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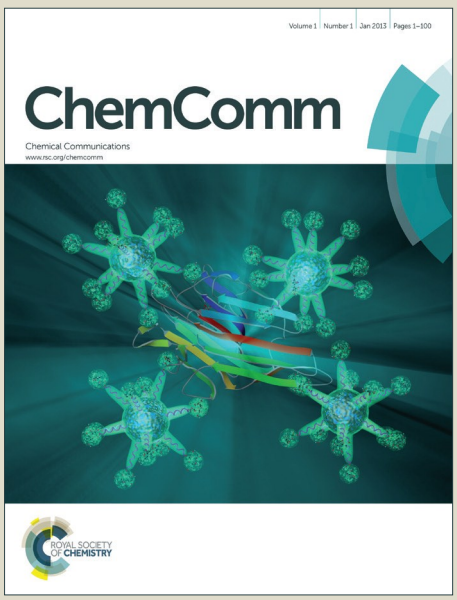

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A Naphthalimide Derived Fluorescence Sensor for Solid-Phase Screening of Cucurbit[7]uril-Guest Interactions

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A napthalimide based fluorescenct sensor displaying a significant increase in emission upon binding CB[7] with excellent pH stability was developed and utilized in a surface-bound displacement assay for the rapid detection of CB[7] encapsulation of therapeutically relevant drug classes. Previously unknown binders with moderate to strong affinities were discovered.

The rapid identification and characterization of macrocycle driven host-guest interactions, especially in competitive aqueous media, is of significant importance since these interactions play a prominent role in many drug delivery systems and chemosensors. [1-5] Further, host-guest interactions are increasingly used for the construction of functional assemblies, polymers, and nanoparticles, with stimuli responsive or self-healing properties.[6-11] Although a number of elegant macrocyclic hosts have been investigated, there is particular interest in the cucurbit[n]uril family of hosts (CB[n], n = 5, 6, 7, 8 & 10) for the design of functional supramolecular constructs due to their facile synthetic preparation and ability to bind selectively with guest molecules in water. [12-15]

Within the CB family, CB[7] is particularly salient as it has high solubility in water (20-30 mM), can display strikingly large affinity constants with guests (e.g., recently a Ka of 10^{17} M^{-1} with diamantane diammonium was discovered) and exhibits low cellular toxicity.[16-20] While CB[7] shows much promise as a host molecule in a variety of applications, it remains a significant challenge to rapidly screen compound libraries for guest molecules that bind to CB[7]. Indeed, CB[7]-guest interactions are typically analyzed using ^{1}H-NMR and isothermal titration microcalorimetry (ITC). [21-24] Both these techniques are time and sample consuming and are not readily amenable to rapid screening via solid-phase assays.

Significant strides have been made in the development of sensitive solution derived assays (based on fluorescent displacement phenomena) to detect CB[7]-guest interactions. [25-29] However, to date, a solid-phase fluorescence-based screening platform capable of rapidly identifying molecules that bind to CB[7] has not been reported. Such an assay would be a boon for a broad range of researchers investigating functional systems centered on the molecular encapsulation ability of CB[7].

The development of a solid-phase fluorescence assay that detects CB[7]-guest interactions remains a formidable challenge as typical fluorophores (e.g., acridine orange, neutral red and dapoxyl) utilized as sensors to detect CB[7] binding do not possess the functional group handles to be readily attached to surfaces. Additionally, the fluorescence of CB[7] binding chromophores are often highly sensitive to pH and thus are difficult to use under a broad range of conditions. [26, 27, 30, 31]

With this communication we first describe the design of a naphthalimide (NMI) based fluorescent sensor **1** (Figure 1A) that exhibits a significant increase (over an order of magnitude) in fluorescence quantum yield when encapsulated by CB[7]. This enhancement is readily reversed upon addition of competing CB[7] binders. Importantly, sensor **1** exhibits robust CB[7] binding and signaling through the pH range 5-10.

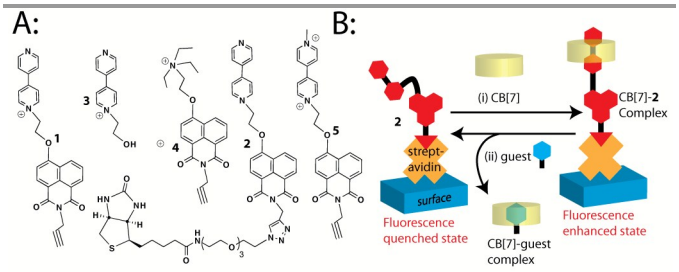

Figure 1. (A) Structures of CB[7]-guest interaction sensors **1** and **2** as well as controls **3, 4** and **5**. (B) Schematic illustrating the fluorescence properties of surface bound **2**. (i) When CB[7] binds to the bipyridine moiety of **2**, there is an increase in the fluorescence of **2**. (ii) When a guest molecule that binds CB[7] is introduced to the **2**:CB[7] complex, CB[7] encapsulates the guest and is released from the surface, resulting in a decrease in the fluorescence of **2**.

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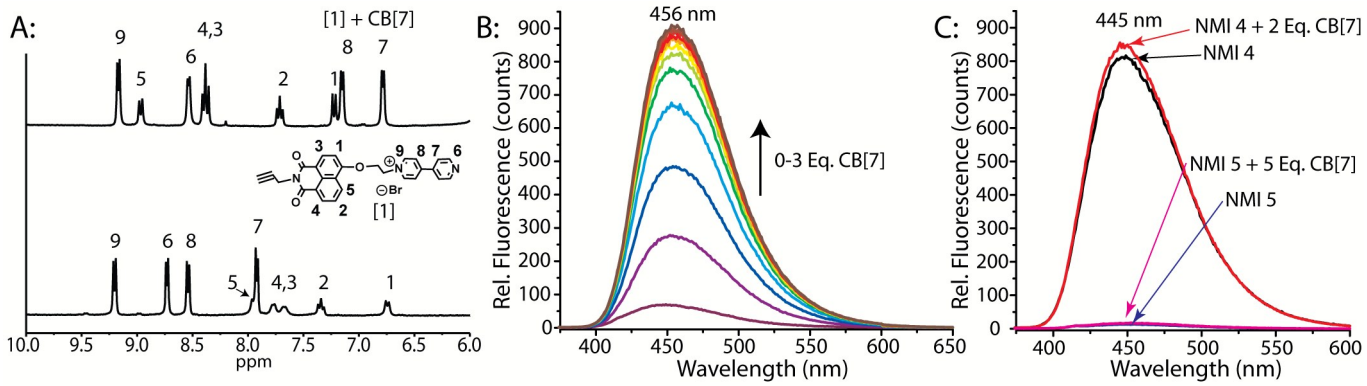

Figure 2. (A) 1H NMR recorded in D_2O for NMI **1** (1.0 x 10^{-3}M) before (bottom) and after (top) addition of 4 eq CB[7]. B) Fluorescence emission spectra of NMI **1** (2 μM) in the presence of increasing concentration (0-3 eq.) of CB[7] (λ_{ex} = 365 nm). (C) Fluorescence profile of controls NMI **4** (1 μM) and NMI 5 (2 μM) in the absence and presence of CB[7] (λ_{ex} = 365 nm). Fluorescence Spectra collected in ultrapure H_2O (18 MΩ).

Second, we discuss the development of a biotinylated congener NMI **2**. Specifically, NMI **2** was attached onto commercially available micro-well plates via biotin-streptavidin interactions that enabled the development of a facile solid-phase derived fluorescence displacement assay to screen libraries of guests for binding to CB[7] (Figure 1B). As a proof of concept, a library containing anti-cancer agents, anti-malarials, and enzyme (carbonic anhydrase II) inhibitors, were screened. From this assay, unknown CB[7] binders were easily identified and categorized.

The core 1,8-napthalimide was chosen due to its strong fluorescence in aqueous media and amenability to functionalization.[32] Although researchers have previously investigated dicationic viologens for binding to CB[7],[33] and such viologens have been utilized as quenchers for fluorophores including napthalimides,[34] the fluorescence increase upon encapsulation with CB[7] to dicationic viologens is minor. [35, 36] In contrast, we found (*vide infra*) that the use of a mono-cationic bipyridine (bpy) that maintains reasonably strong CB[7] binding (K_a of approximately 10^6 M^{-1})[37] provides for much greater enhancement of fluorescence. The connection of these motifs via a short, flexible $O(CH_2)_2$ linker ensured spatial proximity, while functionalization at the imide position (on the opposite side of the molecule) with an alkyne unit allowed a reactive handle for facile surface modification (NMI **2**) with the commercially available biotin-azide. The syntheses of NMIs **1**, **2** as well as controls **3, 4** and **5** are provided in the SI.

The binding of CB[7] with **1** was first probed by examining ^{1}H-NMR chemical shifts (in D_2O) in the naphthalene and bpy moieties upon addition of CB[7]. As shown in Figure 2A, NMI **1** exhibits two sets of protons corresponding to the napthalene core (H1-H5, see inset) and the bpy unit (H6-H9, see inset). Addition of CB[7] into a solution of **1** results in the bpy protons H7 and H8 undergoing significant upfield shifts (Δδ = 1.2 and 1.4 ppm, respectively). The H6 and H9 protons also display upfield shifts but the magnitude is smaller (Δδ = 0.2 and 0.1 ppm, respectively). These results are consistent with the bpy unit being well-encapsulated by CB[7], wherein the interior pyridine protons are buried deep inside CB[7]. [37] In contrast to the bpy resonances, the napthalene protons on **1** undergo sharpening and downfield shifts (Δδ = 0.4-1.0 ppm) when CB[7] is added, likely due to de-stacking of the NMI units. As detailed in the SI, UV-vis spectroscopy provided further evidence that CB[7] binds to the bpy unit of **1**. Additionally, a 1:1 stoichiometry for binding was confirmed by MALDI-TOF MS (observed m/z = 1596.7 Da, calculated m/z = 1596.5 Da) and a Jobs plot via fluorescence spectroscopy.

With evidence in hand for the formation of a discrete stoichiometric NMI **1**:CB[7] complex, the fluorescence properties of **1** were next evaluated. As can be seen from inspection of Figure 2B, the fluorescence emission of **1** shows a dramatic 14 fold enhancement in intensity upon addition of increasing concentrations of CB[7]. This value is consistent with independently determined fluorescence quantum yield values (Table S1). In contrast, control NMI **4** which is water soluble, mono-cationic, and binds to CB[7] via the appended tetraethyl ammonium unit, [38] shows high-intensity emission with or without CB[7] (see Figure 2C). Additionally, the dicationic viologen derivative (NMI **5**) shows negligible native fluorescence and only a small change on encapsulation by CB[7]. These results suggest that the encapsulation of the

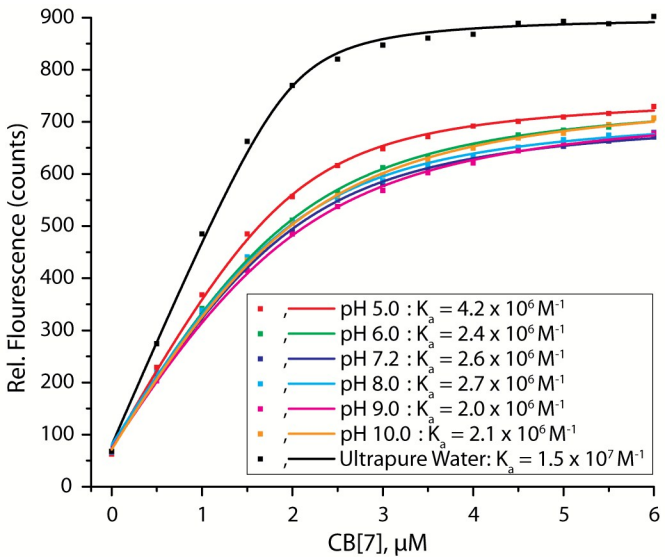

Figure 3. Fluorescence titrations of 2 μM NMI **1** with CB[7] in 10 mM ammonium phosphate buffer at pH 5-10. Also shown is the corresponding titration in ultrapure water. A 1:1 binding model was used to fit the data via non-linear regression analysis.

bipyridine is responsible for the observed fluorescence quenching phenomena, and that the use of the mono-cationic bpy rather than the dicationic viologen significantly increases the magnitude of response on encapsulation. Cyclic voltammetry, luminescence lifetime measurements and literature precedents [34, 36] lead us to suggest that the mechanism of quenching for NMI **1** is via a photoinduced electron transfer process (see Section 1.5.3 in the SI for more details).

Since a drawback of typical CB[7] binding chromophores is their pH sensitivity, we investigated the pH dependence on the fluorescence of **1** in the presence of 10 mM ammonium phosphate buffer (Figure 3). These studies show that in the range of pH 5-10 neither the fluorescence intensity nor the CB[7] binding constant (K_a of 2-4 x 10^6 M^{-1}) change significantly, indicating that the binding and fluorescent properties of **1** are not appreciably influenced within this pH range. In comparison to the buffered spectra, when ultrapure water (without added ammonium phosphate) was used, the binding to CB[7] is appreciably larger (K_a = 1.5 x 10^7 M^{-1}), consistent with cation-induced attenuation of CB[7] binding. [26]

Interestingly, initial solution-phase fluorescence studies indicated that addition of a known CB[7] binder (N-adamantylmethyl-1,4-diamine, AdBut) to the NMI **1**:CB[7] complex results in quenching of NMI **1** via the displacement of CB[7] (Figure S12). Further, the same titration was conducted with acridine orange in order to validate NMI **1** as a sensor. These results showed that both NMI **1** and acridine orange yield a similar binding constant for CB[7]:AdBut (~1 x 10^9 M^{-1}). [29] Based on these results, we were keen to test the utility of the surface-anchorable NMI **2** as a signal OFF sensor for detecting CB[7]:guest interactions in the solid-phase. In order to develop an easy-to-use system that is readily amenable to adaptation in other labs, we introduced biotinylated **2** directly onto the surface of commercially available streptavidin-coated 96 well plates (Figure 4A). Following the removal of any unbound NMI **2**, a surface showing low fluorescence was obtained. Subsequent introduction of CB[7] (100 µM) caused a 10 fold increase in fluorescence intensity.

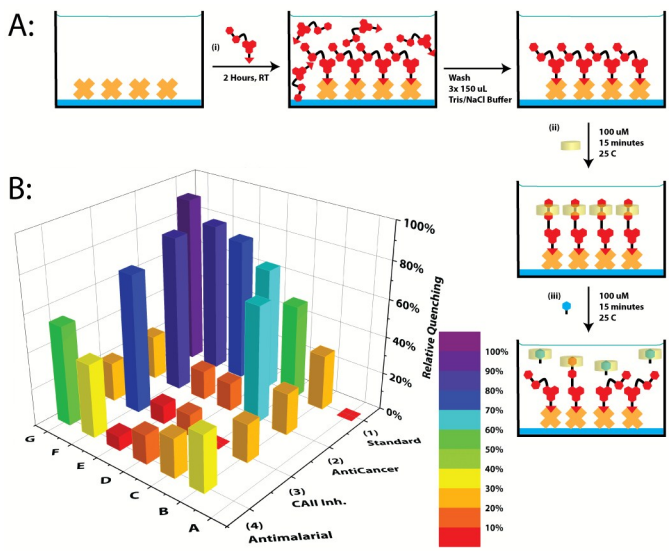

Figure 4. (A) Schematic of multiplate assay. (i) Incubation with NMI **2**, (ii) addition of CB[7], (iii) addition of competitively binding guests. (B) Relative fluorescent quenching of **2**:CB[7] complex on addition of competitively binding guests, averaged over 3 runs.

The above mentioned NMI **2**:CB[7] complex is considered as 0% quenching in NMI **2** (Figure 4B, graph 1A), while the maximum enhancement in quenching (91%) is NMI **2** in the absence of CB[7] (see Figure 4B, graph 1G). The degree of displacement of CB[7] from the surface bound NMI **2** at a given equivalency of guest should lead to an increase in quenching that is proportional to the strength of the CB[7]:guest complex. To test this contention, three panels of different therapeutic classes (each containing six library members) were screened for CB[7] binding (Figure 4B, rows 2-4). Additionally, a panel of internal standards with known solution-phase affinities for CB[7] (with a range of binding constants; K_a = 5x10^5 – 5x10^{12} M^{-1}) [38-41] was run in order to ensure that the solid-phase data was consistent with known solution binding (Figure 4B, graph 1B-1F).

A qualitative comparison of the relative quenching of the standards versus the unknowns showed that *N*-(aminophenyl)-piperidine (4G; an antimalarial fragment)[§] and doxorubicin (2C; an anti-cancer drug) bound CB[7] with moderate binding. Further, 4-(adamantanyl-carboxamido)–benzenesulfonamide (3F; a CA-II inhibitor) and oxaliplatin (2F; an anticancer drug known to bind CB[7]) [42] showed strong binding to CB[7]. The strength of each unknown binding interaction was subsequently quantitated by competitive fluorescence displacement assays of **1** in solution (Figures S14-16). The determined binding constants for *N*-(aminophenyl)-piperidine and doxorubicin were 1 x 10^7 M^{-1} and 2.5 x 10^6 M^{-1}, respectively and adamantanyl-carboxamido)–benzenesulfonamide had a K_a of 1.3 x 10^9 M^{-1}.

In conclusion, we have designed a NMI-based fluorescent sensor **1** displaying over an order of magnitude enhancement in emission upon encapsulation by CB[7], with notable pH stability. Additionally, the biotinylated version of sensor **1** is amenable to surface attachment without loss of activity. A facile microplate assay was developed and proof of concept was shown by testing against a library of therapeutically relevant drug classes, resulting in the discovery of guests for CB[7] possessing strong to moderate binding affinities. This assay is straightforward, using commercial microwell plates (and biotinylated click partner) and is expected to be of general utility for researchers interested on the application of using CB[7] as a host molecule in water.

Acknowledgements

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Notes and references

§ As the full structure is not commercially available, the *N*-(aminophenyl)-piperidine side-arm was used to approximate binding of the cryptolepine derivative described by Paulo et al.[43]

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