# Guest Back-Folding: A Molecular Design Strategy That Produces a Deep-Red Fluorescent Host/Guest Pair with Picomolar Affinity in Water

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Supporting Information

ABSTRACT: One of the major goals of modern supramolecular chemistry, with important practical relevance in many technical fields, is the development of synthetic host/ guest partners with ultrahigh affinity and selectivity in water. Currently, most association pairs exhibit micromolar affinity or weaker, and there are very few host/guest systems with  $K_a >$  $10^9$  M<sup>-1</sup>, apparently due to a barrier imposed by enthalpy/ entropy compensation. This present study investigated the threading of a water-soluble tetralactam cyclophane by a deepred fluorescent squaraine guest with flanking polyethylene glycol chains, an association process that is dominated by a highly favorable enthalpic driving force. A squaraine structure



was rationally designed to permit guest back-folding as a strategy to greatly expand the hydrophobic surface area that could be buried upon complexation. Guided by computational modeling, an increasing number of N-benzyl groups were appended to the squaraine core, so that, after threading, the aromatic rings could fold back and stack against the cyclophane periphery. The final design iteration exhibited an impressive combination of fluorescence and supramolecular properties, including ratiometric change in deep-red emission, picomolar affinity ( $K_a = 5.1 \times 10^{10} \text{ M}^{-1}$ ), and very rapid threading ( $k_{on} = 7.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ) in water at 25 °C. Similar excellent behavior was observed in serum solution. A tangible outcome of this study is a new cyclophane/squaraine association pair that will be a versatile platform for many different types of fluorescence-based imaging and diagnostics applications. From a broader perspective, guest back-folding of aromatic groups is a promising new supramolecular stabilization strategy to overcome enthalpy/entropy compensation and produce ultrahigh affinity [2] pseudorotaxane complexes in water and biological media.

## INTRODUCTION

One of the grand challenges of modern supramolecular chemistry is development of synthetic association partners with ultrahigh affinity and bioorthoganol selectivity in water or complex biological media.<sup>1</sup> After optimization, these synthetic assembly systems are expected to have broad utility in biomedicine, nanotechnology, and advanced materials.<sup>2,3</sup> In addition, the research helps identify the fundamental factors that control noncovalent association in aqueous solvent. To date, most but not  $all^{4-6}$  of the work has focused on derivatives of three classes of macrocyclic host molecules, namely, cyclodextrins, cucurbiturils, and pillarenes.<sup>7-10</sup> In recent years, they have been employed in various proof-of-concept studies involving, for example, drug delivery, surface adhesion, protein targeting, and membrane fusion.<sup>7,11-17</sup> Not only do these published studies highlight their potential practical value, they also reveal their current performance limitations. Most notably, there are presently very few host/guest partners with sufficiently high affinities for successful operation at nanomolar

concentrations. The highest affinities have been recorded for macrocyclic and acyclic cucurbiturils, <sup>18–21</sup> but cucurbiturils, like the other host molecules mentioned above, do not strongly absorb long-wavelength light, which makes it hard to track the location of the molecular complexes or to ascertain if host/ guest association has occurred. Ongoing community research efforts are trying to correct these deficiencies by developing new water-soluble host/guest systems with a synergistic blend of outstanding supramolecular and photonic properties.<sup>22,23</sup> It is especially desirable to produce fluorescent systems that absorb and emit light in the desired window of 650-900 nm, where there is maximum penetration through skin and tissue.<sup>24</sup> But working with deep-red and near-infrared organic dyes is technically challenging, since the extensively conjugated  $\pi$ systems are inherently hydrophobic, which lowers water solubility, promotes fluorescence quenching due to dye self-

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aggregation, and increases off-target association with hydrophobic surfaces.<sup>25,26</sup>

We are developing a new high-affinity association system that we call Synthavidin (synthetic avidin) technology.<sup>1,27,28</sup> The published example shown in Scheme 1a involves threading of

Scheme 1. (a) Chemical Structures of M, SQ1, and  $M\supset$ SQ1 and (b) Two Views of  $M\supset$ SQ1 Highlighting the Complementary Hydrogen Bonding (green lines) and Aromatic Stacking within the Cavity of  $M^a$ 



<sup>*a*</sup>After excitation of the anthracene sidewalls in  $M \supset SQ1$  with 375 nm light there is efficient internal energy transfer to the encapsulated squaraine and subsequent fluorescence emission at 710 nm.

the water-soluble tetralactam cyclophane M by a deep-red fluorescent squaraine dye, SQ1, with flanking polyethylene glycol (PEG) chains to form the threaded [2]pseudorotaxane complex  $M \supset SQ1$ <sup>29</sup> In pure water, the association constant  $(K_{2})$  is ~10<sup>9</sup> M<sup>-1</sup>, but in fetal bovine serum (FBS), a close mimic of the human bloodstream, the  $K_a$  is decreased by almost 3 orders of magnitude. As illustrated in Scheme 1b, formation of the threaded complex is promoted by a combination of hydrogen bonds between the squaraine oxygens and the cyclophane amide groups and hydrophobic stacking of the squaraine against the inner anthracene sidewalls of the cyclophane. Recently, we reported a Synthavidin preassembly method that fabricates targeted molecular probes for fluorescence imaging of biological sites within living subjects.<sup>30–32</sup> Another large set of potential applications involves in situ capture of a squaraine dye.<sup>1</sup> That is, the two partners quickly form a fluorescent threaded complex in quantitative yield when they are mixed or are liberated in situ at low concentration.<sup>33'</sup> The early results are very promising, but further expansion of Synthavidin technology requires nextgeneration ultrahigh-affinity pairs with  $K_a > 10^9 \text{ M}^{-1}$  in water and similar high affinities in biological media. Thus, the specific aim of this study was to significantly increase  $K_a$  while the desired rapid threading kinetics and highly favorable squaraine fluorescence properties are maintained.

Rational design of an organic host/guest pair with  $K_a > 10^9$  $M^{-1}$  in water is presently a daunting undertaking, since most supramolecular systems exhibit enthalpy-entropy compensation; that is, structural modification of the host and/or guest does not lead to an increase in  $K_a$  because an increasingly favorable enthalpic driving force is compensated by an increasingly unfavorable entropic contribution.<sup>34-36</sup> Enthalpy-entropy compensation is not a general phenomenon of weak association,<sup>37</sup> where  $K_a$  is readily enhanced with an increased number of pairwise interactions,<sup>38,39</sup> but ultrahigh binding hosts that overcome the compensatory barrier in water are very rare and almost always involve cucurbiturils.<sup>7,13,40,41</sup> There is presently no general design algorithm that enables researchers to optimize organic host structures to have ultrahigh affinity. A major literature survey concluded that a good predictor of host/guest affinity in water is the hydrophobic surface area that is buried upon complexation.<sup>36,42</sup> One possible way to exploit this concept for increased  $K_{2}$  is to enlarge the surface area of a host's hydrophobic binding cavity, and there are several reports of expanded cyclodextrin<sup>43,44</sup> and calixpyrrole<sup>45,46</sup> host molecules with increased guest affinity. But we chose to keep the structure of M unchanged, since it possessed attractive fluorescent properties and was relatively easy to synthesize. Instead, we were drawn to the alternative and unexplored option of increasing the amount of guest hydrophobic surface area that is buried by complexation. We decided to redesign the squaraine's flanking chains so that after threading they could fold back and form additional secondary interactions with the exterior surface of M. Within the pseudorotaxane literature there is a small number of reports on the effects of guest back-folding on the stability and coconformational dynamics of a threaded complex. Virtually all of these studies examined threading of the well-known host cyclobis(paraquat-p-phenylene) by elongated guests with different types of appended groups, such as glycol,<sup>47–50</sup> aromatic,<sup>51</sup> or  $\pi$ -donor units.<sup>52</sup> In organic solvents with a low dielectric constant, the additional weak interactions gained by guest backing-folding stabilized the threaded complexes by 0.5-1.0 kcal mol<sup>-1</sup>. One study found that stabilizing CH···O interactions due to guest back-folding in acetonitrile were absent when the solvent was changed to water, which has a much higher dielectric constant.<sup>53</sup> To the best of our knowledge, guest back-folding has not been shown to stabilize a [2]pseudorotaxane in water, which is somewhat surprising, since it is well-known that noncovalent association can be strongly enhanced by hydrophobic effects.<sup>54–57</sup> Moreover, aromatic stacking is employed often as a molecular engineering tool to assemble biomaterials,<sup>58</sup> stabilize folded regions within peptides and proteins,  ${}^{59,60}$  and organize synthetic foldamers in aqueous solution.  ${}^{61-63}$  Aromatic stacking is also known to influence the coconformation and folded structures of permanently interlocked rotaxanes,<sup>64,65</sup> and guest back-folding with aromatic stacking facilitates the final bond-forming step in the synthesis of many catenanes.<sup>66</sup> With respect to the Synthavidin association pair in Scheme 1, we hypothesized that judicially located aromatic rings in the squaraine's flanking chains could stabilize the threaded complex by providing favorable secondary interactions with the rim of M (Scheme 2a). A potential caveat with this structural change was the likely increase in squaraine hydrophobicity due to the added aromatic rings, which might promote self-aggregation of the free squaraine and diminish its affinity for M. Thus, we expected that the final version of the modified squaraine structure would

Scheme 2. (a) Complex Stabilization Due to Back-Folding and Aromatic Stacking of Squaraine N-Benzyl Groups against the Periphery of M and (b) Chemical Structures of the New Guest Molecules Employed in This Study with the Squaraine N-Benzyl Groups Shown in Pink



have to employ appropriately substituted aromatic rings that simultaneously permitted rapid threading and favorable stacking interactions with **M** but also prevented self-aggregation of the free squaraine.

With these supramolecular constraints in mind, we employed computational modeling to revise the structure of prototype squaraine SQ1 and design four new squaraines, SQ2-SQ5 (Scheme 2b), with an increasing number of appended aromatic groups. We prepared these new squaraines and quantified the thermodynamics and kinetics for threading of M in water and serum solution. A comparison of the data reveals a systematic enhancement of affinity with no degradation of the favorable kinetic and photophysical properties. The final iteration of the design sequence, SQ5, has picomolar affinity for M in water due to a highly favorable change in enthalpy without any entropic compensation. In addition to some important practical implications, our results show that back-folding of guest aromatic groups after cyclophane threading is an effective new way to produce ultrahigh [2]pseudorotaxane complexes in aqueous solution.

## RESULTS AND DISCUSSION

**Molecular Design and Squaraine Synthesis.** The squaraine design process was guided by computer-based molecular modeling of threaded [2]pseudorotaxane complexes for which the starting coordinates were derived from homologous X-ray crystal structures.<sup>67–69</sup> The initial threaded structure was a gas-phase truncated model of the prototype complex  $M \supset SQ1$  with *N*,*N*-dimethyl units at each end of the squaraine (Scheme 3). The complex structure was systematically modified to have an increasing number of *N*-benzyl groups, and in each case the coconformation was optimized using the semiempirical PM7 method followed by further optimization using DFT at the BLYP-gCP-D3/def2-svp level. The complex that encapsulates unsymmetric SQ2 has two *N*-benzyl groups on one end of the dye, whereas the symmetric

Scheme 3. Computer Models of Threaded Complexes (calculated at the BLYP-gCP-D3/def2-svp level) Highlighting the Back-Folding of Squaraine N-Benzyl Groups and Edge-to-Face  $CH-\pi$  Aromatic Stacking with the Periphery of the Surrounding Host M





Figure 1. (a) Partial <sup>1</sup>H NMR spectrum (500 MHz, D<sub>2</sub>O, 25 °C) of SQ4 (1 mM),  $M \supset$ SQ4 (1 mM), and M (1 mM); purple indicates an overlap of peaks for SQ4 and M. (b) ROESY (500 MHz) of  $M \supset$ SQ4 in D<sub>2</sub>O; blue circles indicate through-space cross-relaxation between M and SQ4. Absorption (c) and fluorescence (d,  $\lambda_{ex} = 600$  nm; e,  $\lambda_{ex} = 375$  nm) spectra of SQ4 (2  $\mu$ M) and  $M \supset$ SQ4 (2  $\mu$ M) in H<sub>2</sub>O.

SQ3 has an N-benzyl group at either end. The third complex encapsulates unsymmetric SQ4 with three appended Nbenzylic groups. The DFT modeling suggested that the appended N-benzyl groups could stabilize the threaded complex by interacting with the periphery of M, specifically face-to-face  $\pi - \pi$  or edge-to-face CH $-\pi$  aromatic stacking with the isophthalamide bridging units or edge-to-face  $CH-\pi$ aromatic stacking with the anthracene sidewalls (Scheme 2a). The models in Scheme 3 specifically highlight the edge-to-face CH- $\pi$  aromatic stacking. Additional space filling depictions of the complexes are provided in Figures S40-S43 of the Supporting Information (SI), along with a reduced density gradient (RDG) analysis of each model showing favorable van der Waals interactions between the appended N-benzyl groups and the periphery of M. The aromatic stacking orientations seen in these computed DFT models are essentially identical to those observed in X-ray crystal structures of related squaraine rotaxanes.68,69

Additional structural and energetic insight was gained by conducting 100 ns molecular dynamic (MD) simulations of each threaded complex utilizing explicit water solvation. Trajectories suggest that virtually all of the coconformational states allow the squaraine N-benzyl groups to stack against the isophthalamide bridging units of the surrounding cyclophane and/or the anthracene sidewalls (movies of the MD simulations can be found in the Supporting Information, with a statistical analysis of aromatic stacking interactions in Table S5). Thus, the N-benzyl groups can explore a sizable conformational volume while favorable enthalpic contact is maintained with the periphery of M. Complexation free energies were calculated from the trajectories of each system using the MM-PBSA/ GBSA methodologies.<sup>70</sup> Both sets of calculations (MM-PBSA and MM-GBSA) indicated the same trend of increased [2] pseudorotaxane stabilization with the number of N-benzyl groups and favorable complexation free energies in the relative

order of  $M \supset SQ4 > M \supset SQ3 > M \supset SQ2 > M \supset SQ1$  (see Table S4 for calculated energies).

The promising modeling results motivated us to prepare SQ2-SQ4 as water-soluble versions of the squaraine dyes for experimental study. High water solubility was ensured by attaching long PEG-2000 chains to the ends of the squaraine structures by means of azide/alkyne cycloaddition chemistry. Another purpose of the appended PEG-2000 chains was to minimize dye self-aggregation and association with albumin proteins. We have previously shown that the rate for threading of **M** is not affected by the length of the appended PEG chains, but it is very sensitive to the size of the second N-substituent on the squaraine.<sup>29</sup> Thus, to maintain rapid threading it was crucial that at least one end of the squaraine structure have a small Nmethyl group. The precursor squaraine bis-alkynes were prepared from appropriate N-benzyl-2-aminothiophene building blocks using methodology developed by Hartmann and coworkers.<sup>71</sup> Detailed synthetic procedures and compound characterization data are provided in the Supporting Information.

Association Studies Using SQ1–SQ4. For each of the dyes, SQ1–SQ4, the structure of the threaded complex was characterized using previously validated one- and two-dimensional NMR, absorption, and fluorescence spectroscopy methods.<sup>29</sup> In each case, threading of M produced the following diagnostic changes in NMR chemical shifts: upfield migration of cyclophane anthracene protons D and E, upfield migration of squaraine protons 1 and 2, and downfield migration of cyclophane isophthalamide protons A and B. For the complexes that encapsulate symmetric dyes SQ1 and SQ3, the cyclophane protons A and B are each single peaks, indicating unambiguously that the encapsulated squaraine adopts a trans conformation with regard the orientation of its thiophene units, an assignment that is consistent with our previous observations.<sup>27,29</sup> With the complexes that encapsulate

Table 1. Bind	ding Constants	and Threading	Rate Constants i	in H <sub>2</sub> O	at 25 °C
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entry	squaraine	$K_{\rm a} (10^9 {\rm M}^{-1})^a$	$\Delta G \; (\text{kcal } M^{-1})^a$	$\Delta H \ (\text{kcal } M^{-1})^{b}$	$T\Delta S$ (kcal M <sup>-1</sup> )	$k_{\rm on}~({\rm M}^{-1}~{\rm s}^{-1})$	$t_{1/2} (s)^{e}$
1	SQ1	$1.0 \pm 0.2$	$-12.3 \pm 0.1$	$-12.3 \pm 0.3$	0.0	$(7.2 \pm 0.3) \times 10^{8}$	0.014
2	SQ2	6.4 ± 1.7	$-13.4 \pm 0.1$	$-13.4 \pm 0.1$	0.0	$(1.2 \pm 0.1) \times 10^8$	0.0083
3	SQ3	$8.5 \pm 1.7$	$-13.5 \pm 0.1$	$-11.9 \pm 0.8$	+1.6	$(7.4 \pm 2.2) \times 10^8$	0.0014
4	SQ4	42 ± 6	$-14.5 \pm 0.1$	с	с	$0.02^{d}$	27
5	SQ5	$51 \pm 1$	$-14.6 \pm 0.1$	$-14.0 \pm 0.6$	+0.6	$(7.9 \pm 0.3) \times 10^7$	0.013
1. 6	11 0	h h		6.604	I TTG I I da		

<sup>*a*</sup>Measured by fluorescent titration. <sup>*b*</sup>Measured by ITC. <sup>*c*</sup>Self-aggregation of **SQ4** prevented ITC analysis. <sup>*d*</sup>First-order threading kinetics with the unit  $s^{-1}$ . <sup>*e*</sup>Half-life for the threading process when the starting concentrations of each component is 1  $\mu$ M.

unsymmetric dyes SQ2 and SQ4, the A and B protons are each observed as two equal-intensity singlets. In principle, this could be due to cis or trans conformations for the encapsulated squaraine, but we assume trans to match the trend seen with the symmetric dyes. The complexation-induced changes in chemical shifts are illustrated in Figure 1a, which compares <sup>1</sup>H NMR spectra for free M, free SQ4, and the M⊃SQ4 complex. Shown in Figure 1b is a 2D NOESY spectrum with the blue circles highlighting peaks indicating spatial proximity of squaraine and cyclophane protons. Moreover, cross-relaxation between the N-benzyl protons of SQ4 and the anthracene and isophthalamide protons of M is additional evidence for close proximity due to aromatic stacking. The same NMR chemical shift changes and NOE signatures were found for the complexes  $M \supset SQ1$ ,  $M \supset SQ2$ , and  $M \supset SQ3$  (Figures S1-S7, SI).

Independent evidence that the associated complexes were threaded structures was gained by observing characteristic changes in the absorption and fluorescence spectra. Shown in Figure 1c,d are representative optical data for  $M \supset SQ4$ . There is a 30 nm red-shift of the squaraine absorption and emission maxima caused by complexation. In addition, there is a very efficient internal energy transfer process, where excitation of the anthracene sidewalls in  $M \supset SQ1$  with 375 nm light produces very strong emission of 710 nm light by the encapsulated squaraine dye (see Scheme 1b for illustration). The same spectral signatures were exhibited by  $M \supset SQ1$ ,  $M \supset SQ2$ , and  $M \supset SQ3$  (Figures S11–S27, SI).

Listed in Table 1 are the thermodynamic and kinetic properties for threading of M by each squaraine dye in water.  $K_{\rm a}$  values were initially measured by conducting direct fluorescence titration experiments, but the values were so high that it was not possible to accurately fit the sharp titration isotherms. More accurate measurements were gained by conducting competitive fluorescence titrations that added M to aqueous solutions containing a squaraine dye and a large excess of the competing guest F1 ( $K_a = 1.6 \times 10^4 \text{ M}^{-1}$ ; see the Supporting Information for all details).<sup>27</sup> Entry 1 in Table 1 shows data for the prototype squaraine guest SQ1 with no appended N-benzyl group and thus is a comparative benchmark with  $K_a = 1.0 \times 10^9$  M<sup>-1.29</sup> The new squaraines SQ2 and SQ3 each have two appended N-benzyl groups, and K<sub>a</sub> was enhanced by factors of 6.4 and 8.5, respectively (see entries 2 and 3). The structure of SQ4 possesses three N-benzyl groups, and there was a 42-fold enhancement in affinity with  $K_a = 4.2 \times$  $10^{10} \text{ M}^{-1}$  ( $K_{d}$  = 24 pM), consistent with an additive N-benzyl effect on  $K_a$ . The association thermodynamics were measured using isothermal titration calorimetry (ITC). The association constants were too high for accurate determination using our microcalorimeter, but the change in enthalpy ( $\Delta H$ ) for each threading system was determined by a single injection experiment that added a stoichiometric excess of squaraine to

a solution of M in water and measured the heat generated. In each case, the free energy for threading of M is dominated by a highly favorable enthalpic driving force with a very small or negligible entropic penalty.

The threading rate constant  $(k_{on})$  for each system was measured by monitoring the change in squaraine fluorescence over time after rapid mixing with M in a stopped-flow device. The value of  $k_{on}$  for SQ3  $(k_{on} = 7.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1})$  was the same as the value for SQ1, implying that passage of M over the flanking N-methyl-N-benzyl unit at either end of SQ3 does not raise the kinetic barrier for threading. In the case of unsymmetric SQ2,  $k_{on}$  was six times slower. Most likely this is a statistical effect since M cannot pass over the flanking N,Ndibenzyl unit but instead it must access the squaraine core of SQ2 from the opposite end. In contrast to the rapid threading of M by SQ1-SQ3, which in each case was complete in less than 1 s when the concentration of each component was 1  $\mu$ M, the high-affinity threading of M by SQ4 was substantially slower and took about 150 s to finish. Moreover, the threading curve fitted best to a first-order kinetic model instead of the second-order model (Figure 2a) exhibited by the other



**Figure 2.** Kinetic profiles showing the change in fluorescence ( $\lambda_{ex} = 690 \text{ nm}$ ,  $\lambda_{em} = 710 \text{ nm}$ ) during threading of **M** by **SQ4** in (a) H<sub>2</sub>O or (b) FBS. (c) Cartoon showing the slow escape of monomeric **SQ4** from an aggregate in H<sub>2</sub>O as the rate-determining step followed by rapid threading of **M** to form **M** $\supset$ **SQ4**.

squaraines (see the Supporting Information). It is apparent that threading of M by SQ4 occurs by a different mechanism than that of SQ1–SQ3. The absorption spectrum of free SQ4 in water shows a very broad and strongly blue-shifted peak indicative of extensive squaraine self-aggregation (Figure 1c), and this conclusion was confirmed by observation, using

dynamic light scattering, of soluble aggregates with hydrodynamic diameter ~70 nm and larger (Figure S25, SI). We rationalize this collection of results with the two-step kinetic model in Figure 2c. That is, rapid threading of M by monomeric SQ4 is preceded by slow and rate-determining release of the SQ4 from a micelle-like aggregate. To test this hypothesis, we examined the threading of M by SQ4 in FBS, where the squaraine aggregation band is absent because SQ4 associates with the albumin and other proteins in the serum. Under these conditions, the time-dependent threading curve fitted best to a second-order kinetic model (Figure 2b). The change in the threading kinetic order was confirmed by independent measurements of threading half-life. In pure water, the threading half-life was observed to be independent of the concentration of M and SQ4, which is consistent with a firstorder process (Figure S24, SI). In contrast, the threading halflife in FBS was proportional to the concentrations of M and SQ4, thus indicative of a second-order process (Figure S26, SI). Although the threading of M with SQ4 in FBS obeyed secondorder kinetics, it should be noted that  $K_a$  was reduced to 8.5  $\times$  $10^6 \text{ M}^{-1}$ .

Association Studies Using SQ5. While the very high  $K_a$  for threading of M by SQ4 in pure water was pleasing, there were two supramolecular performance limitations with hydrophobic SQ4, (a) self-aggregation of SQ4 in water, which greatly slowed  $k_{on}$ , and (b) association of SQ4 with albumin and other proteins in FBS, which greatly lowered  $K_a$ . To obviate these drawbacks we designed SQ5 with a PEG-2000 chain attached to each of the three *N*-benzylic groups and acquired the appropriate spectral data confirming formation of M $\supset$ SQ5 (Figures S8 and S28–S32, SI). The absorption band for free SQ5 in water was much narrower than that for SQ4 (compare Figures 1a and 3a), indicating that the attached PEG-2000



Figure 3. (a) Absorption and fluorescence ( $\lambda_{ex} = 600 \text{ nm}$ ) in H<sub>2</sub>O at 25 °C. (b) Comparison of normalized competitive titration curves for threading of M by SQ1 or by SQ5 each in the presence of excess competitor F1 in H<sub>2</sub>O at 25 °C (see Figures S11 and S30 of the SI for details).

chains greatly reduced squaraine self-aggregation, and we were delighted to find extremely high affinity in water ( $K_a = 5.1 \times 10^{10} \text{ M}^{-1}$ ,  $K_d = 20 \text{ pM}$ ) combined with very rapid threading  $k_{on} = 7.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  (Table 1). Thus, the structural optimization process that systematically converted **SQ1** into **SQ5** led to a dramatic 50-fold increase in  $K_a$  for the threading of **M** (see Figure 3b for a visual comparison of the normalized titration curves) with no decrease in  $k_{on}$ . This exciting result prompted us to quantify the threading processes in FBS, which is known to strongly attenuate the association of partially hydrophobic host/guest pairs.<sup>20,72</sup> As shown in Table 2, the values for  $K_a$  and  $k_{on}$  for threading of **M** by **SQ5** in FBS were substantially higher than the values observed for **SQ4**. More

Table 2. Association Constants  $(K_a)$  and Threading Rate Constants  $(k_{on})$  in FBS at 25 °C

entry	squaraine	$K_{\rm a} \ (10^6 \ { m M}^{-1})$	$k_{\rm on} \ (10^4 \ {\rm M}^{-1} \ {\rm s}^{-1})$	$t_{1/2} (s)^{a}$		
1	SQ1	$1.4 \pm 0.1$	$63 \pm 8$	1.6		
2	SQ4	$8.5 \pm 0.8$	$0.6 \pm 0.03$	167		
3	SQ5	$72 \pm 22$	$7.8 \pm 0.6$	13		
"Half-life for threading process when the starting concentrations of						
both components is 1 $\mu$ M.						

specifically,  $K_a$  was about 9 times higher and  $k_{on}$  was about 13 times faster, demonstrating the effectiveness of the two additional PEG-2000 chains in **SQ5** to shield the squaraine and block competitive association with the abundant salts, small molecules, amphiphiles, and various proteins that are present in the FBS (the composition of FBS is listed in Table S3, SI).<sup>73</sup> The high potential of **M** and **SQ5** as a next-generation affinity pair for in situ capture applications in biological media is highlighted by the fact that formation of **M** $\supset$ **SQ5** in FBS is quantitative and essentially complete within a few minutes when the concentration of each component is greater than 200 nM. Furthermore, the squaraine capture process is easily detected by a large ratiometric change in the deep-red fluorescence spectrum.

The monomeric, unaggregated nature of SQ5 in water allowed determination of  $\Delta H$  for threading of M using ITC, and the observed value of  $-14.0 \pm 0.6$  kcal M<sup>-1</sup> was 1.7 kcal  $M^{-1}$  more exothermic than  $\Delta H$  for threading of M by SQ1. Thus, the extra aromatic stacking within M⊃SQ5 provides the threaded [2]pseudorotaxane complex with additional enthalpic stabilization without incurring a compensatory entropic penalty. We reasoned that if the aromatic stacking was driven by attractive electrostatic and dispersion interactions within  $M \supset SQ5$ , then the same stabilization effect should be seen in a low dielectric organic solvent.<sup>74</sup> Therefore, we measured  $K_a$  for threading of an organic soluble version of M by SQ1 and SQ5 in chloroform at 25 °C and found that the K<sub>2</sub> values of (2.5  $\pm$  $(0.7) \times 10^7$  and  $(3.8 \pm 0.2) \times 10^7$  M<sup>-1</sup> were almost identical (Figures S33 and S34, Table S2, SI). It appears that the stabilization provided by the additional aromatic stacking within  $M \supset SQ5$  is a hydrophobic effect, where presumably the low entropic cost to achieve guest back-folding and aromatic stacking is offset by release of solvating water molecules into bulk solution.<sup>55</sup> As illustrated in Scheme 4, the three N-benzyl groups in SQ5 surround M and stabilize the threaded complex. However, the stabilization is not solely due to a single, lowenergy threaded structure with three simultaneous aromatic stacking interactions. The MD trajectory for  $M{\supset}SQ4$  (see the Supporting Information), which is a model of  $M \supset SQ5$ , shows that the [2]pseudorotaxane populates multiple coconformations with one or more stacking interactions. Thus, complex stabilization due to guest back-folding is favored statistically by the high effective molarity of N-benzyl groups that surround M and provide dynamic stacking interactions without incurring a major entropic penalty.75,76

Looking forward, it seems likely that guest back-folding with concomitant aromatic stacking, especially edge-to-face  $CH-\pi$  stacking, can become a general molecular design strategy to stabilize the threaded complexes of many structurally related cyclophane hosts<sup>19,77–80</sup> or alternatively host macrocycles that are not cyclophanes but have appended aromatic arms.<sup>44</sup> Conceptually, guest back-folding is related to the better known approach of multivalent association using oligomeric guest

Scheme 4. Stabilization of M $\supset$ SQ5 Due to Guest Back-Folding Is Favored Statistically by the High Effective Molarity of N-Benzyl Groups That Surround M<sup>a</sup>



<sup>*a*</sup>The threaded complex can sample multiple co-conformations, each stabilized by one or more stacking interactions.

structures and complementary multicavity hosts.<sup>81</sup> With both design strategies, the structure of the linker that connects the multiple binding units is crucial, with high affinity favored by linkers that balance structural preorganization with adaptability.<sup>82,83</sup>

# CONCLUSION

The tangible outcome of this study is the discovery of a new deep-red fluorescent dye, SQ5, that threads M in water and forms a 2 pseudorotaxane complex with picomolar affinity ( $K_a$ =  $5.1 \times 10^{10} \text{ M}^{-1}$ ,  $K_{\text{d}} = 20 \text{ pM}$ ) and an extremely rapid rate of threading ( $k_{\text{on}} = 7.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ). The high affinity and rapid threading seen in FBS ( $K_a = 7.2 \times 10^7 \text{ M}^{-1}$ ,  $k_{on} = 7.8 \times 10^4 \text{ M}^{-1}$  $s^{-1}$ ) is a performance milestone for an organic host/guest system in biological fluid and is especially noteworthy because the binding interface does not involve salt bridges. The impressive supramolecular and deep-red fluorescence properties of SQ5 will facilitate future efforts to exploit Synthavidin technology for a wide range of imaging and diagnostics applications.<sup>1</sup> The expected orthogonality with other strong binding pairs such as biotin/(strept)avidin and cucurbituril/ alkylammonium should allow them to be used simultaneously in the same sample. From a more fundamental perspective, this study is the first to show how guest back-folding can be used as a supramolecular strategy to stabilize a threaded [2]pseudorotaxane complex in water. The computer-guided rational design process started with SQ1 and systematically added three N-benzylic groups to eventually produce SQ5, which exhibited a 50-fold increase in  $K_a$  ( $\Delta\Delta G = 2.3$  kcal  $mol^{-1}$ ) for threading of **M** with no decrease in the extremely rapid rate of complex assembly. The appended N-benzyl groups on SQ5 form favorable face-to-face  $\pi - \pi$  and edge-to-face CH- $\pi$  stacking interactions with the periphery of M that provide enthalpic stabilization with negligible entropic penalty. MD simulations show that all three N-benzyl groups rarely contact M at the same time. Rather, the [2]pseudorotaxane complex populates multiple coconformations, each with its own number of stabilizing stacking interactions, and the complex is favored statistically by the high effective molarity of N-benzyl groups that surround M. It seems likely that  $K_a$  can be improved even more with additional rounds of squaraine

optimization to increase the surface area of the appended aromatic groups and alter the linkers. However, it needs to be emphasized that this optimization process is a multiparameter problem, the solution of which must simultaneously consider several factors. For example, any aromatic groups appended to the squaraine dye must not cause strong intramolecular folding or intermolecular aggregation of the dye before threading of M and must not sterically inhibit the rate of threading. With thoughtful design, these potential caveats can be avoided and we are hopeful that guest back-folding will become a general strategy for stabilizing threaded [2]pseudorotaxanes in water. Moreover, guest back-folding will increasingly be used to control the tertiary structures of threaded and permanently interlocked molecules, which in turn will influence the molecular dynamics (e.g., pirouetting, shuttling)<sup>84-86</sup> and ultimately the performance of these systems as molecular machines.

## EXPERIMENTAL SECTION

**Synthesis.** See the Supporting Information for synthetic procedures and compounds characterization.

**Molecular Modeling.** The starting coordinates for the computer modeling were derived from X-ray crystal structures homologous to closely related [2]pseudorotaxane and [2]rotaxane structures.<sup>67–69</sup> The truncated structures used for modeling are provided in the Supporting Information. In each case, the starting structure of the threaded complex was optimized by the semiempirical method at the PM7 level using the MOPAC program,<sup>87</sup> followed by DFT optimization at the BLYP-D3-gCP/def2-SVP level using the ORCA program.<sup>88</sup> Reduced density gradient analysis was performed by the Multiwfn program and the noncovalent interaction surface was visualized with Visual Molecular Dynamics.<sup>89</sup>

Molecular dynamics (MD) simulations started with the gas-phase DFT-optimized structures. Restrained electrostatic potential charges for the macrocycle and the encapsulated squaraine were derived using the R.E.D. Development Server<sup>90</sup> and atom types assigned using the antechamber module of AmberTools 16.91 The xleap module was used to set up each calculation. A TIP3P water box extending 12 Å in each direction was added and used for all MD simulations, which employed NAMD (version 2.11)<sup>92</sup> with the gaff2 force field. A cutoff of 10 Å was used for the treatment of nonbonded interactions. Prior to the MD simulations, a two-step minimization procedure was used. First, the solvent was minimized while the solute was constrained. Then, the entire system was minimized over 5000 conjugate gradient steps. Heating to 300 K was performed in the NVT (constant number of particles, volume, and temperature) ensemble with constraints on the solute. This was followed by 2 ns of equilibration and a 100 ns production run in the NPT ensemble (constant number of particles, pressure, and temperature). Simulations used 2 fs time steps and the SHAKE algorithm was used to constrain bonds between heavy atoms and hydrogen. Coordinates were written every picosecond. MM-PBSA and MM-GBSA postprocessing of the trajectories was performed using the MMPBSA.py script of AmberTools 16. Analysis was performed on 2000 equally spaced snapshots of the simulation, and default settings were used. The raw and relative binding energies are provided in Table S4 (SI). A statistical analysis of coconformations that lacked any aromatic stacking interactions examined 100 000 frames of the simulation, and the results are listed in Table S5 (SI). A stacking interaction was counted when the distance between any phenyl carbon atom in a squaraine N-benzyl ring was less than 4 Å from any atom in M.

Association Measurements. Previously described methods were employed.<sup>27,29</sup> Absorption spectra were collected on an Evolution 201 UV/vis spectrometer with Thermo Insight software. Fluorescence spectra were taken and analyzed on a Horiba Fluoromax-4 fluorometer with FluoroEssence software. Spectra were obtained using spectrophotometric grade solvent at 25 °C with either glass or quartz cuvettes (1 mL, 10 mm path length) accordingly. Fetal bovine serum was

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purchased from Atlanta Biologicals (catalogue number S11150). Quantum yield measurements were performed by using methylene blue ( $\Phi_f = 0.02$  in H<sub>2</sub>O) as standard. The concentrations of methylene blue and squaraine dyes were adjusted to the absorption value 0.08 at 600 nm. The fluorescence spectrum of each solution was obtained with excitation at 600 nm, and the integrated area was used in the quantum yield calculation. The estimated error of this method is  $\pm 10\%$ .

For direct fluorescence titration experiments, stock solutions of squaraine guest, generally  $0.01-3 \mu M$ , were prepared, and stock solutions of host M (0.6-60  $\mu$ M) were made by using guest solution as solvent (to keep the concentration of guest constant during the titration). A solution of guest (1 mL) was placed in the cuvette (maintained at 25 °C) and M was added in increments. After each addition, the fluorescence intensity ( $\lambda_{ex} = 690 \text{ nm}$ ,  $\lambda_{em} = 710 \text{ nm}$ ) was measured. Association constants ( $K_a$ ) were calculated by fitting the titration data with a nonlinear least-squares fitting equation for 1:1 binding within Origin Lab 8.6 software. For competitive fluorescence titration experiments, stock solutions of a M/F1 mixture, generally 0.01–0.5  $\mu$ M, were prepared and stock solutions of squaraine guest  $(0.2-10 \,\mu\text{M})$  were made by using the mixed M/F1 solution as solvent (to keep the concentration of host constant during the titration). A solution of M/F1 mixture (1 mL) was placed in the cuvette and guest solution was injected as small aliquots. The fluorescence intensity ( $\lambda_{ex}$ = 690 nm,  $\lambda_{em}$  = 710 nm) was measured. At this excitation wavelength, the squaraine/macrocycle complex is fluorescent, and the macrocycle and squaraine dye are transparent. The association constants were calculated by fitting the titration data with a nonlinear least-squares fitting equation for competitive binding within Origin Lab 8.6 software.

**Kinetic Measurements.** The rate constant for squaraine threading of **M** ( $k_{on}$ ) was measured using a SFA-20 M stopped-flow device (empirical dead time <8 ms) and a previously described method.<sup>27</sup> Equal volume solutions of squaraine and **M** were mixed by the device (maintained at 25 °C) and the fluorescence ( $\lambda_{ex} = 680 \text{ nm}$ ,  $\lambda_{em} = 695 \text{ nm}$ ) intensity was measured over time. The kinetic profiles were fitted using either first-order and/or second-order kinetics equations by Origin Lab 8.6 software.

**Isothermal Titration Calorimetry.** A solution of M ( $300 \ \mu$ L, 0.03 mM) in pure water was injected into the sample cell (volume 200  $\mu$ L) of a MicroCal PEAQ-ITC at 25 °C. A solution of squaraine guest (1.5 mol equiv) in pure water was injected into the cell. The heat generation was monitored over time and the peak integrated to give the overall heat. To remove the heat effect due to dilution, an identical aliquot of squaraine guest solution was added to pure water and the heat generation was monitored over time. The binding enthalpy ( $\Delta$ H) was obtained by subtracting the solvent dilution heat from the overall heat.

# ASSOCIATED CONTENT

#### **S** Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b12991.

Synthetic procedures, compound characterization and spectral data, experimental methods, composition of FBS, and molecular modeling details (PDF)

A total of 1000 snapshots from a 100 ns MD simulation of a complex comprised of host **M** threaded by squaraine guest **SQ1** (AVI)

A total of 1000 snapshots from a 100 ns MD simulation of a complex comprised of host M threaded by squaraine guest SQ2 (AVI)

A total of 1000 snapshots from a 100 ns MD simulation of a complex comprised of host M threaded by squaraine guest SQ3 (AVI)

A total of 1000 snapshots from a 100 ns MD simulation of a complex comprised of host M threaded by squaraine guest SQ4 (AVI)

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#### Notes

The authors declare no competing financial interest.

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