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High Affinity Macrocycle Threading by a Near-Infrared Croconaine Dye with Flanking Polymer Chains

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Abstract

Croconaine dyes have narrow and intense absorption bands at ~800 nm, very weak fluorescence, and high photostabilities, which combine to make them very attractive chromophores for absorption-based imaging or laser heating technologies. The physical supramolecular properties of croconaine dyes have rarely been investigated, especially in water. This study focuses on a molecular threading process that encapsulates a croconaine dye inside a tetralactam macrocycle in organic or aqueous solvent. Macrocycle association and rate constant data are reported for a series of croconaine structures with different substituents attached to the ends of the dye. The association constants were highest in water ($K_a \sim 10^9 \text{ M}^{-1}$), and the threading rate constants (k_{on}) increased in the solvent order $\text{H}_2\text{O} > \text{MeOH} > \text{CHCl}_3$. Systematic variation of croconaine substituents located just outside the croconaine/macrocycle complexation interface hardly changed K_a but had a strong influence on k_{on} . A croconaine dye with *N*-propyl groups at each end of the structure exhibited a desirable mixture of macrocycle threading properties; that is, there was rapid and quantitative croconaine/macrocycle complexation at relatively high concentrations in water, and no dissociation of the pre-assembled complex when it was diluted into a solution of fetal bovine serum, even after laser induced photothermal heating of the solution. The combination of favorable near-infrared absorption properties and tunable mechanical stability makes threaded croconaine/macrocycle complexes very attractive as molecular probes or as supramolecular composites for various applications in absorption-based imaging or photothermal therapy.

INTRODUCTION

The high value of the biotin-streptavidin association pair in numerous biotechnology applications is due to the extremely strong affinity ($K_a \sim 10^{13-15} \text{ M}^{-1}$) under a wide range of aqueous environments,^{1–4} but the large size and immunogenicity of the streptavidin protein is problematic in certain biomedical situations.⁵ One of the major goals of supramolecular chemistry is to create synthetic host-guest pairs that associate with similarly strong affinity

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ASSOCIATED CONTENT

Supporting Information Available

Synthesis and compound characterization data (Figures S1-S9), absorption maxima (Table S1), association and kinetic plots (Figures S10-S25, Table S2), absorption spectra before and after laser heating (Figure S26), computed energies (Table S3) and absorption spectra (Figure S28).

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in water. To date, the highest binding constants have been achieved using macrocyclic cucurbituril molecules as host systems.^{6–8} Alternative approaches based on synthetic cyclophane systems have the potential advantage that the binding partners can be optically or redox active,^{9–12} but they are presently limited by relatively weak association constants in water.^{13,14} Recently, we discovered an unusually effective supramolecular process that threaded a tetralactam macrocycle onto a fluorescent squaraine dye (Scheme 1) that was flanked by two long polyethylene glycol (PEG) chains.¹⁵ The nanomolar dissociation constants in water were attributed to the excellent shape complementarity of the squaraine dye with the cavity of the tetralactam macrocycle. The macrocycle threading kinetics hardly changed with the length of the PEG chains attached to the nitrogen atoms at each end of the squaraine dye, but the threading rates were sensitive to the size of the second *N*-substituent (identified in Scheme 2).¹⁶ Threaded squaraine/macrocycle complexes have outstanding near-infrared fluorescent properties and thus they have great promise as molecular probes for fluorescence imaging of biological samples.^{17–19}

This present report focuses on croconaine dyes (Scheme 1), which are the larger 5-ring homologues of squaraines.^{20,21} Croconaines (unlike squaraines) are very weakly fluorescent, and their sharp and intense absorption bands at ~800 nm make them very attractive dye systems for near-infrared absorption-based imaging or laser heating applications.^{22–24} Previous association studies of organic soluble croconaines in organic solvent have shown that they can be encapsulated inside the tetralactam macrocycle **M1** to form croconaine/macrocycle complexes (**M** $\supset$ **C**) whose structures are analogous to the threaded croconaine complexes (Scheme 2).^{25,26} The threaded complexes are stabilized by hydrogen bonds between the dye oxygen atoms and the four macrocycle NH residues, with simultaneous coplanar stacking of the dye aromatic surfaces against the anthracene sidewalls of the macrocycle. In this current study, we describe an extended series of new croconaine dyes and report the association and kinetic parameters for dye encapsulation inside macrocycles **M1** and **M2** in four different solvents: CHCl₃, MeOH, H₂O, and FBS solution. While we expected that the croconaine binding properties would be similar to the previously studied squaraine/macrocycle systems, it was not obvious at the start of the study if nanomolar dissociation constants in water would be observed. In addition, we needed to ascertain if a generalizable pre-assembly paradigm could be developed to create a threaded croconaine/macrocycle complex with sufficiently high mechanical stability for subsequent operation as a near-infrared photothermal heating agent in biological solution.

2. EXPERIMENTAL SECTION

a. Materials

Commercially available solvents and chemicals were purchased from Sigma-Aldrich and VWR international and used without further purification unless otherwise stated. Fetal bovine serum (Cat. No.: S11150) was purchased from Atlanta Biologicals and used directly without dilution. Water was deionized and micro filtered.

b. Synthesis

The synthetic procedures, spectral data, and proof of purity are provided in the supporting information.

c. Instrument Details

^1H and ^{13}C NMR spectra were recorded at 25 °C on Bruker AVANCE III HD 400, 500 MHz or Varian INOVA-600 MHz spectrometers. Chemical shift was presented in ppm and referenced by residual solvent peak. Mass spectra of small molecules were acquired using a Bruker microTOF II spectrometer with electrospray ionization (ESI). Mass spectrometry of macromolecules modified with PEG chains were acquired using a Bruker Autoflex III spectrometer with MALDI using a literature procedure.²⁷ Gel permeation chromatography (GPC) was performed with a system including a Waters 515 HPLC pump, a Waters 2414 refractive index detector and three Polymer Standards Services (PSS) columns (GRAM, 104, 103, and 102 Å). Linear PEO standards were used for calibration and data was analyzed with PSS WinGPC Unity software. Absorption spectra were collected on Evolution 201 UV/Vis Spectrometer with Thermo Insight software. Spectra were obtained using spectrophotometric grade solvent at 20 °C with glass cuvette (1 mL, 10 mm path length). Kinetic profiles were measured using a UV/Vis spectrometer equipped with a SFA-20M Stopped-Flow accessory. A pneumatic Drive system, OPT-20P, was used to operate the stopped flow device at the recommended pressure (4 bar) with an empirical dead-time of 8 ms and a flow tube observation path length of 1 cm.

d. Association Measurements by UV/Vis Titration

A stock solution of guest (croconaine dye, 0.5-5 μM) was prepared. In addition, a stock solution of host (macrocycle, 20-100 times higher concentration than the guest solution) was made by using the guest solution as solvent (to keep the concentration of guest constant during the titration). An aliquot of guest (1.0 mL) was placed in a cuvette at 20 °C and the host solution was added in small increments. The absorption was recorded after each addition. Association constants in CHCl_3 , MeOH, and FBS were measured by conducting standard titration experiments and the titration isotherms were fitted with a 1:1 binding model.²⁸ **M2** threading by croconaine dyes with *N*-methyl substituents (**C2-Me**, **C3-Me**, **C4-Me**) in H_2O was too strong to be measured by direct titration. Therefore, a competitive titration experiment was performed as described in the supporting information.

e. Kinetic Measurements by Stopped Flow

Equal volumes of host and guest solutions (concentration range of 1 - 10 μM) were mixed by stopped flow and the absorption maxima was monitored over time using a UV/Vis spectrometer at 20 °C. The kinetic profiles were fitted to the standard second order kinetic equation using Origin LabTM 8.6 software.¹⁵

f. Computer Molecular Modelling

The computed structures of croconaine and croconaine/macrocycle complexes were optimized by the semi-empirical method at PM7 level executed by MOPAC program and then further optimized by DFT method at BLYP-D3-gCP/def2-SVP level executed by

ORCA program. The croconaine structure can adopt three possible conformations based on the relative orientations of the thiophene units, and the computed stabilities of each conformation are almost identical (Table S3). Calculations of the croconaine/macrocyclic complex started with the encapsulated dye in a cis conformation. The final optimized structures were used to calculate UV/Vis absorption spectra using the TD-DFT method at B3LYP/6-31++G** level executed by Gaussian09 program. The calculated spectra were visualized by Gabedit program with Lorentzian lineshape conversion.²⁹

3. Results and Discussion

As shown in Scheme 2, eight croconaine dyes and two tetralactam macrocycles were studied. The modified croconaine dyes were prepared by attaching PEG chains to the precursor bisalkyne **C1** via copper catalyzed azide-alkyne cycloaddition chemistry. Croconaine/macrocyclic complexation was studied in four solvents: chloroform, methanol, water and FBS solution. Consistent with previous observations, croconaine encapsulation by the macrocycle was indicated by a diagnostic ~30 nm red-shift in croconaine absorption maxima (Figure 1, Table S1).^{25,26} Additional structural detail was provided by the ¹H NMR spectra in Figure 2, which showed the changes in chemical shift that occurred upon encapsulation of **C3-Me** by **M2** to form **M2** ⊃ **C3-Me**. The croconaine thiophene protons 1 and 2 were shifted upfield, whereas the macrocycle protons B were shifted downfield. A ROESY spectrum confirmed the interlocked structure by showing through space correlations between thiophene protons 1 and two different macrocycle proton signals (see supporting information).

As shown in Scheme 3, there are three possible conformational isomers for croconaine dye based on the orientation of the thiophene units. But the ¹H NMR spectral patterns in Figure 2 indicate that only two of the dye conformations are encapsulated within the **M2** ⊃ **C3-Me** complex. For example, there are two sets of ¹H NMR signals for macrocycle protons C (atom labels are shown in Scheme 2). The major signal is comprised of two, equal intensity singlets and corresponds to a complex containing a dye in a cis conformation, whereas, the minor signal is comprised of two doublets and corresponds to a complex containing a dye in a trans conformation. To further visualize the threaded croconaine/macrocyclic complex, a structural optimization was performed by conducting density functional theory calculations at the BLYP-D3-gCP/def2-SVP level.^{30,31} The optimized structure (Figure 3) indicates that two of the croconaine oxygen atoms form bifurcated hydrogen bonds with the NH residues in the tetralactam macrocycle, which adopts a macrocyclic boat conformation. On the NMR time-scale, this structure is in rapid exchange with the mirror-image, degenerate co-conformational isomer that has the macrocycle hydrogen bonded to the other side of the croconaine dye. The optimized croconaine structure was further used to calculate the absorption spectrum for free dye and complex. Reassuringly, the calculated spectra show a red shift (see supporting information) in the croconaine absorption upon encapsulation inside the macrocycle, which agrees with experimental observation, and supports the validity of the calculated structures.

The croconaine/macrocyclic association constants were measured by titration experiments that monitored the changes in croconaine absorption maxima. The titration isotherms were

fitted to a 1:1 binding model using nonlinear least square regression analysis and a typical titration curve is shown in Figure 4. The affinities for **M2** threading by croconaine dyes with *N*-methyl substituents (*i.e.*, **C2-Me**, **C3-Me**, **C4-Me**) in H₂O were too high to be determined accurately by direct titration, so a competitive titration method was employed. The croconaine dye was added to a solution containing **M2** and a large excess of a fumaride guest that was known to have moderate affinity for the macrocycle (see supporting information). The macrocycle threading rates were measured using a stopped flow device that monitored changes in croconaine absorption maxima as a function of time and the kinetic profiles fitted well to a standard second order model (Figure 4). The observed association constants and second order rate constants in CHCl₃, MeOH, H₂O, and FBS solution are listed in Table 1. Overall, the association and kinetic data is quite similar to the previously reported values for analogous squaraine/macrocycle association.¹⁶ To summarize the trends, the values for K_a increased in the solvent order H₂O > CHCl₃ > MeOH and the values for k_{on} increased in the solvent order H₂O > MeOH > CHCl₃. Compared to MeOH, the croconaine/macrocycle affinity is higher in CHCl₃ but the kinetics are slower. This suggests that host/guest hydrogen bonding plays an important thermodynamic and kinetic role in organic solvent, since stronger host/guest hydrogen bonding in the less polar CHCl₃ produces a more stable complex, but a higher barrier for complex association/dissociation. The nanomolar dissociation constants in H₂O are notable and consistent with a hydrophobic effect driving highly favorable aromatic stacking of the encapsulated croconaine dye against the anthracene sidewalls of the macrocycle. The croconaine structural trends in H₂O (Table 1, entries 9-13) show that changes in the size of the substituents attached to the nitrogen atoms at each end of the croconaine dye had very little influence on K_a , which was expected since the substituents are located outside the macrocycle binding cavity. The values for k_{on} were relatively insensitive to the length of the chain attached to the nitrogen atoms, but very sensitive to the size of the second *N*-substituent. For example, a comparison of entries 10 and 13 in Table 1 shows that decreasing the size of the second *N*-substituent from *N*-propyl to *N*-methyl increases k_{on} by more than 10,000. The same steric effect was also observed in CHCl₃, MeOH and FBS solution (Table S2). Thus, a rate determining step for the threading process is passage of the macrocycle over the nitrogen atom at one end of the croconaine dye. In comparison, threading of the macrocycle onto the chain attached to the croconaine dye (intron effect),³² and passage along the chain is relatively fast. There is no kinetic evidence for a chain entanglement effect in any of the solvents. For example, extending the length of the PEG chains from 2 to 111 ethylene glycol units (*i.e.*, changing from **C2-Me** to **C4-Me**) only slowed k_{on} by a factor of two at the most (see Table 1 and compare entries 2 and 4, 6 and 8, 9 and 11, 14 and 16). Thus, even with the smallest *N*-methyl substituent and longest PEG chain, there is no evidence that any intermolecular or intramolecular PEG chain interactions^{33,34} are a kinetically significant factor.

With an eye on future applications in biological systems, we examined croconaine/macrocycle association in FBS, and found that K_a was about 500 times weaker than in H₂O (Table 1). Because of this weakened affinity, a sample of **M2** $\supset$ **C3-Et** that was pre-assembled in water and subsequently diluted in FBS showed spectral evidence for slow dissociation over several hours (Figure 5). This potential limitation was overcome by increasing the size of the *N*-alkyl substituent on the croconaine dye. We found that the

higher steric barrier for the *N*-propyl group in **C3-Pr** led to a **M2** $\supset$ **C3-Pr** complex that could be pre-assembled in quantitative yield by simply mixing the two components for two hours at 1 mM concentration in water at 20 °C. Because of its higher mechanical stability, the complex did not show any evidence for dissociation (Figure 5) over 16 hours when diluted to 1 μ M in FBS. Furthermore, the diluted solution of **M2** $\supset$ **C3-Pr** (1.5 μ M) in FBS was compatible with extended photothermal heating. For example, irradiation of the sample with an 808 nm diode laser (8 W/cm²) for 5 minutes raised the temperature from 20 °C to 40 °C but produced no change in the absorption spectrum, indicating no photobleaching or unthreading of the complex (see supporting information). The results in FBS strongly suggest that pre-assembled croconaine/macrocyclic complexes with threaded structures that are analogous to **M2** $\supset$ **C3-Pr** should have high enough mechanical stability in biological solutions to enable new absorption-based imaging methods using spatially modulated spectroscopy,²³ or photoacoustic imaging²⁴ and also become the basis for new therapeutic strategies that involve laser induced photothermal heating.²² The fact that the same macrocycle system can be used to encapsulate a highly fluorescent squaraine dye (absorption maxima ~680 nm) or a photothermally efficient croconaine dye (absorption maxima ~815 nm) with the same affinity raises interesting possibilities in supramolecular design. For example, it should be possible to devise a two-step theranostic procedure that utilizes a pre-assembled squaraine/macrocyclic complex as a fluorescent probe to locate a specific biomedical target, and then employ the analogous pre-assembled croconaine/macrocyclic complex for laser-induced photothermal therapy of the target site. Alternatively, various supramolecular light harvesting or diagnostics strategies can be envisioned that exploit energy transfer from a fluorescent squaraine/macrocyclic complex (energy donor) to a nearby croconaine/macrocyclic complex (energy acceptor).

CONCLUSIONS

This study quantified the threading of a tetralactam macrocycle by a near-infrared croconaine dye. Association and kinetic constants were measured for a series of related croconaine structures in organic and aqueous solvent. Overall, the trends were similar to a previous study that examined a structurally related, but photophysically different, squaraine dye system. The association constants were highest in water ($K_a \sim 10^9 \text{ M}^{-1}$), and the threading rate constants (k_{on}) increased in the solvent order $\text{H}_2\text{O} > \text{MeOH} > \text{CHCl}_3$. Systematic variation of the croconaine substituents located just outside the croconaine/macrocyclic complexation interface hardly changed K_a but had a strong influence on k_{on} . Most notably, a croconaine dye with *N*-propyl groups at each end of the structure exhibits a desirable combination of macrocycle threading properties that enable a new pre-assembly paradigm for rapid fabrication of mechanically stable molecular probes for near-infrared laser heating of biological samples. From a broader perspective, the combination of favorable near-infrared absorption properties and tunable supramolecular association should lead to novel croconaine-based materials for a range of absorption-based imaging or photothermal heating applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Absorption maxima of **C3-Me** (2 μM, 20 °C) during titration with **M1** in CHCl₃, undergo a red shift from 792 nm to 820 nm.

Figure 2. Partial ^1H NMR (600 MHz, 25 °C) spectrum of (a) croconaine **C3-Me**, (b) croconaine/macrocycle complex **M2** $\supset$ **C3-Me** and (c) macrocycle **M2** in D_2O . Atom labels are shown in Scheme 2. *designates NMR signals for the minor **M2** $\supset$ **C3-Me** complex with encapsulated **C3-Me** in a trans conformation.

Figure 3. Calculated structure of threaded croconaine/macrocyclic complex (blue: croconaine; red: tetralactam macrocycle; green dashed lines: hydrogen bonds).

Figure 4.

Representative absorption data: (*left*) Titration of **C3-Me** with **M1** in CHCl_3 . (*right*)

Threading of **M1** by **C3-Me** over time in CHCl_3 . Red lines are the fitted curves. $T = 20^\circ\text{C}$.

Figure 5.

(*left*) Absorption spectra indicating partial dissociation of **M2 C3-Et** (1 μM) in FBS over 2 h (spectra acquired every 3 min) at 20 $^{\circ}\text{C}$. (*right*) Absorption spectra indicating no dissociation of **M2 C3-Pr** (1 μM) in FBS over 16 h at 20 $^{\circ}\text{C}$ (spectra acquired every 30 min).

Scheme 1.
Structures of squaraine and croconaine dyes.

Scheme 2.

Molecules studied. The X group represents the second *N*-substituent.

Scheme 3.

Conformational isomers (two cis and one trans) for; (*top*) croconaine dye, (*bottom*) croconaine/macrocycle complex.

Table 1

Association constant (K_a) and threading rate constant (k_{on}) for croconaine dye and tetralactam macrocycle at 20 °C.

entry	solvent	croconaine	macrocycle	K_a (M ⁻¹)	k_{on} (M ⁻¹ s ⁻¹)
1	CHCl ₃	C1-Me	M1	(1.6±0.2)×10 ⁷	(3.1±0.1)×10 ⁴
2	CHCl ₃	C2-Me	M1	(1.9±0.3)×10 ⁷	(3.3±0.1)×10 ⁴
3	CHCl ₃	C3-Me	M1	(5.6±2.2)×10 ⁶	(1.7±0.1)×10 ⁴
4	CHCl ₃	C4-Me	M1	(4.0±1.6)×10 ⁶	(1.4±0.1)×10 ⁴
5	MeOH	C1-Me	M2	(4.0±1.0)×10 ⁵	(8.1±1.5)×10 ⁵
6	MeOH	C2-Me	M2	(3.0±0.2)×10 ⁵	(1.0±0.1)×10 ⁶
7	MeOH	C3-Me	M2	(2.3±0.2)×10 ⁵	(1.6±0.1)×10 ⁵
8	MeOH	C4-Me	M2	(2.1±0.3)×10 ⁵	(1.7±0.1)×10 ⁵
9	H ₂ O	C2-Me	M2	(1.4±0.1)×10 ⁹	>10 ⁶
10	H ₂ O	C3-Me	M2	(1.4±0.2)×10 ⁹	>10 ⁶
11	H ₂ O	C4-Me	M2	(5.2±0.3)×10 ⁸	>10 ⁶
12	H ₂ O	C3-Et	M2	(0.9±0.1)×10 ⁸	>10 ⁶
13	H ₂ O	C3-Pr	M2	(0.9±0.1)×10 ⁸	83±9
14	FBS	C2-Me	M2	(5.5±0.6)×10 ⁶	(6.6±0.6)×10 ⁵
15	FBS	C3-Me	M2	(3.2±0.6)×10 ⁶	(4.2±0.4)×10 ⁵
16	FBS	C4-Me	M2	(1.6±0.1)×10 ⁶	(3.9±0.1)×10 ⁵