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Self-Assembly of Fluorescent Inclusion Complexes in Competitive Media Including the Interior of Living Cells

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Abstract

Anthracene containing tetralactam macrocycles are prepared and found to have an extremely high affinity for squaraine dyes in chloroform (log K_a = 5.2). Simply mixing the two components produces highly fluorescent, near-infrared inclusion complexes in quantitative yield. An X-ray crystal structure shows the expected hydrogen bonding between the squaraine oxygens and the macrocycle amide NH residues, and a high degree of cofacial aromatic stacking. The kinetics and thermodynamics of the assembly process are very sensitive to small structural changes in the binding partners. For example, a macrocycle containing two isophthalamide units associates with the squaraine dye in chloroform 400,000 times faster than an analogous macrocycle containing two 2,6-dicarboxamidopyridine units. Squaraine encapsulation also occurs in highly competitive media such as mixed aqueous/organic solutions, vesicle membranes, and the organelles within living cells. The highly fluorescent inclusion complexes possess emergent properties; that is, compared to the building blocks, the complexes have improved chemical stabilities, red-shifted absorption/emission maxima, and different cell localization propensities. These are useful properties for new classes of near-infrared fluorescent imaging probes.

Introduction

The selective and reversible self-assembly of biomolecules is a central requirement for cell function as demonstrated by the recognition properties of DNA, bilayer membranes, enzyme/substrates, and protein receptor/ligand complexes. Indeed, a major goal of the pharmaceutical industry is to discover “biologically active” drug molecules that selectively inhibit or activate these recognition processes. In addition, there is an articulated need to develop synthetic host:guest systems that can operate in biological media.[1] A notable success has been the invention of synthetic chemosensors for cell imaging; that is, small fluorescent host molecules that can recognize and sense the presence of cellular analytes such as metal cations, anions, and biomolecules.[2] In contrast to this success, there are comparatively few examples of synthetic host:guest partners that can selectively self-assemble in complicated biological environments and form discrete complexes with well-defined structures. The focus of this article is on uncharged organic molecules with limited water solubility,[3] which means that in biological samples they will be confined to hydrophobic environments such as protein surfaces,[4] micelles,[5] and the interior of bilayer membranes.[6] Programmed host-guest assembly under these competitive conditions is particularly challenging because the association must be driven by the relatively weak non-covalent interactions of hydrogen bonding, aromatic stacking, and dispersion forces.[7]

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Furthermore, it is difficult to prove unambiguously that assembly has occurred as designed because common spectroscopic methods (*e.g.*, NMR, UV absorption, etc) are generally not applicable in these complex matrices.[8] Therefore, it is particularly helpful if the host:guest complex can be identified *in situ* by a unique and sensitive spectroscopic signature.[9]

Here, we describe a novel, photoactive host:guest system whose self-assembly can be monitored directly by the complexation-induced changes in its photophysical properties.[10] The guest molecules are squaraines, intensely fluorescent near-infrared dyes that have been investigated for potential applications in many photonic based technologies.[11,12] Recently, we described the synthesis of permanently interlocked squaraine-rotaxanes, **1** (Chart 1) and demonstrated that encapsulation of the squaraine fluorophore within a phenylene tetralactam macrocycle provides substantial steric protection of the squaraine chromophore and inhibits aggregation induced self-quenching, as well as attack by biological nucleophiles.[13] In addition, we have shown that squaraine-rotaxanes have tremendous promise as extremely stable molecular probes for fluorescence bioimaging.[14] These first generation squaraine-rotaxanes were synthesized in yields of 10–30% using a Leigh-type clipping reaction (a templated macrocyclization around the squaraine dye).[15] Preparation by a direct self-assembly of the two components (macrocycle and squaraine) does not work with the phenylene tetralactam macrocycle (*i.e.*, the macrocycle in rotaxane structure **1**) because it is quite insoluble.[16] To circumvent this problem we designed the novel anthrylene tetralactam macrocycles **2a** and **2b**.[17] We find that these macrocycles are soluble in many different solvents, and they can associate strongly with squaraine dyes in weakly polar organic solvents and aqueous/organic mixtures. Furthermore, the assembly process occurs in highly competitive biological media such as bilayer membranes and the organelles within a living cell. The assembly process produces host:guest inclusion complexes that possess emergent properties such as improved chemical stability, red-shifted absorption/emission maxima, and altered intracellular targeting. Since one of the practical goals of this research is to produce new classes of near-infrared fluorescent imaging probes, these properties are highly desired.[18]

Results

Structure of Inclusion Complexes

The novel 2+2 macrocycle **2a**, with 2,6-dicarboxamidopyridine and 9,10-anthrylene components, was prepared in 30% yield by a straightforward, pseudo-dilution macrocyclization reaction. Simply stirring a mixture of macrocycle **2a** (9 mM) and an excess of poorly soluble squaraine **3a** in $CDCl_3$ produces the corresponding inclusion complex **2a⊃3a** in quantitative yield as judged by ^{1}H NMR spectroscopy. The solution-state ^{1}H NMR spectrum exhibits a number of upfield chemical shifts that are highly characteristic of dye encapsulation inside the macrocycle (see supporting information). Additional structural characterization was gained from single crystal X-ray diffraction analysis of **2a⊃3a** (Figure 1). Notable features of the solid-state structure include the expected hydrogen bonding between the squaraine oxygens and the macrocycle amide NH residues, and the high degree of cofacial aromatic stacking.[19] An unexpected feature is the macrocyclic conformation, which is almost perfectly flat and not the usual chair conformer that is seen with the phenylene macrocycle in previously published squaraine-rotaxane structures (the macrocyclic conformation is discussed further below).[13,20]

Self-Assembly in Weakly Polar Organic Solvent

Inclusion complex **2a⊃3a** is classified as a pseudo-rotaxane because there is partial dissociation when a chloroform solution is diluted to 1 μM. To inhibit dissociation, we prepared the symmetrical bis-alkyne squaraine **3b**, which has relatively larger and more

flexible end-groups. As expected,[21] a 1:1 mixture of **2a** and **3b** in chloroform forms **2a⊃3b** in quantitative yield, but with a very slow rate constant (second order rate constant is 2×10^{-4} M^{-1} s^{-1} at 25° C, the half-life is 8.5 days). However, the assembly process can be pushed to completion within a few days by heating the sample at 120° C.[22] A room temperature solution of rotaxane **2a⊃3b** does not dissociate upon extreme dilution in organic solvents, which allows its photophysical properties to be measured. A listing of the relevant absorption and emission maxima is provided in Table 1. It is apparent that encapsulation of the squaraine dye inside the anthrylene macrocycle induces a much larger red-shift effect than the phenylene macrocycle. Shown in Figure 2A is the absorption spectrum for anthrylene rotaxane **2a⊃3b**, and the fluorescence emission that is observed upon excitation at 580 nm (squaraine chromophore). Shown in Figure 2B are the fluorescence emission upon excitation at 350 nm (anthrylene chromophore), overlaid with the emission spectrum for an equimolar mixture of unassembled anthrylene macrocycle **2a** and squaraine **3b**. While the free macrocycle is highly fluorescent, there is no similar anthrylene emission band when it is part of the rotaxane.[23,24]

While the quantitative formation of stable rotaxane **2a⊃3b** from a mixture of **2a** and **3b** is very useful, the slow kinetics of the self-assembly process is a potential limitation for many eventual applications. We suspected that a contributing factor was the known propensity of the 2,6-dicarboxamidopyridine unit in **2a** to form a locked and less-flexible macrocyclic conformation due to internal hydrogen bonding between the pyridyl nitrogen and the amide NH residues.[21] Therefore, we prepared the 2+2 isophthalamide macrocycle **2b** with the expectation that it would be more flexible and exhibit faster association kinetics. We were gratified to find that simply dissolving a 1:1 mixture of macrocycle **2b** and squaraine **3b** in $CDCl_3$ (6 mM each) at room temperature leads to extremely rapid and quantitative formation of **2b⊃3b**. Like **2a⊃3b**, the absorption and emission maxima for **2b⊃3b** are significantly red-shifted when compared to the parent squaraine **3b** (Table 1); however, unlike the stable **2a⊃3b**, there is measurable dissociation upon dilution of a chloroform solution (*i.e.*, **2b⊃3b** is a pseudo-rotaxane). The encapsulation-induced change in absorption maxima allowed direct measurement of association kinetics and thermodynamics. The association constant for a mixture of **2b** and **3b** to form **2b⊃3b** is $(1.8\pm0.4)\times10^5$ M^{-1} in chloroform and the initial second order rate constant is 80 M^{-1} s^{-1} at 25° C. In other words, association to form the pseudo-rotaxane **2b⊃3b** is essentially quantitative when the concentrations of **2b** and **3b** are above 100 μM. Furthermore, the rate of association of squaraine **3b** with isophthalamide macrocycle **2b** is 400,000 times faster than the association with pyridyl macrocycle **2a**.[25]

Self-Assembly in Polar Solvents

There was no spectrometric evidence for squaraine encapsulation by macrocycle **3b** in the polar aprotic solvents acetonitrile and DMSO, which agrees with previous studies of uncharged inclusion processes that are driven primarily by hydrogen bonding and aromatic stacking.[26]

We next examined the self-assembly process in water/acetonitrile mixtures, although only semi-quantitatively, because the water slowly attacks and bleaches the unprotected monodisperse squaraine dye. In 1:1 water:acetonitrile there was no evidence for encapsulation of symmetric squaraine **3b** by macrocycle **2b**; however, the admixture of asymmetric squaraine **3c** and macrocycle **2b** produced substantial amounts of **2b⊃3c**, as judged by the time-dependent, red-shift in squaraine absorption and fluorescence maxima. The spectra in Figure 3 indicate that the binding equilibrium was reached after approximately seventeen minutes and that the association constant was $>10^5$ M^{-1}. The enhanced stability of asymmetric inclusion complex **2b⊃3c** compared to symmetric **2b⊃3b** is attributed to favorable stacking interactions in **2b⊃3c** between the bridging

isophthalimide units on the macrocycle and the terminal N,N′-dibenzyl rings on the squaraine (Figure 4). This structural model is supported by unpublished X-ray diffraction evidence, from three different squaraine-rotaxane crystal structures, that the encapsulating macrocycle can easily adopt a macrocyclic boat conformation.[27]

Self-Assembly in Vesicle Membranes

The uncharged squaraine dye **3b** is essentially non-fluorescent in water (due to aggregation and self-quenching) but it fluoresces strongly upon insertion into vesicle membranes. Figure 5 shows time-dependent absorption and fluorescence emission spectra after addition of **3b** to vesicles composed of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). The dye undergoes slow chemical bleaching, due to hydrolysis, over a period of several hours. In Figure 6 are the absorption and fluorescence emission obtained after **3b** is added to vesicles that contain the lipophilic macrocycle **2b**. In this case, the absorption and fluorescence maxima undergo time-dependent red-shifts of 17 and 35 nm, respectively, reflecting the bimolecular formation of **2b⊃3b**. The spectra for this inclusion complex hardly change with time, indicating that squaraine **3b** is strongly protected inside the macrocycle **2b**. A graphic illustration of the enhanced stability is the photograph in Figure 7, which shows the vesicles from Figures 5 and 6 after they have been allowed to sit for 24 h. At this point in time the vesicles have settled to the bottom of the tubes. The vesicles containing only squaraine **3b** have completely lost color due to hydrolytic bleaching; whereas, the vesicles containing the highly stable inclusion complex **2b⊃3b** retain their blue color.

Self-Assembly Inside Living Cells

The complexation-induced red-shift in absorbance and emission allows the self-assembly process to be monitored by multichannel epifluorescence microscopy. The appearance of an inclusion complex can be observed using a far-red filter set where there is no interference from the assembly components (macrocycle or squaraine). The anthrylene chromophore does not fluoresce after it forms an inclusion complex, so only the uncomplexed macrocycle (**2**) appears in the blue channel. In contrast, the uncomplexed squaraine dye (**3**) cannot be observed selectively because of bleed-through emission from the inclusion complex. Typical cell microscopy results are provided in Figure 8, which shows micrographs of live CHO (Chinese Hamster Ovary) cells 24 hours after treatment with separate aliquots of macrocycle **2b** and asymmetric squaraine **3c**. The blue-emitting, uncomplexed anthrylene macrocycle is seen in panel B, the far-red emitting **2b⊃3c** complex appears in panel C, and an overlay of the two is in panel D. It is apparent that both the uncomplexed macrocycle and the inclusion complex are inside the cells but they are not colocalized. The far-red emission of lipophilic **2b⊃3c** is confined to small punctate compartments; whereas, the blue emission of the uncomplexed macrocycle **2b** is distributed fairly evenly throughout the cell. The far-red staining due to **2b⊃3c** is quite photostable, and allows the acquisition of real time fluorescence movies (see supporting information). They indicate that the punctate compartments are vesicle-like structures, trafficking slowly inside the living cells.[28] Flow cytometry was used to show that the far-red fluorescence of **2b⊃3c** appears over time, as expected for a bimolecular association process. Shortly after treating the cells with separate aliquots of macrocycle **2b** and squaraine **3c**, the fraction of cells with far-red emission due to **2b⊃3c** was negligible, but it increased substantially after 24 hours (Figure 9). These live cell staining experiments were repeated with macrocycle **2b** and symmetric squaraine dye **3b** with a similar outcome (see supporting information).

Discussion

The X-ray structure of **2a⊃3a** shows that squaraine dyes **3** and anthrylene macrocycles **2** are a highly complementary host:guest pair (Figure 1). The very strong association in

chloroform solvent (K_a = 1.8 × 10^5 M^{-1}) is driven by the formation of four hydrogen bonds and extensive overlap of aromatic surfaces. Association cannot be detected in polar aprotic solvents like acetonitrile and DMSO because these solvents have a combination of high polarity that disrupts hydrogen bonds and moderate polarizability that disrupts aromatic stacking.[26] However, association is very strong (K_a > 10^5 M^{-1}) if the solvent includes significant amounts of water which provides a strong hydrophobic driving force for aromatic stacking.[29] Inclusion of the squaraine inside the macrocycle is a "snug fit" as reflected by the large changes in association kinetics that are induced by small structural variations in the bridging unit of the macrocycle (**2a** vs **2b**) or the terminal groups on the squaraine (**3a** vs **3b**). Although the macrocyclic hosts (**2**) have not yet been fully evaluated for guest selectivity, it is notable that the two assembly partners (*e.g.*, macrocycle **2b** and squaraine **3c**) can find each other within the extreme molecular complexity of a living cell. In other words, macrocycle/squaraine association does not appear to be greatly inhibited by the presence of biomolecules that contain aromatic rings or functional groups that can act as hydrogen bond acceptors.

The encapsulation of squaraine dyes (**3**) by macrocycles (**2**) produces inclusion complexes with emergent properties;[30] that is, the complexes have properties (*i.e.*, improved chemical stabilities, red-shifted absorption/emission maxima, different cell localization propensities) that are not the linear sum of an uncomplexed mixture of binding partners.[31] Squaraines **3b** and **3c** have terminal acetylene residues and in a future publication we will describe how click chemistry (copper catalyzed Huisgen cycloaddition) can be used to cap these end groups with azide-containing stopper groups and produce permanently interlocked squaraine-rotaxanes. Our eventual goal is to convert these highly stable dyes with bright, near-infrared fluorescence into new families of molecular probes for cell and whole animal imaging. The first generation squaraine-rotaxanes, **1**, with a phenylene tetralactam macrocycle have absorption/emission profiles (Table 1) that closely match the highly popular cyanine dye, Cy-5, whereas squaraine-rotaxanes with an anthrylene macrocycle have a red-shifted absorption/emission that matches the homologous cyanine, Cy-5.5.[32] The different emission colors of these two squaraine-rotaxane systems can easily be resolved by commercial fluorescence microscopes, flow cytometry instruments, and in vivo animal imaging stations using common filter sets. Furthermore, it may be possible to employ these two families of squaraine-rotaxane dyes as a donor/acceptor pair for photonic technologies that employ FRET (fluorescence resonance energy transfer), potentially inside living organisms.[33] Finally, the self-assembly process described here can be employed to make various interlocked fluorescent switches and shuttles, with applications in materials science and nanotechnology.[34]

Experimental

See supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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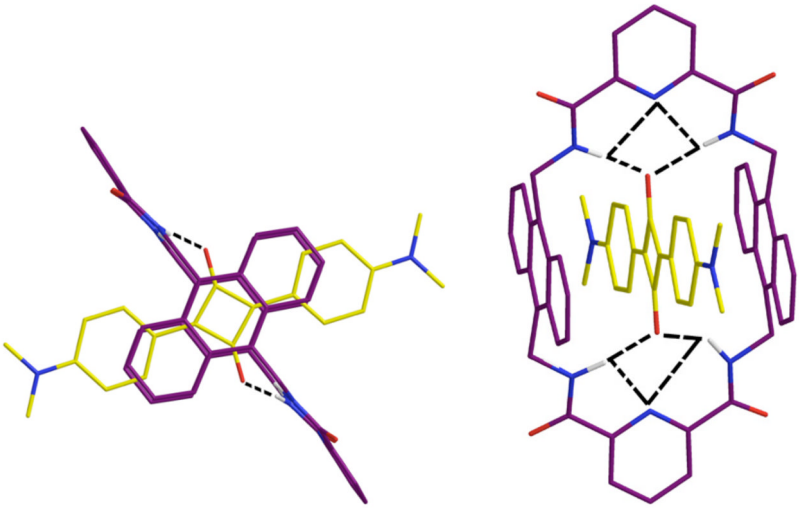

Figure 1.
Two views of the X-ray crystal structure of **2a⊃3a**.

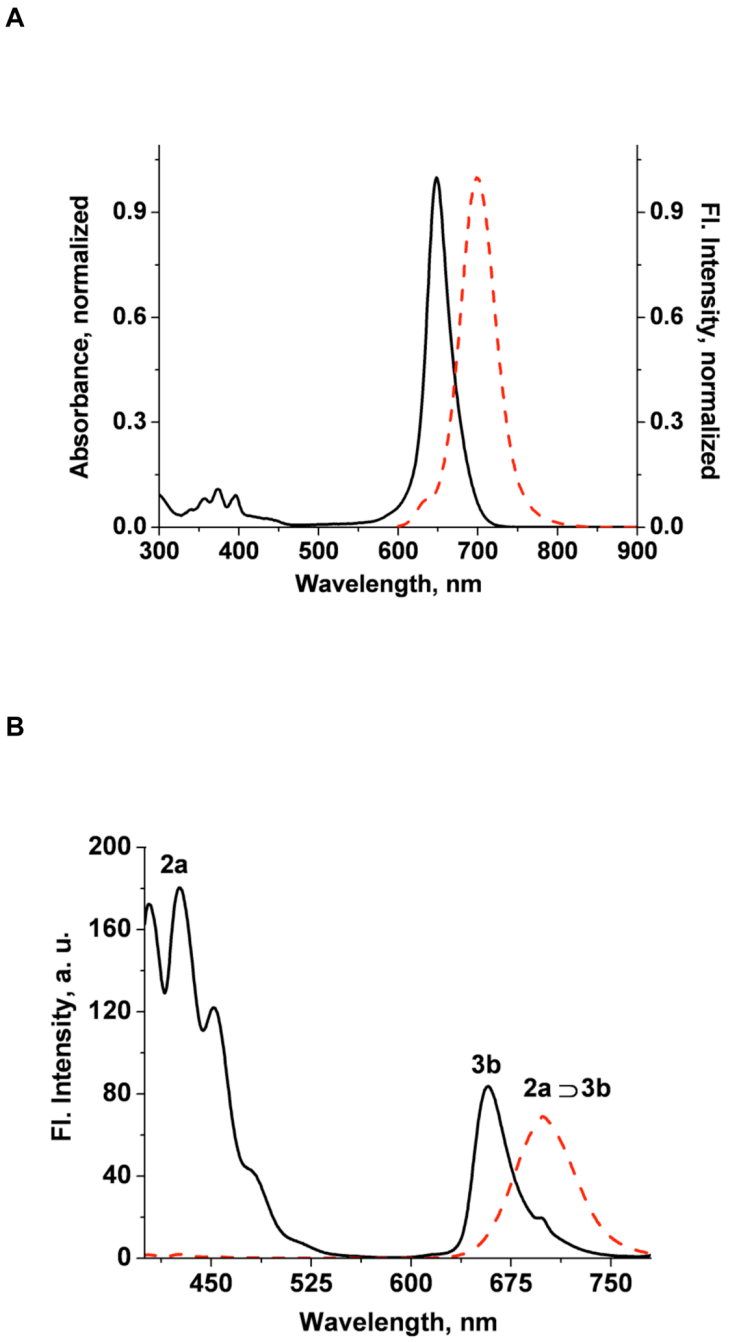

Figure 2.

(A) Absorption (black full line) and fluorescence emission (red dashed line, ex: 580 nm) spectra for rotaxane **2a⊃3b** in chloroform. (B) Fluorescence emission spectra (ex: 350 nm) for an equimolar mixture of **2a** and **3b** (both 5 μM) (black full line), and a solution of **2a⊃3b** (red dashed line) in chloroform.

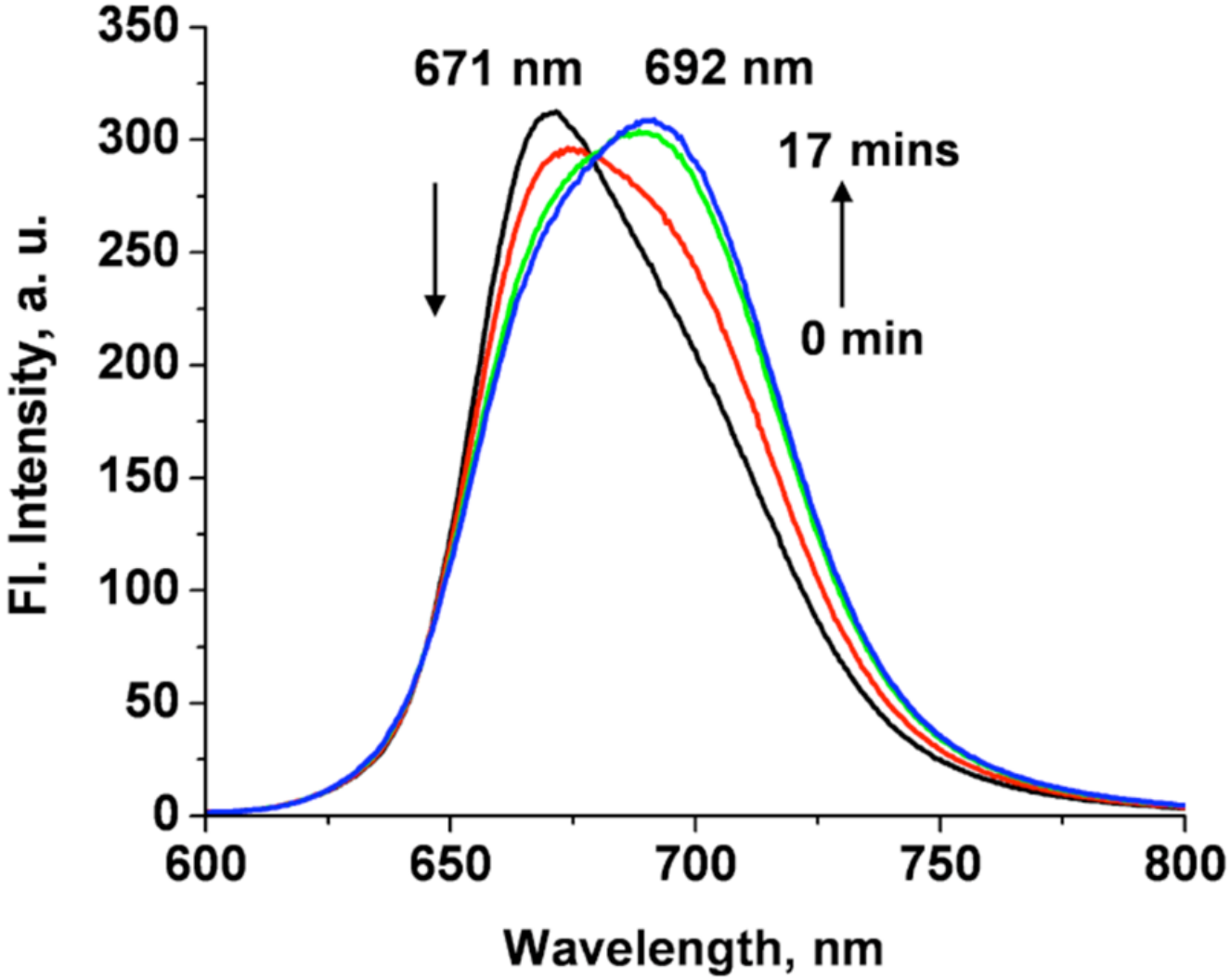

Figure 3.
Time dependent changes in fluorescence emission for association of unsymmetrical squaraine **3c** (10 μM) and macrocycle **2b** (10 μM) to form **2b⊃3c** in 1:1 water/acetonitrile at 25° C.

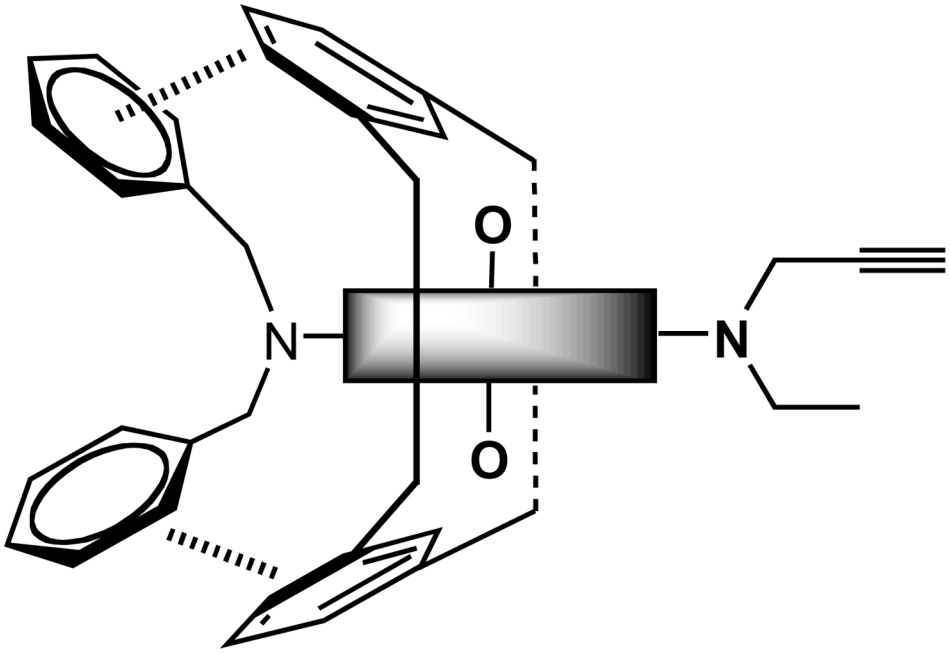

Figure 4.
The relative kinetic and thermodynamic stability of pseudo-rotaxane **2b⊃3c** is attributed to aromatic stacking interactions that are absent in **2b⊃3b**.

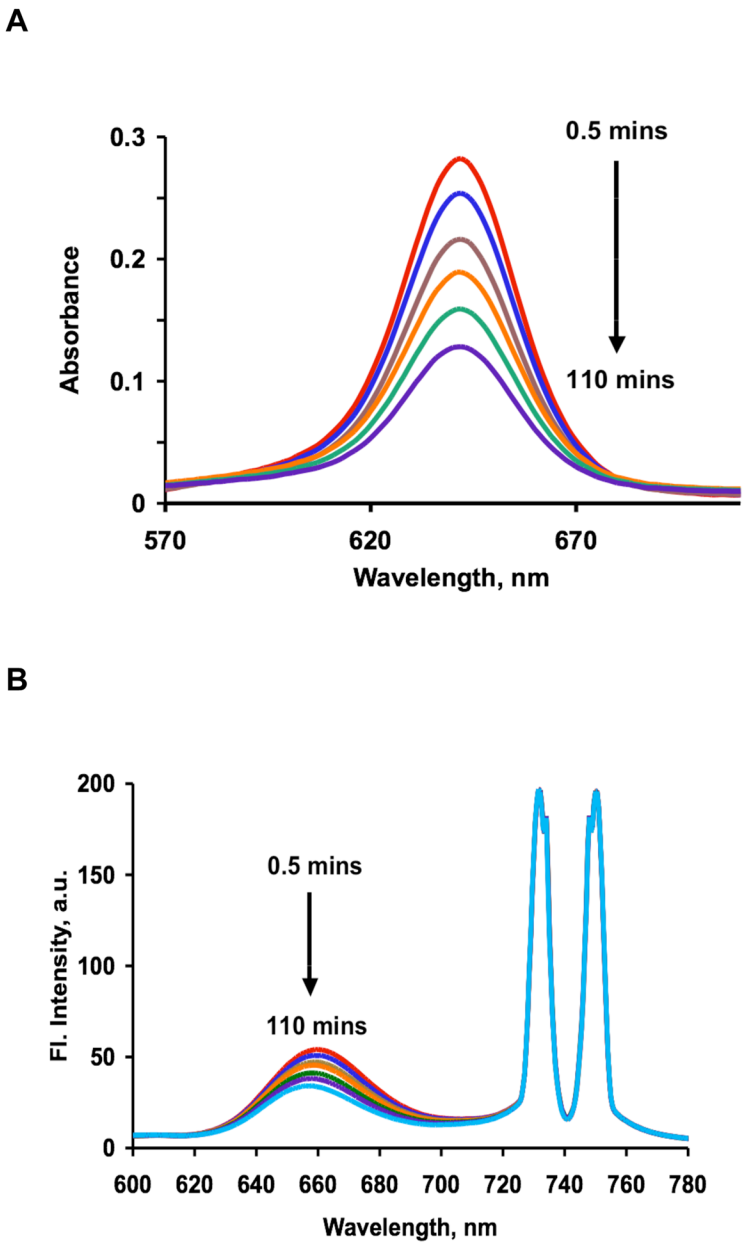

Figure 5.
Time dependent changes in absorbance (A) and fluorescence (B) maxima for squaraine **3b** (1.9 μM) after addition to vesicles (200 nm diameter) composed of POPC. The two fluorescence bands centered at 740 nm are artifacts due to the excitation at 370 nm.

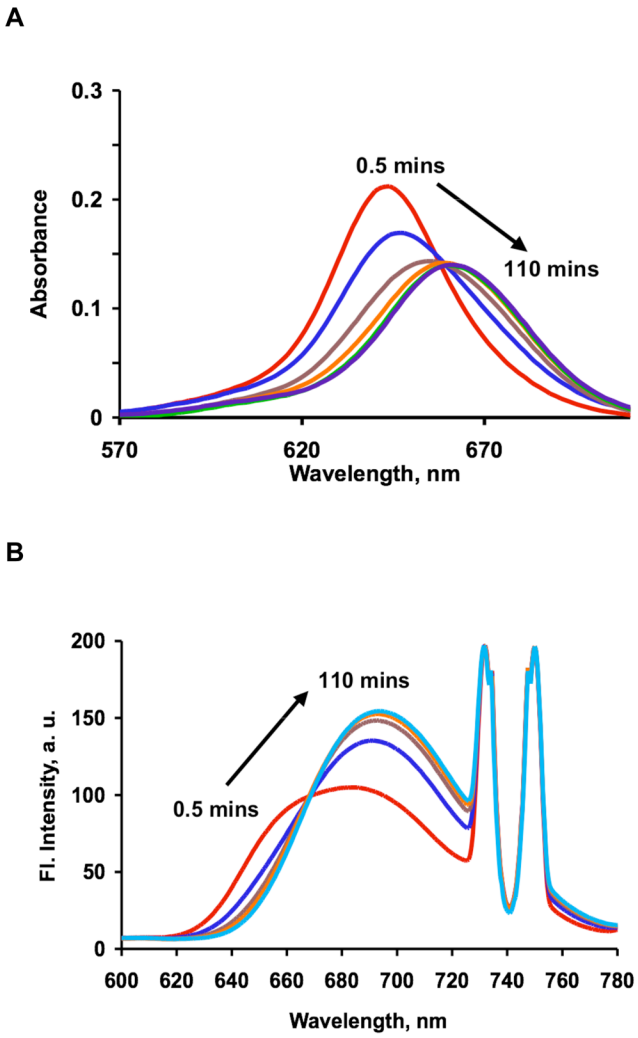

Figure 6.

Time dependent changes in absorbance (A) and fluorescence (B) maxima for squaraine **3b** (1.9 μM) after addition to vesicles (200 nm diameter) composed of 99.5:0.5 POPC: **2b**. The two fluorescence bands centered at 740 nm are artifacts due to the excitation at 370 nm.

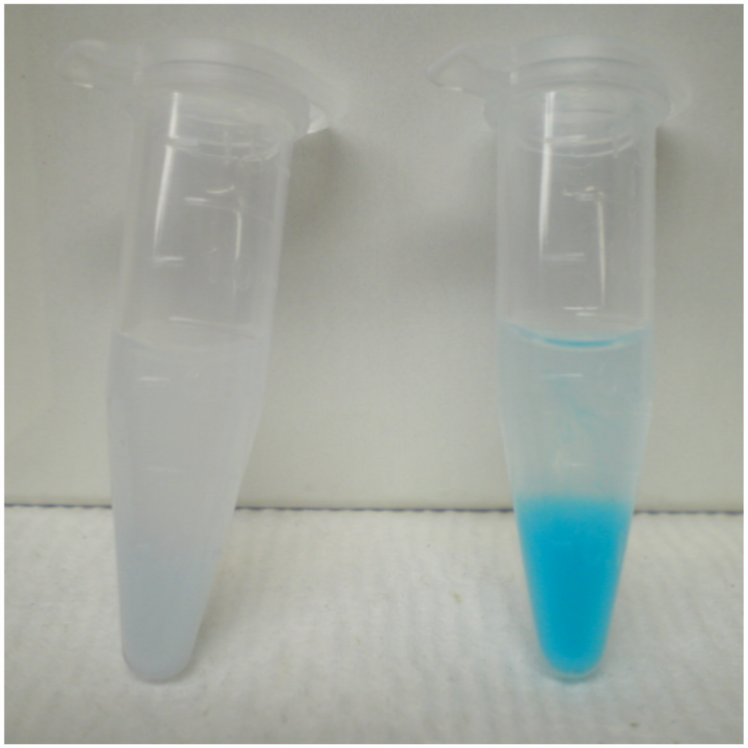

Figure 7.
Vesicles containing squaraine **3b** (left) or a mixture of **3b** and macrocycle **2b** (right) after 24 h. In both cases the vesicles have settled to the bottom of the tubes. The vesicles containing only **3b** have completely lost color due to hydrolytic bleaching (left); whereas, the vesicles containing the highly stable **2b⊃3b** retain the blue color (right).

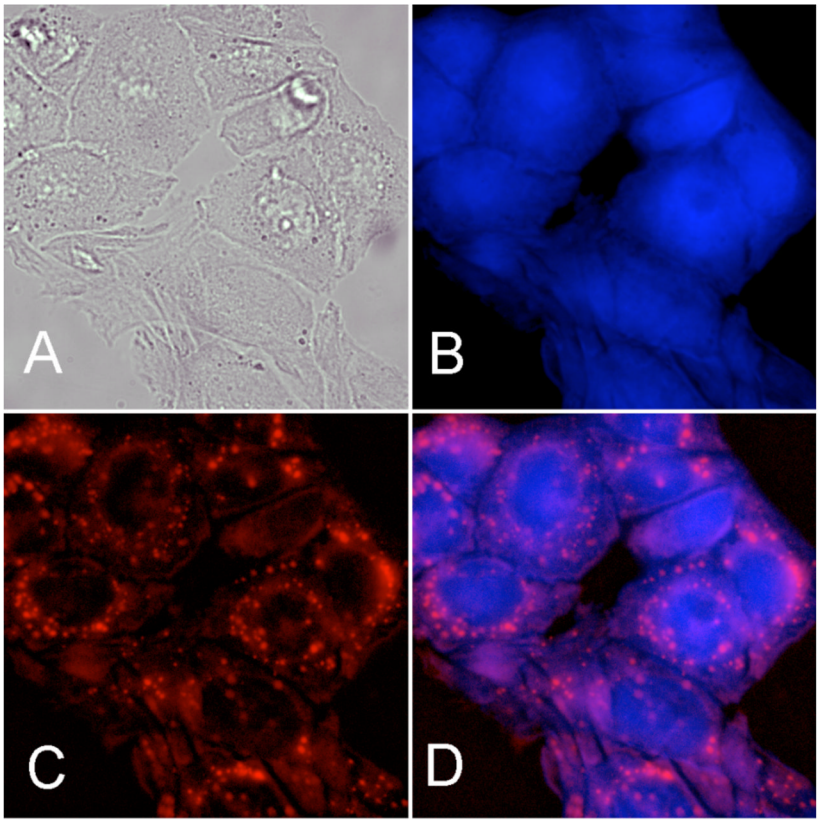

Figure 8.
Fluorescence microscopy images of live CHO cells treated with separate aliquots of **3c** (10μM) and **2b** (10μM). Panel A: Phase contrast image. Panel B: Blue emission of uncomplexed **2b**. Panel C: Far-red emission of **2b⊃3c**. Panel D: Overlay of panels B and D.

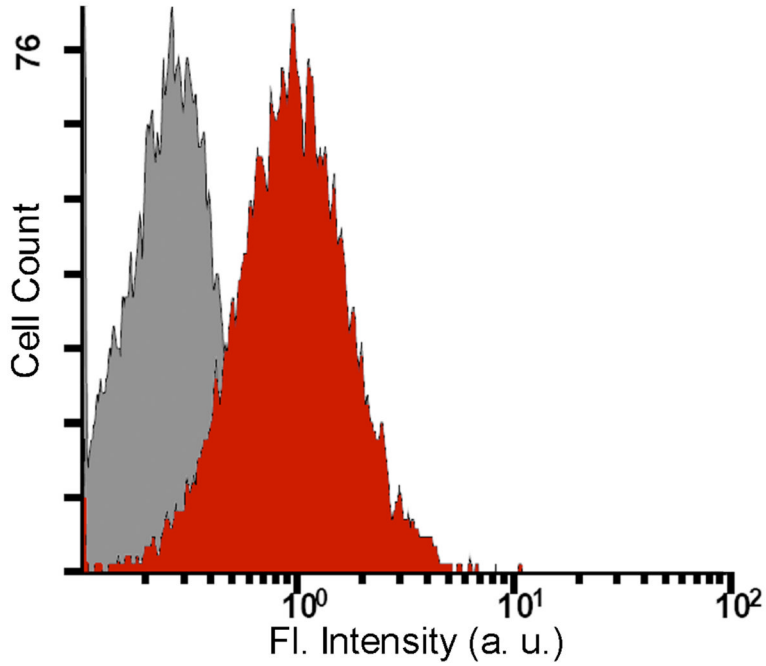

Figure 9.
Flow cytometry of CHO cells at time zero (grey), and 24 h (red) after treatment with squaraine **3c** and macrocycle **2b**. The filter set only detect cells that emit far-red fluorescence due to time-dependent formation of **2b⊃3c**.

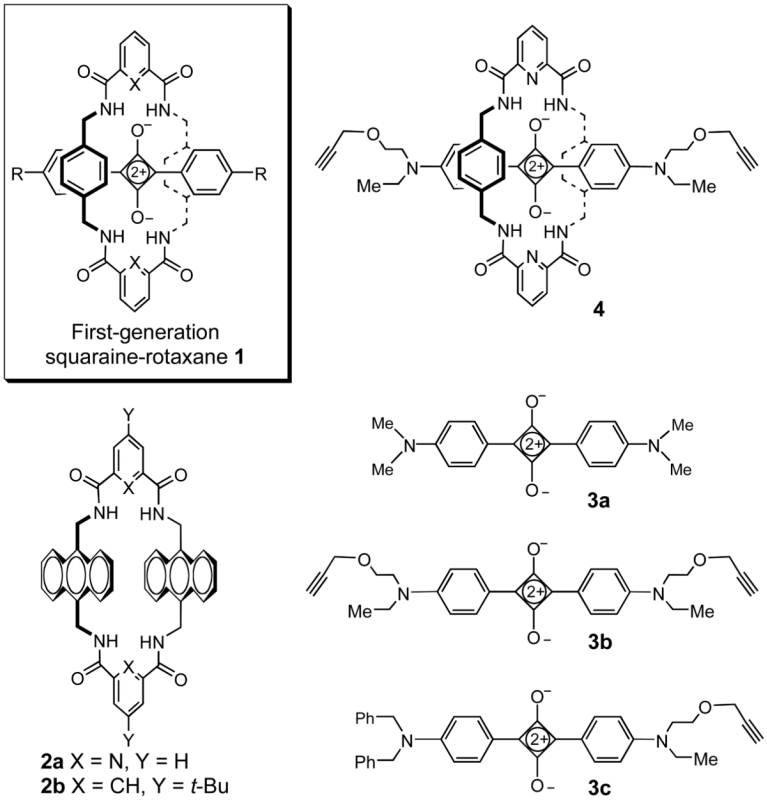

Chart 1.

Table 1

Absorption and emission maxima for selected squaraine derivatives.[a]

	λ_{abs} (nm)	log ε	λ_{em} (nm)	φ_f[b]
3b	630	5.65	657	0.60
2a⊃3b	648	5.45	699	0.45
2b⊃3b	658	5.44	695	0.45
4	642	5.70	667	0.60

[a] Chloroform solvent.

[b] Solutions were excited at 580 nm and emission monitored in the region 600–800 nm for estimating Φ_f. Fluorescence quantum yields were determined using 4,4-[bis-(N,N-dimethylamino)phenyl]squaraine dye as the standard (Φ_f = 0.7 in $CHCl_3$), error limit ± 5%.