, tissue penetration, and lack of ionizing radiation.1 MRI relies on detecting the energy released over time by water protons returning to magnetic equilibrium after a radio frequency pulse has been applied. This relaxation rate—or relaxivity—is highly dependent on the chemical environment of the water. Since water intrinsically possesses low sensitivity to magnetic fields, contrast agents are used to increase the contrast between different features in the final image2,4 with the majority of modern MRI contrast agents based on Gd4 complexes.5,7 Although Gd has performed8-10 remarkably in clinical settings, concerns about its toxicity11-15 especially for patients with impaired renal functionality, have catalyzed efforts to design alternatives to Gd and other metal-based MRI contrast agents. Several types of metal-free contrast agents have been investigated16-23 with organic radical contrast agents (ORCAs) based on paramagnetic aminoxyl moieties showing significant promise. Aminoxyl ORCAs are distinguished by their compatibility with existing MRI techniques, enabling facile implementation in current clinical settings, and have low cytotoxicity and high biodegradability, reducing the potential for side effects.24, 25 However, two major issues prevent aminoxyl-based ORCAs from replacing traditional contrast agents based on Gd: (i) their single unpaired electron provides weaker contrast compared to the seven unpaired electrons of Gd and (ii) they are reduced rapidly to MRI-silent hydroxyamines in physiological conditions by compounds including ascorbate, saccharides, and cysteine-rich proteins.24

Contrast issues in ORCAs have improved greatly in recent years by attaching them to polymeric or biomacromolecular nanoparticle systems26-42 that both create high local concentrations of aminoxyl moieties and decrease the diffusional and rotational motion of the attached ORCAs. Because rotational correlation time is inversely proportional to relaxivity, attaching contrast agents onto large macromolecules will—in general—improve their performance. On the other hand, sensitivity to reduction in vivo has been more difficult to address as the reduction-oxidation (REDOX) potentials of aminoxyl radicals are such that they are quickly reduced in the high physiological concentrations of ascorbate. Strategies to overcome this have primarily focused on mitigating reduction by installing sterically hindered moieties around the aminoxyl radical and incorporating aminoxyl-containing molecules into macromolecular systems that shield the radical.43 Problematically, creating more steric bulk may also preclude water from interacting with the free electron, which is detrimental to good contrast; consequently, synthetic strategies to prevent reductants from reacting with the aminoxyl radical must conceptually titrate good shielding of the relatively large reductants while not greatly inhibiting access of water.

In this work, we utilize a supramolecular strategy to overcome ORCAs’ poor relaxivity and high sensitivity toward reduction by fabricating a viral nanorod-based ORCA inclusion complex—specifically a pseudorotaxane—wherein the macrocycle cucurbit[8]uril (CB[8]) binds with nanomolar affinities to TEMPO moieties that have been conjugated onto the exterior surface of an anisotropic virus-like particle (VLP). We also show that this architecture is effective at shielding the radicals from reduction by ascorbate while still allowing the exchange of water and providing high contrast in vivo. These results indicate that shielding of aminoxyl radicals with macrocycles is a promising strategy in the pursuit of a persistent ORCA viable for clinical applications.

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To achieve maximum contrast, many types of nanomaterials have been investigated for their potential as platforms for aminoxyl-based MRI contrast agents. Relative to silica, synthetic polymer, or metallic nanoparticles, VLPs offer the advantages of monodispersity, high functionalizability, and high biodegradability. VLPs are self-assembled macromolecular structures composed of tens to thousands of individual protein subunits. The particular VLP utilized in this study is the tobacco mosaic virus (TMV), a 300 × 18 nm rod-shaped plant virus with a central 4 nm pore along its axis of symmetry. The ease of synthetic modification and its resilience under a wide range of temperatures, solvents, and pH values has allowed TMV to function as a versatile platform in VLP technology for applications involving biomedicine and stimulus-responsive materials. Each virus is composed of 2130 identical self-assembled 17.5 kDa coat proteins (Scheme 1) of which, tyrosine 139 (Y139), located on the outer surface of the rod is solvent exposed and available for functionalization. To this exposed residue, we planned to conjugate a derivative of the aminoxyl radical TEMPO, compound 6, featuring an ammonium for enhanced binding of CB[8].

Various derivatives of TEMPO have been shown to bind CB[8] and we predicted that the amine in 6 would offer enhanced binding through an extra ion-dipole interaction with the oxygens in the crown of the macrocycle. Molecular dynamics equilibrium and free energy simulations of the CB[8]·6 inclusion complex establish the stability and precise location of TEMPO within the CB[8] cavity and quantify the accessibility to solvent water molecules of the TEMPO oxygen radical (see S1). Through equilibrium, adaptive biasing force, and umbrella sampling simulations we computed the free energy to reversibly remove TEMPO from the CB[8] cavity (see Fig. S13). From these data the equilibrium position of the TEMPO ring is 0.85 Å above the plane of the CB[8] ring and centered within it. From equilibrium simulations we observe that water hydrogen atoms are found preferentially a distance of 2 Å from the TEMPO oxygen radical (see Fig. S14). On average, one water hydrogen atom is found within 2.6 Å of the TEMPO oxygen radical. A representative snapshot of the CB[8] and TEMPO molecules along with the water molecule containing this hydrogen atom is shown in Scheme 1D and Figure S15. Since this water molecule is surrounded by other solvent water molecules, the water exchange needed to generate the MRI contrast is readily apparent. Prior to the conjugation of 6 to TMV, the binding of 6 to CB[8] was probed via isothermal titration calorimetry (ITC) (Figure S9). The $K_d$ value for the CB[8]·6 complex was determined to be $1.5 \times 10^{-8}$ M—this nanomolar affinity between 6 and CB[8] is on par with many antibody-substrate binding affinities. This suggests CB[8]·6 should remain associated under concentrations and time scales relevant for MRI contrast agents in murine models.

![Scheme 1](image)

**Scheme 1.** (A) Structure of TMV highlighting the solvent exposed amino acid residues of a single coat protein. (B) Installation of alkyne functionality on Y139 via diazonium coupling followed by the conjugation of 6 via CuAAC. (C) Structural formula of CB[8] (D) CB[8]·6 inclusion complex showing the TEMPO oxygen is accessible by water (green) but still embedded within the macrocycle and protected from reduction.

![Figure 1](image)

**Figure 1.** Characterization of TMV after bioconjugation reactions. (A) TEM image of TMV-6. (B) Bioconjugation of the TEMPO radical to TMV was confirmed via ESI-MS. The peak at 17534 m/z corresponds to native TMV. The peak at 17662 m/z corresponds to TMV-Aky. The peak at 17958 m/z corresponds to TMV-Aky. The spectrum corresponding to TMV-6 confirms complete conversion of TMV-Aky to TMV-6. (C) The integrity of the TMV rods after modification was confirmed by SEC. The single peak in the chromatograms (at 260 nm) of the modified TMV samples demonstrates that the bioconjugation reactions did not compromise the morphology of the TMV rods. (D) X-band EPR spectra of 6 and TMV-6. The samples were prepared in capillary tubes to minimize interactions between high dielectric aqueous solvent and the electric field of the incident microwave radiation.

Compound 6 was attached to TMV by first conjugating an alkyne handle to Y139 (Scheme 1) via a diazonium coupling reaction to produce TMV-Aky. Following this, a copper-
catalyzed azide-alkyne cycloaddition (CuAAC) between TMV-Aky and 6 produced TMV-6. As seen in Figure 1B, ESI-MS confirmed quantitative conversions of the TMV coat proteins while TEM (Figure 1A) and SEC (Figure 1C) show that the size and morphology of the TMV rods were unaltered following bioconjugation. Finally, the EPR spectrum of the TMV-6 conjugate (Figure 1D) shows a characteristic triplet centered at a \( g \)-value of 2.007 corresponding to \( ^{14}\text{N} \) (\( I = 1; A \approx 45 \text{ MHz} \)). The spectrum of 6 contains sharp peaks and isotropic \( g / A \) values that are characteristic of the rotational averaging found in small molecules. The spectrum of TMV-6 contains broad peaks, anisotropic \( g / A \) values, and a lower S/N ratio relative to the spectrum of 6. These differences between the spectra are attributed to the reduction of rotational and translational mobility upon attachment of 6 to the TMV rod. The high density of aminoxyl radicals on the surface of TMV can also allow for dipole spin exchange, which also results in peak broadening.

Figure 2. Plots of (A) \( 1/t_1 \) (s\(^{-1}\)) and (B) \( 1/t_2 \) (s\(^{-1}\)) versus [TEMPO] (mM) for TMV-6 and compound 6 in the absence and presence of CB[8] at 43 MHz in KP buffer (0.1 M, pH 7.4) \( @ \ 310 \text{ K} \).

Since the properties of small molecules can change upon conjugation to proteins, we investigated the binding of CB[8] to TMV-6 following the confirmation of the TMV-6 conjugate. Since the solutions of TMV-6 at concentrations required for ITC characterization were subject to viscosity and adhesion issues, a fluorescence titration was used instead to determine a \( K_d \) value for the CB[8]–TMV-6 complex was determined to be 3.8 \( \pm 0.5 \times 10^{-7} \) M. The reasons for the modest decrease in binding affinity of the CB[8]–TMV-6 complex relative to the CB[8]–6 complex is not yet fully known, but the binding is still high enough to be relevant for the purposes of the ORCA design. Taken as a whole, the fluorescence titration experiments suggest that CB[8] molecules can bind to the TEMPO moieties conjugated onto the exterior surface of TMV to form a pseudorotaxane.

After establishing that CB[8] binds strongly to TMV-6, the relaxation behavior of the TEMPO moieties was evaluated to determine the suitability of the pseudorotaxane as an ORCA. Relaxation behavior is dependent on several factors, with magnetic field strength and solvent exchange being major examples. While maintaining a constant magnetic field strength of 1 T, longitudinal (\( T_1 \)) and transverse (\( T_2 \)) relaxation values were obtained for varying concentrations of TMV-6 in the presence and absence of CB[8] (Figure 2). Relaxivity values (\( r_1 \) and \( r_2 \)) were derived from a linear fit of the inverse relaxation data. When compared to the small molecule TEMPO control, the TMV-6 conjugate exhibits a five-fold increase in \( r_1 \) going from a modest 0.6 mM\(^{-1}\) to 2.9 mM\(^{-1}\) — rivaling that of clinically available Gd(DOTA) (Table S2). For the supramolecular systems, the small molecule complex CB[8]–6 has very low \( r_1 \) values, which are similar to that of bulk water\(^{66} \) (0.2 \( \pm 0.1 \) mM\(^{-1}\)); however, conjugating CB[8]–6 to TMV yields an order of magnitude enhancement in relaxivity (to \( 1.9 \pm 0.1 \) mM\(^{-1}\)). These enhancements from conjugation to TMV are readily explained by the limited molecular motion provided by the attachment of TEMPO moieties to TMV. The modest difference in \( r_1 \) values between TMV-6 and CB[8]–TMV-6 likely arises from of restriction of exchange of bulk water to the TEMPO radical by the CB[8]. Likewise, \( r_2 \) values were enhanced by more than an order of magnitude from conjugation of CB[8]–6 to TMV (Table S2). Curiously, the CB[8] had a much more pronounced attenuating effect on \( r_2 \) relative to \( r_1 \). Nevertheless, the transverse and longitudinal relaxivities obtained by our experiments are comparable to clinically available Gd(DOTA), demonstrating that our pseudorotaxane provides the contrast required to function in vivo.

Having characterized the NMR relaxation properties of the pseudorotaxane and establishing its ability to function as an ORCA, we sought to investigate the shielding performance of the pseudorotaxane architecture. The rapid reduction of aminoxyl radicals to hydroxylamines in the presence of physiologically relevant reducing agents is well known. Ascorbate is one example of these reducing agents and is commonly utilized for reduction experiments owing to its ubiquity in the human body and its extensively studied redox properties. Upon the addition of a sodium ascorbate (10 equivalents per TEMPO moiety) solution in KP buffer (0.1 M, pH 7.4) to solutions of TMV-6 in the absence and presence of CB[8] (10 equivalents per TEMPO moiety), EPR spectra of the TMV conjugates were collected over 2 h (Figure 3). EPR intensities were fitted under pseudo-first-order conditions. The pseudo-first-order rate constant for the reduction of TEMPO, \( k' \), was determined to be 1.6 \( \pm 0.1 \times 10^{-3} \text{s}^{-1} \) (\( t_{1/2} = 7.2 \text{ min} \)) for TMV-6 in the absence of CB[8] and 2.0 \( \pm 0.1 \times 10^{-3} \text{s}^{-1} \) (\( t_{1/2} = 577.6 \text{ min} \)) for TMV-6 in the presence of CB[8]. To the best of our knowledge, this two-order of magnitude rate reduction in the pseudorotaxane is far lower than that of any reported aminoxyl-based ORCAs thus far (Table S1) by at least 20-fold. The
substantial decrease in the reduction rate of TEMPO suggests strongly that the CB[8] in the pseudorotaxane architecture effectively shields the aminoxyl radical from ascorbate. We were able to fully demonstrate the high contrast enhancement and stability of ORCA in vivo as shown in Figure 3C. We injected equal concentrations of CB[8] ↔ TMV-6 (Figure 3 green circle) and TMV-6 (orange circle) into the thigh of a Balb/C mouse. Both probes initially showed bright contrast in the muscle; however, TMV-6 began to lose contrast quickly, with most signal gone after 2 h, in agreement with our EPR results.

In conclusion, we have demonstrated the enhancement of the survivability of aminoxyl radicals in the presence of ascorbate via shielding with CB[8]. The attachment of TEMPO derivatives onto the exterior surface of TMV results in a conjugate that provides enhanced T1 and T2 relaxation properties relative to small molecule aminoxyl radicals. The addition of CB[8] to the TMV conjugate forms a pseudorotaxane ORCA where the TEMPO moieties are encaged by CB[8]. The CB[8] can sterically shield the aminoxyl radical from reduction by ascorbate while still allowing for the exchange of water. Although the CB[8] also reduces the contrast strength of the ORCA compared to its unshielded form, the contrast strength is still higher than that of small molecule aminoxyl radicals and close to that of Gd(DOTA) (Table S2). Most importantly, the ORCA can survive in the presence of ascorbate for periods of time that exceed—to the best of our knowledge (table S1)—currently known aminoxyl-based ORCAs by over two orders of magnitude.40-42,67,68 Our results indicate that with the combined approach of (i) utilizing macromolecules for steric shielding to inhibit reduction in vivo along with (ii) enhancing T1 relaxivity by conjugating these supramolecular agents to biomacromolecular scaffolds, ORCAs are moving closer to clinically viable contrast agents.

Figure 3. EPR spectra for the reduction of TMV-6 (2.6 mg/mL) with sodium ascorbate (10 equivalents per TEMPO moiety) in (A) absence and (B) presence of CB[8] (10 equivalents per TEMPO moiety). Data were collected at 10 min intervals over 2 h. (C) T1 weighted images of CB[8] ↔ TMV-6 (green circle) vs TMV-6 (orange circle) injected into the thigh muscle of a mouse.

Conflicts of interest
There are no conflicts to declare.

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Supramolecular and Biomacromolecular Enhancement of Metal-Free Magnetic Resonance Imaging Contrast Agents

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Materials
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), ThermoFisher Scientific (Pittsburgh, PA), Alfa Aesar (Ward Hill, MA), TCI America (Portland, OR), VWR International (Radnor, PA), and used without further purification.

Instrumentation
$^1$H and $^{13}$C NMR spectra were obtained using a 600 MHz Bruker Avance NMR spectrometer with residual solvent peaks as a reference for all NMR spectra. Thiophenol (10-20 eq) were added to samples containing aminoxyl radicals to reduce them to hydroxylamines so that the compound could be observed via NMR. ESI-MS were acquired using a Agilent 1100 HPLC with a PLRP-S column for separation and an ABSciex 4000 QTRAP system for detection. Size exclusion chromatography (SEC) was conducted using an Agilent 1100 HPLC with a Phenomenex PolySep-GFC-P Linear 300 × 7.8 mm column. Isothermal titration calorimetry (ITC) was conducted using a Malvern Microcal iTC200. Transmission electron microscopy (TEM) was conducted using a JEOL JEM-1400Plus transmission electron microscope. Fluorescence data were obtained using a BioTek Synergy H4 Hybrid microplate reader. EPR spectroscopy was performed using a Bruker EMX ER041XG X-band spectrometer with a Bruker ER 4119HS resonator. NMR relaxometry experiments were performed with a 43 MHz Magritek Spinsolve NMR spectrometer operating with a magnetic field strength of 1 T.
Synthesis

Scheme S1. Synthesis of 6.

Synthesis of Imidazole-1-sulfonyl azide hydrogen sulfate

NaN₃ (10 g, 154 mmol) was stirred in dry EtOAc (150 mL) at 0 °C, 0.5 h. Sulfuryl chloride (20.7 g, 12.4 mL, 153 mmol) was added dropwise while stirring was maintained. The resulting mixture was stirred at RT, 24 h. The reaction mixture was cooled to 0 °C in an ice bath. Imidazole (20 g, 294 mmol) was slowly added over 5 min. The resulting mixture was stirred at 0 °C, 5 h. A saturated NaHCO₃ solution (aq.) (300 mL) was added to the reaction mixture. The organic fraction was isolated, washed with H₂O (3×100 mL) and dried with MgSO₄. The organic fraction was filtered and the filtrate was collected. The resulting solution was cooled to 0 °C in an ice bath while stirring was maintained. H₂SO₄ (18 M, 8.4 mL, 151 mmol) was added dropwise over 5 min while vigorous stirring was maintained. The resulting solution was stirred vigorously at RT until colorless or white precipitate was formed. The reaction mixture was filtered and the feed was washed with EtOAc (0 °C). The feed was collected and solvent was removed under reduced pressure to yield the product as a white solid. Yield: 35.3 g, 85 %. ¹H NMR (600 MHz, DMSO-d₆) δ ppm 7.67 (s, 1 H), 8.01 (s, 1 H), 9.06 (s, 1 H), 10.43 (s, 1 H), 14.28 (s, 1 H). ¹³C NMR (150 MHz, DMSO-d₆) δ ppm 129.8, 134.4, 137.9.
Figure S1. $^1$H NMR spectrum of imidazole-1-sulfonyl azide hydrogen sulfate. Asterisks indicate decomposition products.

Figure S2. $^{13}$C NMR spectrum of imidazole-1-sulfonyl azide hydrogen sulfate.
Synthesis of 6-Azidohexanoic acid (2)

Compound 1 (5.0 g, 25.6 mmol), and NaN₃ (4.998 g, 76.9 mmol) were stirred in DMF (20 mL) at 85 °C overnight. Upon cooling the reaction to RT, DCM (40 mL) was added to the reaction mixture and stirred. The reaction mixture was then washed with aqueous HCl (0.1 M, 3×20 mL) and dried with MgSO₄. The organic fraction was isolated and the solvent was removed under reduced pressure to yield the product as a clear pale yellow oil. Yield: 3.85 g, 96 %. ¹H NMR (600 MHz, CDCl₃) δ ppm 1.43 (quint, J = 7.2 Hz, 2 H), 1.61 (quint, J = 7.2 Hz, 2 H), 1.67 (t, J = 7.2 Hz, 2 H), 2.37 (quint, J = 7.2 Hz, 2 H), 3.27 (t, J = 6.4 Hz, 2 H).

Figure S3. ¹H NMR spectrum of 2.
Synthesis of 2,5-Dioxopyrrolidin-1-yl 6-azidohexanoate (3)

Compound 2 (2.0 g, 12.7 mmol), N-hydroxysuccinimide (NHS) (4.395 g, 38.2 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (5.929 g, 38.2 mmol) were dissolved in DMF (15 mL) and stirred at RT, 48 h. DMF was then removed under reduced pressure. The remaining residue was dissolved in ethyl acetate (EtOAc) (25 mL). The organic layer was washed with H2O (3×40 mL), saturated brine (3×40 mL), and dried with MgSO4. The solvent was removed under reduced pressure to yield the crude product. The crude product was further purified via column chromatography (silica gel) with Hexanes:EtOAc (100:0-0:100). The fractions corresponding to the product were combined and the solvent was removed under reduced pressure to yield the product as a clear pale yellow oil. Yield: 2.80 g, 87 %. 1H NMR (600 MHz, CDCl3) δ ppm 1.49 (quint, J = 6.8 Hz, 2 H), 1.63 (quint, J = 7.1 Hz, 2 H), 1.77 (t, J = 7.0 Hz, 2 H), 2.61 (quint, J = 7.1 Hz, 2 H), 2.82 (s, 4 H), 3.28 (t, J = 6.4 Hz, 2 H).

Figure S4. 1H NMR spectrum of 3.
Synthesis of 6-Azido-N-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)hexanamide (4)

4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl (2.0 g, 11.7 mmol), 3 (5.94 g, 23.3 mmol), and TEA (1.18 g, 11.7 mmol) were dissolved in dry (need not be anhydrous) DMF (25 mL). Potassium carbonate (1.61 g, 11.7 mmol) was added and the mixture was stirred at RT, OVN. DCM (50 mL) and H₂O (20 mL) were added and the reaction mixture was washed with H₂O (3×50 mL). The aqueous fraction was extracted with DCM (2×25 mL) and the organic fractions were combined. The organic fraction was washed with H₂O (3×50 mL) and dried with MgSO₄. The solvent was removed under reduced pressure. The crude product was further purified via column chromatography (silica) with DCM:MeOH (100:0 - 90:10). The solvent was removed under reduced pressure to yield the product as a red liquid. Yield: 6.52 g, 90 %. ¹H NMR (600 MHz, CDCl₃) δ ppm 0.90-2.30 (m, 26 H), 3.28 (t, 1 H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 19.83, 25.15, 25.20, 26.44, 28.73, 36.62, 41.21, 45.57, 51.35.

Figure S5. ¹H NMR spectrum of 4.
Synthesis of 4-((6-Aminohexyl)amino)-2,2,6,6-tetramethylpiperidin-1-oxyl (5)

Compound 4 (2.0 g, 6.4 mmol) was dissolved in Et₂O (100 mL) and LiAlH₄ (2.45 g, 64 mmol) was slowly added portionwise to the reaction solution. The reaction mixture was stirred at RT, OVN. The reaction mixture was cooled to 4 °C in an ice bath. H₂O (1.22 mL), followed by an aqueous NaOH solution (15% w/v, 1.22 mL), followed by H₂O (3.66 mL) were slowly added to the reaction mixture under stirring. Stirring was continued for 15 min. The reaction mixture was dried with MgSO₄ and stirred for 15 min. The resulting mixture was filtered and the feed was washed with Et₂O (100 mL). The organic fractions were combined. The solvent was removed under reduced pressure. The crude product was further purified via column chromatography (alumina) with DCM:MeOH (100:0 - 90:10). The solvent was removed under reduced pressure to yield the product as a light orange liquid. The crude compound was used without further purification. Yield: 1.27 g, 73%. 

Figure S6. $^{13}$C NMR spectrum of 4.
Synthesis of 4-((6-Azidohexyl)amino)-2,2,6,6-tetramethylpiperidin-1-oxyl (6)

Compound 5 (2.0 g, 7.4 mmol) was dissolved in MeOH (50 mL) and the reaction solution was cooled to 4 °C in an ice bath. 1H-Imidazole-1-sulfonyl azide hydrogen sulfate (2.21 g, 8.1 mmol), CuSO₄ · 5H₂O (0.02 g, 0.074 mmol), and K₂CO₃ (2.04 g, 14.8 mmol) were added and the reaction mixture was stirred at RT, OVN. The solvent was removed under reduced pressure. H₂O (25 mL) was added to the remaining residue and the resulting mixture was filtered. The feed was washed with H₂O (25 mL) and EtOAc (25 mL). The filtrate was collected and H₂O (25 mL) was added. The organic fraction was collected. The aqueous fraction was extracted w/ EtOAc (2×25 mL) and the organic fractions were combined. The combined organic fraction was washed with NaHCO₃ (aq.) (4% w/v, 2×30 mL), and saturated brine (2×30 mL). The organic fraction was collected and dried with MgSO₄. The solvent was removed under reduced pressure to yield the crude product. The crude product was further purified via column chromatography (alumina) with DCM:MeOH (100:0 - 90:10). The solvent was removed under reduced pressure to yield the product as a red liquid. Yield: 1.84 g, 84 %. ¹H NMR (600 MHz, CDCl₃) δ ppm 1.15-20 (m, 12 H), 1.41 (m, 4 H), 1.62 (m, 4 H), 1.87 (m, 4 H), 3.17 (m, 1 H), 3.27 (t, 2 H), 3.65 (t, 2 H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 20.45, 20.47, 25.21, 26.32, 27.76, 28.62, 36.29, 39.38, 42.80, 51.28, 67.32, 67.97.

Figure S7. ¹H NMR spectrum of 6.
**Figure S8.** $^{13}$C NMR spectrum of 6.
Isothermal Titration Calorimetry

Figure S9. ITC data for 6 at 25 °C in 10 mM sodium phosphate (pH 7.0). 6 (250 µM) was titrated into a solution of CB[8] (50 µM). The top portion of the figure is a plot of power versus time. The bottom portion of the figure are integrated enthalpy values versus the molar ratio of 6:CB[8]. The $K_d$ value for the CB[8]⸧6 complex was determined to be $1.5 \pm 0.1 \times 10^{-8}$ M. The enthalpy and entropy values were determined to be $-1.0 \times 10^4 \pm 118.2$ cal/mol and 16.0 cal/mol/deg respectively. These data were fit to a one-binding site model using Origin 8.0 software.
Protocol for Expression of TMV

TMV particles were isolated from *Nicotiana benthamiana* plants. Tobacco plants were grown, infected with a solution of TMV from a stock source, collected (~10 d after infection), and stored at −80 °C until needed. The leaves (~100 g) were blended with cold (4 °C) extraction buffer (KP buffer (0.1 M, 1000 mL, pH 7.4) with 2-mercaptoethanol (0.2% (v/v))), followed by thorough grinding with a mortar and pestle. The mixture was filtered through cheesecloth to remove the plant solids, and the filtrate centrifuged at 11,000 ×g (4 °C, 20 min). The supernatant was filtered through cheesecloth again, and an equal volume of 1:1 chloroform/1-butanol mixture was added and stirred (4 °C, 30 min). The mixture was centrifuged at 4500 ×g for 10 min. The aqueous phase was collected, followed by the addition of NaCl (final concentration of 0.2 M), PEG 8000 (8% (w/w)), and Triton X-100 surfactant (1% (w/w)). The mixture was stirred on ice for 30 min and stored (4 °C, 1 h). The solution was centrifuged at 22,000 ×g (4 °C, 15 min). The supernatant was discarded, and the pellet resuspended in KP buffer (0.1 M, pH 7.4) (4 °C, OVN). The supernatant was carefully layered on a 40% (w/v) sucrose gradient (that had undergone at least one freeze-thaw cycle to create a moderate sucrose gradient) in KP buffer (0.01 M, pH 7.4) in ultracentrifuge tubes and centrifuged in a swing bucket rotor for 2 h at 96,000 ×g. An LED light positioned under the transparent centrifuge tube was used to illuminate the colloidal suspension, which appears blue from Mie scattering. The light-scattering region was collected and centrifuged at 360,562 ×g for 1.5 h. The supernatant was discarded, and the pellet resuspended in KP buffer (0.01 M, pH 7.4) (4 °C, OVN). The solution was portioned equally into microcentrifuge tubes and centrifuged at 15,513 ×g for 15 min. The supernatant was collected as the final TMV solution. UV-Vis measurements were taken at 260 nm (RNA) and 280 nm (protein). A ratio of A260/A280 around 1.23 indicates intact TMV. Using the Beer-Lambert Law with ε = 3 the concentration of the solution was determined.

Bioconjugation of TMV

*Synthesis of TMV-Aky*

Solutions of 3-ethynylaniline in acetonitrile (0.68 M, 75 μL) and NaNO₂ (aq.) (3.0 M, 25 μL) were added to a cold (4 °C) solution of p-toluenesulfonic acid (aq.) (0.3 M, 400 μL) and mixed well. The resulting solution was mixed in light-free conditions (4 °C, 1 h) to form the diazonium salt. A solution of TMV (20 mg/mL, 100 μL, 0.1 μmol) was diluted in borate buffer (0.1 M, pH 8.8, 862 μL) and the resulting solution was chilled to 4 °C. The diazonium salt solution (70 eq per coat protein of TMV, 76 μL) was added to the solution of TMV and the resulting solution was mixed in light-free conditions (4 °C, 45 min). The resulting product was purified via either size exclusion chromatography using a GE Healthcare PD-10 Desalting Column or centrifuge filtration using an EMD Millipore Amicon Ultra Centrifugal Filter Unit (10,000 MW Cutoff) (4,303 ×g).
**Synthesis of TMV-6**

Compound 6 (1.7 mg, 5.7 μmol) was dissolved in DMSO (1 mL). Cold (4 °C) KP buffer (0.1 M, 3 mL, pH 7.4) was added to the resulting solution and mixed well. A cold (4 °C) solution of TMV-Aky (20 mg/mL, 100 μL, 0.1 μmol) in KP buffer (0.1 M, pH 7.4) was added to the resulting solution and mixed well. An aqueous solution of copper sulfate pentahydrate (0.1 M, 10 μL) was added to the resulting solution and mixed well. An aqueous solution of sodium ascorbate (0.2 M, 10 μL) was added to the resulting solution and mixed well. The reaction was left to proceed at RT for 24 h. The resulting product was purified via either size exclusion chromatography using a GE Healthcare PD-10 Desalting Column or centrifuge filtration using an EMD Millipore Amicon Ultra Centrifugal Filter Unit (10,000 MW Cutoff) (4,303 xg).

**Fluorescence and EPR Kinetics Experimental Details**

Fluorescence titrations of TMV-6 into CB[8]∙PF were performed using Greiner 384-well, black, flat-bottomed plates. Solutions of TMV-6 were prepared by serial dilutions of a stock solution of TMV-6 (200 μM in terms of TEMPO) in sodium phosphate buffer (0.01 M, pH 7.0). TMV-6+CB[8]∙PF solutions were prepared by mixing the appropriate TMV-6 (20 μL) solution with the solution of CB[8]∙PF (0.6 μM, 10 μL). This resulted in solutions with final TMV-6 concentrations from 0-20 μM and a final CB[8]∙PF concentration of 0.2 μM. The solutions were mixed by pipetting before reading the fluorescence intensities on the plate reader (top reading mode; 400 nm excitation, 10 nm bandwidth; 510 nm emission, 20 nm bandwidth). Z-depth and gain were optimized on the first scan and then exact values were used in subsequent scans. For the titrations of native TMV (nTMV) into CB[8]∙PF, all methods and parameters were identical to the titrations of TMV-6 into CB[8]∙PF, except for the use of nTMV instead of TMV-6. For the titrations of TMV-6 into PF, all methods and parameters were identical to the titrations of TMV-6 into CB[8]∙PF, except for the use of PF instead of CB[8]∙PF.

Since no other significant interactions between TMV, 6, CB[8], and PF were observed (Figure S10, S11) and assuming the conservation of mass, the relationship and equilibria of the titration components can be described by the following equation:

\[
[\text{CB}[8]\cdot\text{PF}]+[6]=\text{[CB}[8]]+[\text{PF}]+[6]=\text{[CB}[8]\cdot\text{PF}]+[\text{PF}]
\]  

(Eq. 1)

Therefore, the two dissociation constants, \( K_a \) for the complex consisting of CB[8] and PF and \( K_b \) for the complex consisting of CB[8] and 6 can be represented by the following equations:

\[
K_a = \frac{[\text{CB}[8]][\text{PF}]}{[\text{CB}[8]\cdot\text{PF}]}
\]  

(Eq. 2)
\[ K_b = \frac{[\text{CB}[8]] [6]}{[\text{CB}[8] > 6]} \]  
\hspace{1cm} (Eq. 3)

Equations 1-3 can then be combined to form the following cubic equation:

\[ [\text{CB}[8]]^3 + a [\text{CB}[8]]^2 + b [\text{CB}[8]] + c = 0 \]  
\hspace{1cm} (Eq. 4)

where

\[ a = K_a + K_b + [\text{PF}]_0 + [6]_0 - [\text{CB}[8]]_0 \]  
\hspace{1cm} (Eq. 5)

\[ b = K_b ([\text{PF}]_0 - [\text{CB}[8]]_0) + K_a ([6]_0 - [\text{CB}[8]]_0) + K_a K_b \]  
\hspace{1cm} (Eq. 6)

\[ c = -K_a K_b [\text{CB}[8]]_0 \]  
\hspace{1cm} (Eq. 7)

and \([\text{PF}]_0\), \([6]_0\), and \([\text{CB}[8]]_0\) denote the total concentration of each respective compound. The change in the observed fluorescence intensity can be directly linked to the binding constants \(K_a\) and \(K_b\) by solving Equation 4 for the real root. The relationship between the observed fluorescence intensities and the binding constants are described by the equation:

\[ F = F_{\text{Min}} + (F_{\text{Max}} - F_{\text{Min}}) \cdot \frac{2 \cdot \sqrt{(a^2 - 3b) \cdot \cos^2 \theta - a}}{3K_b \cdot \frac{2 \cdot \sqrt{(a^2 - 3b) \cdot \cos^2 \theta - a}}{2 \cdot \sqrt{(a^2 - 3b)^3}}} \]  
\hspace{1cm} (Eq. 8)

where

\[ \theta = \cos^{-1} \frac{-2a^3 + 9ab - 27c}{2 \cdot \sqrt{(a^2 - 3b)^3}} \]  
\hspace{1cm} (Eq. 9)

and \(F\), \(F_{\text{Min}}\), and \(F_{\text{Max}}\) denote the observed fluorescence intensity at any given point in the titration, the minimum observed fluorescence intensity during the titration, and the maximum observed fluorescence intensity during the titration, respectively. Upon fitting
Equation 8 to the observed fluorescence intensities, the $K_d$ value for the CB[8]::$\text{TMV-6}$ complex was determined to be $3.8 \pm 0.5 \times 10^{-7}$ M.

**Figure S10.** Fluorescence titration data for control experiment with nTMV and CB[8]::$\text{PF}$. nTMV (0-20 μM in terms of TMV coat protein) was titrated into solutions of CB[8]::$\text{PF}$ (0.2 μM). Since no significant changes in fluorescence were observed, it is demonstrated that TMV does not compete with 6 to bind inside the cavity of CB[8].

**Figure S11.** Fluorescence titration data for control experiment with TMV-6 and PF. TMV-6 (0-20 μM in terms of TEMPO) was titrated into solutions of PF (0.2 μM). Since no significant changes in fluorescence were observed, it is demonstrated that TMV does not quench the fluorescence of 6.
Figure S12. Fluorescence titration data for TMV-6. TMV-6 (0-20 μM in terms of TEMPO) was titrated into solutions of CB[8]⇌PF (0.2 μM). The $K_d$ value for the CB[8]⇌TMV-6 complex was determined to be $3.8 \pm 0.5 \times 10^{-7}$ M.

**EPR Spectroscopy**

All EPR measurements were obtained using the following instrumental conditions:
- Microwave Power: 4.54 mW
- Microwave Frequency: 9.38 GHz
- Modulation Frequency: 100 kHz
- Modulation Amplitude: 0.4 mT (4 G)
- Temperature: 298 K
- Center Field: 334 mT (3340 G)
- Sweep Range: 8 mT (80 G)

Samples were prepared by filling a double-ended glass capillary tube (1 mm internal diameter) with the appropriate solution, sealing the capillary tube with paraffin laboratory film, and then placing the capillary tube in a quartz EPR tube (4 mm internal diameter).
Table S1: Kinetics of the reduction of nitroxides with excess of sodium ascorbate. Numerical fits to pseudo-first order rate equation ($k'$) for the relaxometric data of the reaction.

| Agent                                      | Reduction with ascorbate $k'$ (×10^{-5} s^{-1}) | Reference |
|--------------------------------------------|-------------------------------------------------|-----------|
| TMV-6 with CB[8]                          | 2.0                                             | This work |
| exTEMPO-TMV,                               | 170.0                                           | 1         |
| TEMPO-conjugated branched-bottle brush polymer* | 791.0                                           | 2         |
| Chex-MIM*                                  | 37.9                                            | 3         |
| P1*                                       | 27.0                                            | 3         |
| Dendrimer*                                 | 57.8                                            | 4         |
| TEMPOL*                                    | 633.3                                           | 5         |

*Determined with the integrated peak height (IPH) EPR data and for the initial kinetic fit (<1 hour).
Relaxometry

Both longitudinal and transversal relaxation times were determined using 43 MHz Magritek Spinsolve NMR spectrometer operating with a magnetic field strength of 1 T. Relaxivity was obtained using linear regression analysis of the relaxation rates of four solutions (0 – 0.14 mM). The NMR measured all samples at 310 K.

Table 1. Comparison of relaxivity values between contrast agents. *Direct comparison of relaxivity rates at different field strengths is difficult as \( r_1 \) values are depressed and \( r_2 \) values enhanced at higher fields. These values are included for completeness and to acknowledge the contributions of others in this area.

| Contrast Agent (CA) | CA per Particle | \( r_1 \) per CA (mM\(^{-1}\) s\(^{-1}\)) | \( r_2 \) per CA (mM\(^{-1}\) s\(^{-1}\)) | \( r_1 \) per Particle (mM\(^{-1}\) s\(^{-1}\)) | \( r_2 \) per Particle (mM\(^{-1}\) s\(^{-1}\)) | \( r_2/r_1 \) | Field (T)* | ref |
|---------------------|-----------------|-------------------------------|-------------------------------|---------------------------------|---------------------------------|-----------------|-------------|-----|
| TMV-6               | ~2130           | 2.8 ± 0.1                     | 10.3 ± 0.1                    | ~5964                           | ~21939                          | 3.7             | 1           | This work |
| TMV-6+CB[8]         | ~2130           | 1.9 ± 0.1                     | 3.1 ± 0.1                     | ~4047                           | ~6603                           | 1.6             | 1           | This work |
| TEMPO-NH\(_2\)      | 1               | 0.6 ± 0.1                     | 2.0 ± 0.1                     | 0.6 ± 0.1                       | 2.0 ± 0.1                       | 3.3             | 1           | This work |
| TEMPO-NH\(_2\)+CB[8]| 1               | 0.2 ± 0.1                     | 0.2 ± 0.1                     | 0.2 ± 0.1                       | 0.2 ± 0.1                       | 1               | 1           | This work |
| Gd-DOTA             | 1               | 3.0                           | 5.0                           | 3.0                             | 5.0                             | 1.7             | 1           | 6             |
| exTEMPO-TMV         | ~2130           | 1.5 ± 0.1                     | 4.7 ± 0.1                     | ~3195                           | ~10011                          | 3.13            | 1.5         | 1             |
| inTEMPO-TMV         | ~3919           | 0.4 ± 0.1                     | 1.7 ± 0.1                     | ~1567.6                         | ~6662.3                         | 4.25            | 1.5         | 1             |
| 3-CP                | 1               | 0.15                          | 0.17                          | 0.15                            | 0.17                            | 1.13            | 7           | 7             |
| Chex-MM             | N/A             | 0.21                          | 0.3                           | N/A                             | N/A                             | 1.42            | 7           | 3             |
| Chex-dendrimer      | N/A             | 0.44                          | 0.86                          | N/A                             | N/A                             | 1.95            | 7           | 4             |
| Chex-bottlebrush    | N/A             | 0.32                          | 0.82                          | N/A                             | N/A                             | 2.56            | 7           | 3             |
| BASP-ORCA1          | N/A             | 0.41                          | 4.67                          | N/A                             | N/A                             | 11.39           | 7           | 7             |
| Dy-DTPA-PcHexPh2    | 1               | 0.11                          | 3                             | 0.11                            | 3                               | 27              | 7           | 8             |

MRI In Vivo Studies

All animal procedures were reviewed by the UT Southwestern IACUC committee and accepted under protocol # 2016-101780. Mice were placed under anesthesia and a heater was used to keep the temperature around the mice at 30 °C for the duration of the study. Each mouse was injected intramuscularly with 50 µL of TMV-6 without CB and TMV-6 with CB. The mice were placed in a 9.4 T Varian MRI scanner and the bladder was positioned to be in the center. 3D T1-weighted gradient echo multi slice scans were taken before injection (\( TE = 4.00 \) ms and \( TR = 256.92 \) ms, Matrix = 128 × 138 × 128) and at 1 min, 30 min, and 2 h after injection.

Modeling

The molecular dynamics (MD) simulations included one TEMPO molecule, one CB[8] molecule, 1877 water molecules, and one chloride anion in the unit cell under periodic boundary conditions. The force field parameters for CB[8] were obtained from CGenFF\(^9,\)\(^10\) using the online server at https://cgenff.paramchem.org. The force field parameters for
TEMPO were obtained from the SLH moiety in work by Sezer et al. The TIP3P water model was used. Note that the TIP3P water model was found to yield better agreement with experimental cucurbituril-guest binding enthalpies than competing water models such as TIP4P-Ew.

![Figure S13](image)

**Figure S13.** The free energy profile as a function of the insertion depth of the TEMPO ring in the CB[8] cavity.

All simulations were run using the NAMD software package with the following parameter choices: temperature 300 K enforced with a Langevin thermostat with damping parameter 1.0 ps\(^{-1}\); pressure 1 atm enforced with a Langevin barostat with a period of 100 fs and a decay time of 50 fs; cutoff distance 12 Å for the van der Waals interactions and the changeover from real space to reciprocal space for the electrostatic interactions; time step 1.0 fs; particle mesh Ewald grid spacing of 1 Å.

![Figure S14](image)

**Figure S14.** Radial distribution functions and their integrals for the distance between the TEMPO oxygen radical and water hydrogen and oxygen atoms.

Three simulations were run, namely an equilibrium simulation of 100 ns, an adaptive biasing force simulation of 100 ns, and an umbrella sampling simulation of 100 ns. The
equilibrium simulation quantifies, among other things, the accessibility of the TEMPO oxygen radical to solvent water molecules. This data is shown in Fig. S14. We see that water hydrogen atoms (black line) are found preferentially about 2 Angstroms from the TEMPO oxygen radical, and water oxygen atoms (red line) are found preferentially about 3 Angstroms from the TEMPO oxygen radical. In terms of numbers, the green and blue curves in Fig. S14 show that, on average, one water hydrogen and one water oxygen are found within 2.6 and 3.2 Angstroms, respectively, of the TEMPO oxygen radical. A water molecule including this hydrogen and oxygen atom is shown in Fig. S15. This water molecule is surrounded by other solvent water molecules, thus allowing exchange to generate the MRI contrast. In addition, due to the CB[8] cavity, we see that the radial distribution functions do not plateau until about 15 Angstroms.

**Figure S15.** Representative snapshot from the equilibrium MD simulation showing the location of the TEMPO molecule with respect to CB[8] and the TEMPO oxygen radical’s access to solvent water. Only one solvent water molecule is shown.

The two free energy simulations quantify the CB[8]—TEMPO host—guest binding free energy and also show exactly where the TEMPO molecule prefers to sit in the CB[8] host cavity. The data is shown in Fig. S13. We can notice two main observations. First, the equilibrium position of the TEMPO ring is about 0.9 Å above the plane of the CB[8] ring. Second, the binding free energy is very strong at over 20 kcal/mol. Previous literature suggests that computational methods typically overestimate the binding free energy for cucurbit[n]uril host-guest systems\textsuperscript{14} but nonetheless the CB[8]—TEMPO association is clearly very strong.

Although we do not attempt to decompose the binding free energy into contributions from separate factors, we note that previous studies have attributed significant effects from the change in solvation of the ammonium unit upon binding,\textsuperscript{15} and from the release of water molecules inside the CB[8] cavity upon binding.\textsuperscript{16} For the binding free energy calculation, we used five collective variables to control the insertion of TEMPO into the CB[8] cavity and ensure reversibility of the transformation.
The first two collective variables have no effect on the energy, the 3rd and 4th ones do have a minor effect on the energy, and the 5th one is the reaction coordinate. The 1st collective variable constrains the center of mass of CB[8] to the origin. The 2nd collective variable constrains the plane of the CB[8] ring to the x-y axis. These two collective variables have no effect on the system properties since periodic boundary conditions are used; their purpose is to make the choice of reaction coordinate simpler. The 3rd collective variable weakly constrains the center of mass of the TEMPO ring to the z-axis. This is done so that the TEMPO molecule does not drift sideways when it is separated from CB[8]. The 4th collective variable does not allow the terminal carbon atom in the TEMPO tail chain to be more than 3 Å below the center of mass of the TEMPO ring. This is done to prevent the TEMPO molecule from rotating 180 degrees which would otherwise potentially let it insert backwards after being separated from CB[8]. Although the entropic cost of the 3rd and 4th collective variable constraints could be estimated analytically, we simply neglected their contribution to the binding free energy. The 5th collective variable is defined as the z-distance between the center of mass of CB[8] and the center of mass of the TEMPO ring. This is our reaction coordinate for measuring the binding free energy. For the umbrella sampling run, it was controlled using 40 umbrellas between reaction coordinate values of -3 and +8 with a spring constant of 10 kcal/mol/Å² over a 100 ns simulation. For the ABF run we used the default values. We also computed the binding free energy, or at least the accessible portion, from the equilibrium simulation. This was done by taking kT times the negative of the logarithm of the normalized histogram of the reaction coordinate values visited during the equilibrium simulation.

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