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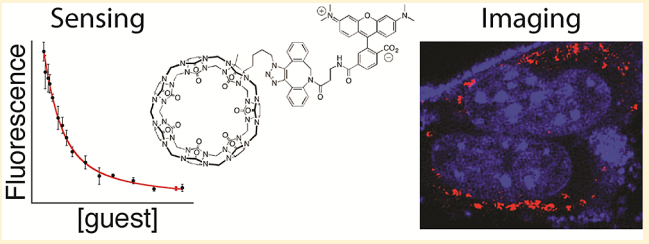
Cucurbit[7]uril–Tetramethylrhodamine Conjugate for Direct Sensing and Cellular Imaging

Andrew T. Bockus,[†] Lauren C. Smith,[†] Amy G. Grice,[†] Omar A. Ali,[†] Carolyn C. Young,[†] William Mobley,[†] Ashley Leek,[‡] James L. Roberts,[‡] Brittany Vinciguerra,[§] Lyle Isaacs,[§] and Adam R. Urbach*[,†]

[†]Department of Chemistry and [‡]Department of Biology, Trinity University, 1 Trinity Place, San Antonio, Texas 78212, United States
[§]Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, United States

Supporting Information

ABSTRACT: This paper describes the design and synthesis of a conjugate (Q7R) comprising the synthetic host cucurbit[7]-uril (Q7) linked to the fluorescent dye tetramethylrhodamine (TMR), and the characterization of its optical and guest-binding properties as well as its cellular uptake. Q7R was synthesized in two steps from monofunctionalized azidobutyl-Q7 and NHS-activated TMR. The fluorescence of Q7R is quenched upon guest binding, and this observable was used to determine equilibrium dissociation constant (K_d) values. Unexpectedly, the K_d values for guests binding to Q7R and to unmodified Q7 were essentially identical. Therefore, Q7R can directly report binding to Q7 without an energetic penalty due to the conjugated fluorophore. This result demonstrates a potentially general strategy for the design of single-component host–indicator conjugates that respond sensitively to analytes without perturbing the binding properties of the host. The unique properties of Q7R enabled measurement of K_d values across 3 orders of magnitude and at concentrations as low as 0.7 nM. This result is particularly relevant given the unmatched range of guests and binding affinities demonstrated for Q7. Confocal fluorescence microscopy of live and fixed HT22 neurons revealed the cellular uptake of Q7R and its punctate localization in the cytoplasm. Q7R did not alter cell growth at concentrations up to 2.2 μM over 4 days. These experiments demonstrate the feasibility of Q7R as a direct sensor for guest binding and as a cell-permeable compound for imaging applications.



INTRODUCTION

The development of novel fluorescent compounds and fluorescence-based assays continues to drive advancements in many areas of basic and applied science. In particular, the field of supramolecular chemistry has benefited from the practical use of fluorescent dyes to measure the thermodynamics of host–guest binding and the kinetics of enzymatic reactions.[1] These assays rely on an indicator-displacement strategy, in which the target analyte competitively displaces a fluorescent guest, or vice versa, and results in a change in fluorescence intensity.[2] Indicator displacement assays (IDAs) do not require the covalent modification of host or guest, thus preserving their binding properties and obviating the need for chemical synthesis. The noncovalent association of host and indicator, however, necessitates the tuning of their binding affinity and working concentrations in order to ensure competitive binding conditions. Most hosts have a limited range of affinities and thus a limited range of working conditions for competitive binding assays.[3] Moreover, IDAs have limited utility under continuous flow conditions (e.g., flow sensing or separations) or in biological imaging applications in which the working concentrations are well below the K_d value of the host–indicator complex.[2]

Covalent conjugation of a host to an indicator removes the dependence of their association on concentration. Careful design of a conjugate can yield single-component, direct sensors capable of detecting guest binding over a wide range of concentrations.[4−8] Covalent modification, however, is likely to alter the host binding properties. Given the narrow scope of binding affinities available to most host compounds, the advantages of covalent host–indicator conjugates do not typically justify the additional effort required for design, synthesis, characterization, and optimization. One synthetic receptor, cucurbit[7]uril (Q7), may be an exception.

Q7 is a barrel-shaped, water-soluble, organic macrocycle composed of seven glycoluril units linked by pairs of methylene groups.[9−13] Binding is driven by displacement of frustrated water molecules and electrostatic attraction of cationic groups on the guest to C=O dipoles on the host.[14] Q7 is unique among synthetic receptors in the breadth of its guests and the corresponding range of K_d values, which span the millimolar to attomolar range in aqueous solution.[15−18] Accordingly, Q7 has

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found many applications, including sensing, separation, catalysis, and drug delivery, to name a few.[11−13,17,19−21]

There are many fluorescent guests for Q7,[1] and myriad IDA-based studies of Q7 have been reported. Q7 is difficult to modify covalently,[22] but recent synthetic approaches to monofunctionalization have enabled the synthesis of several discrete conjugates.[23−27] To the best of our knowledge Kim and co-workers have reported the only Q7−fluorophore conjugate.[28] Their Q7−Cy3 conjugate was used to detect vesicle fusion via fluorescence resonance energy transfer (FRET) between Cy3 and its FRET partner, Cy5, which was conjugated to a high affinity guest, adamantane ammonium. Their conjugate, however, was not studied in a living system. In parallel with these efforts, we describe here the synthesis of a conjugate between Q7 and tetramethylrhodamine (TMR) and the characterization of its optical, guest binding, and cellular uptake properties.

RESULTS AND DISCUSSION

Design and Synthesis. Our conjugate design took into account several considerations. A complementary pair of monofunctionalized Q7 and monofunctionalized TMR were chosen to ensure a single point of conjugation to Q7. A Hüisgen 1,3-dipolar cycloaddition[29] was chosen to make use of a readily available monofunctional azidobutyl Q7[23] and to avoid amine-based coupling strategies (Q7 binds amines). A strained dibenzocyclooctyne (DBCO) linker was chosen to avoid Cu-based catalysts,[30] which may also bind Q7. A dye with modest affinity for Q7 was chosen to limit the competition with target analytes.

On the basis of these considerations, the 6-isomer of the Q7·TMR conjugate (Q7R) was synthesized in two steps from monofunctional azidobutyl Q7 (Q7-N_3)[23] (Figure 1) (see

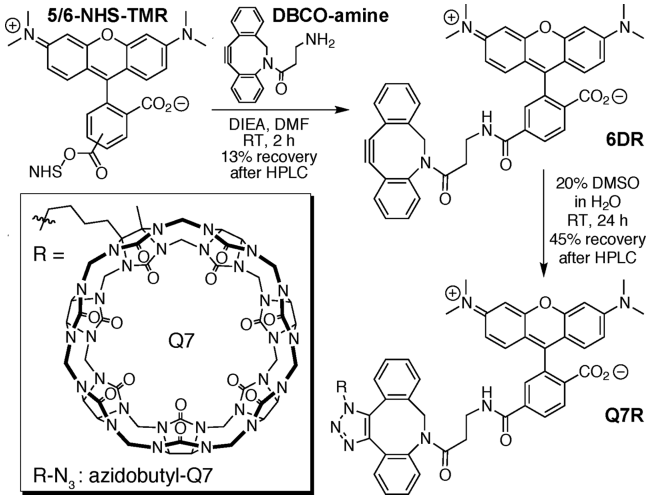

Figure 1. Synthesis of the cucurbit[7]uril-tetramethylrhodamine conjugate (Q7R).

Supporting Information for experimental details). First, an equimolar quantity of 5/6-NHS-TMR was treated with the DBCO-amine cross-linker in Hünig's base to produce a mixture of 6-DBCO-rhodamine (6DR) and its constitutional isomer (5DR), which were obtained in 13% yield each following HPLC purification. The identities of the 5DR and 6DR were confirmed by HPLC coinjection with authentic samples synthesized from the more expensive 5-NHS-TMR or 6-NHS-TMR isomers (Figure S2). Coupling of 6DR with Q7-N_3 was performed in 20% aqueous DMSO to yield a 1:1 mixture of the 1,4- and 1,5-substituted triazole isomers of Q7R (Figures S7 and S8) in 45% recovery following HPLC purification. This mixture of triazole isomers of the 6-substituted Q7R conjugate was used for all subsequent studies.[31]

Solubility and Optical Properties. Among the cucurbit-[*n*]uril homologues, Q7 displays the highest aqueous solubility (20−30 mM).[10] Accordingly, it has been used as a solubilizer for hydrophobic pharmaceuticals,[32] and it has been shown to enhance the solubility of free rhodamine.[33] As expected, the solubility of Q7R in pure water at room temperature (860 ± 20 μM) was 45-fold greater than that observed for TMR (19 ± 3 μM), demonstrating the solubilizing ability of the Q7 group. Despite the measured solubility of Q7R near 1 mM, we observe the slow formation of Q7R precipitate at high μM concentrations, thus necessitating the acquisition of NMR spectra at 150 μM. Even at this concentration, the spectrum of Q7R shows significant peak broadening, perhaps due to the formation of soluble aggregates. The addition of excess guest reduces peak broadening significantly (Figure S9).

As a baseline for subsequent binding studies, absorption and fluorescence spectra of Q7R were acquired in the absence of guest (Figures S10 and S11). The visible absorbance spectrum of Q7R was nearly identical to that of tetramethylrhodamine ethyl ester (TMRE). A 3 nm blue-shift in the wavelength of maximum absorbance (λ_{max}) of Q7R relative to that of TMRE was observed. Similar peak shifts have been reported for xanthene dyes upon binding to Q7.[1,32,34] The fluorescence intensity of Q7R, however, was significantly higher than that of TMRE, as would be expected for binding of the dye to Q7.[1]

Guest Binding. Guests **1**−**4** (Figure 2) were selected for binding studies because they bind to Q7 with K_d values that

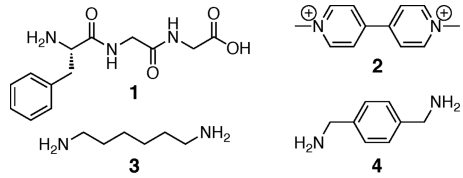

Figure 2. Formulas of the guests used in this study.

span the useful micromolar to nanomolar range.[15,35,36] The addition of guest to Q7R resulted in no change in Q7R absorbance, but the fluorescence intensity of Q7R was quenched significantly in the presence of each guest (Figure 3a). Using fluorescence quenching as an observable for guest binding, we carried out equilibrium binding titrations for guests **1**−**4** in 10 mM sodium phosphate-buffered water, pH 7.0. In all cases, concentration-dependent quenching of Q7R fluorescence was observed (Figures 3b and S17−S19). The data were fit to a simple 1:1 host−guest equilibrium binding model to determine K_d values (Table 1). Complex stoichiometry was confirmed to be 1:1 (host:guest) by isothermal titration calorimetry and mass spectrometry experiments (Figures S12−S16).[37]

Initially, we were puzzled that guests **1**−**4** bind to Q7R and to free Q7 with essentially identical K_d values. We were cognizant that prior work with Q7 and free TMR established a K_d value of 71 μM for the Q7·TMR complex and an increase in the quantum yield of fluorescence upon complex formation.[1,34] Given that our binding assays were conducted at concentrations well below the K_d for intermolecular association, we surmised

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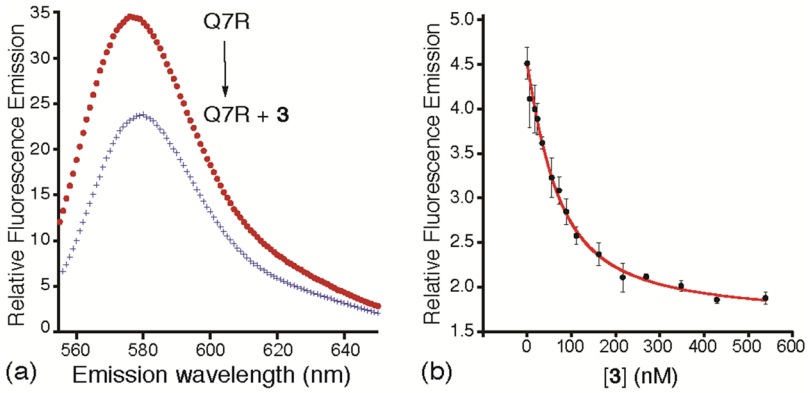

Figure 3. Representative fluorescence data for the binding of Q7R with guest **3** at 25 °C in 10 mM sodium phosphate buffer, pH 7.0. (a) Fluorescence emission spectra of Q7R in the presence (gray +) and absence (red •) of **3**. (b) Titration of Q7R (50 nM) with **3**, showing the fluorescence emission intensities at 577 nm (λ_{ex} = 550 nm) as a function of total guest concentration. Data points are average values from three experiments; error bars are standard deviations. The red line is the best fit to a binary equilibrium binding model.

Table 1. Binding constants of Q7R and Q7 with 1−4

guest	Q7R K_d (M)[a]	Q7 K_d (M)[b]
1	3.6 (±1.0) × 10^{-7}	3.6 (±0.1) × 10^{-7}
2	1.6 (±1.8) × 10^{-7}	1.5 (±0.1) × 10^{-7}
3	4.6 (±1.8) × 10^{-8}	1.1 (±0.2) × 10^{-8}
4	6.5 (±2.7) × 10^{-10}	5.4 (±1.0) × 10^{-10}

[a]Fluorescence titrations were performed at 25 °C in 10 mM sodium phosphate, pH 7.0, at λ_{ex} = 550 nm and λ_{em} = 577 nm with Q7R concentrations fixed at 1000, 600, 50, and 10 nM for titrations with **1**, **2**, **3**, and **4**, respectively. [b]Reported literature values for Q7.[15,35,36]

that the observed fluorescence quenching of Q7R upon guest binding was due to competitive displacement of the TMR group of Q7R from the cavity of Q7 within a self-complexed Q7R molecule. Theory tells us that the observed K_d values for guest binding must be reduced by the intramolecular self-association constant, which again was puzzling until we realized that an intramolecular self-association constant near unity could explain the observed behavior. Accordingly, we hypothesize that Q7R exists in two nearly isoenergetic conformations that possess very different fluorescence properties; we do not believe the TMR group of Q7R is bound significantly within the Q7 cavity but may be loosely associated near the C═O portals. Binding of a guest inside the Q7 cavity of Q7R then favors the conformation with the lower fluorescence intensity. We believe this represents a new and potentially general strategy for the design of single-component host−indicator conjugates that respond sensitively to analytes without perturbing the innate molecular recognition properties of the host.

Imaging. In addition to sensing applications, covalent host-indicator conjugates should also be preferable to noncovalent host−indicator complexes for imaging applications. The covalent connection ensures that the indicator is attached to the host and that the host is being observed directly. Moreover, the lack of free dye reduces background signal.[2] To assess compatibility with cell imaging applications, we treated HT22 neurons with Q7R and observed cellular uptake and punctate localization to the cytoplasm via live cell (Figure 4) and fixed cell (Figure S20a) confocal fluorescence microscopy. Live cell imaging with organelle markers (Figure S22) shows no pattern of localization to endoplasmic reticulum, golgi apparatus, lysosomes, or mitochondria. TMRE was not visibly cell

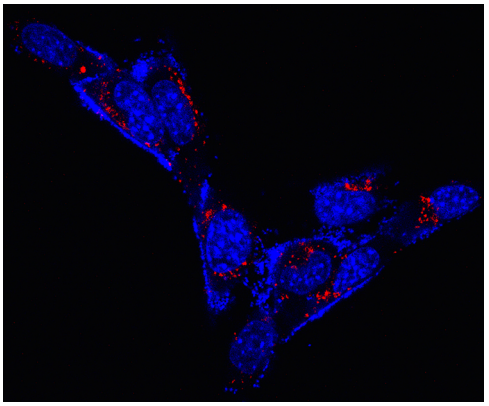

Figure 4. False color confocal fluorescence micrograph of live HT22 cells treated with 91 μM Q7R (red) and Hoechst 33342 (blue).

permeable (Figure S20b) at the same concentration as Q7R (91 nM), and therefore the Q7 group was required for uptake. Excess unlabeled Q7 did not inhibit the uptake of Q7R (Figure S21), and therefore receptor-mediated transport is likely not involved. Although not confirmed unambiguously, we believe that Q7R internalizes via endocytosis and localizes to endosomes. As might be expected from prior work on tolerance to Q7,[38] the cells showed no perturbation in morphology or confluency from treatment with Q7R at concentrations up to 2.2 μM over 4 days, and therefore Q7R did not appear to alter cell growth (Figures S23−S26).

■ CONCLUSIONS

This paper describes the design, synthesis, and characterization of the guest binding and cell uptake of a single-component, direct optical sensor based on Q7. The Q7−rhodamine conjugate, Q7R, displays concentration-dependent fluorescence quenching upon binding to guests, and yet its binding affinities match those of unmodified Q7. The covalent attachment of host and indicator allowed us to measure K_d values across 3 orders of magnitude. The ability to measure guest binding with unmitigated affinity allowed us to directly determine K_d values at concentrations as low as 0.7 nM. These properties demonstrate the versatility of this compound for sensing applications and the use of Q7R as a Q7 surrogate for the direct measurement of guest binding over a wide range of concentrations. This work presages the use of Q7R for

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continuous sensing applications and as a single component reporter for supramolecular enzyme assays.[39]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b11140.

Experimental details and optical, NMR, and mass spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*aurbach@trinity.edu

ORCID

Lyle Isaacs: 0000-0002-4079-332X

Adam R. Urbach: 0000-0003-3426-4760

Notes

The authors declare no competing financial interest.

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