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Design of a Fluorescent Dye for Indicator Displacement from Cucurbiturils: A Macrocycle-Responsive Fluorescent Switch Operating through a pK_a Shift

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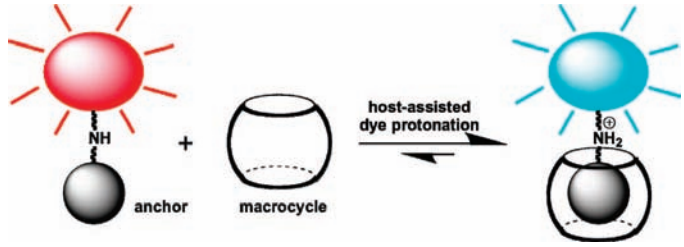
ABSTRACT



The derivatization of 3-amino-9-ethylcarbazole with a diamino-alkyl anchor affords a fluorescent dye suitable for indicator displacement from cucurbituril macrocycles. The novel compound 1 shows, due to a complexation-induced pK_a shift, a large and predictable dual fluorescence response (100-fold increase at 375 nm and 9-fold decrease at 458 nm) upon supramolecular encapsulation and a strong affinity for cation-receptor macrocycles, in particular cucurbit[6]uril (CB6). A direct application is presented by monitoring the enzymatic activity of lysine decarboxylase.

There is presently an increasing interest in the development of fluorescent dyes which show a high affinity to macrocycles in aqueous media and a concomitant fluorescence change upon host−guest complexation. Possible applications of the resulting fluorescent dye−macrocycle complexes lie in the area of indicator displacement, wherein an analyte displaces the fluorescent dye from the complex, resulting in a readily detectable fluorescence response.[1−9] Frequently, suitable fluorescent indicators have been identified by a systematic screening of established commercial dyes. Several dyes with a favorable combination of affinity and fluorescence response characteristics toward different macrocycles,[10,11] including calixarenes,[4,12,13] cyclodextrins,[12,14] and, most recently, cucurbiturils[15−17] have been identified. However, the factors responsible for a high affinity of a dye are not always

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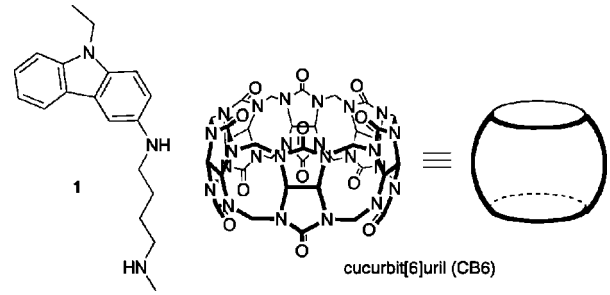
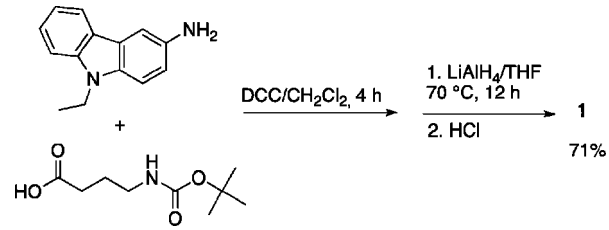
accurately predictable, and the fluorescence response upon complexation is frequently not understood in photophysical detail. In most cases, environmental changes are held responsible; i.e., the change in fluorescence upon inclusion into the less polar cavity of macrocycles resembles that observed upon going from an aqueous solution to solvents such as alcohols.[17−20] Anilinonaphthalene sulfonate (ANS) is the classical example of such a polarity-sensitive fluorescent dye (enhancement factors up to 200).[18,21] To bypass the uncertainty of low affinity, derivatized dyes with a strongly binding anchoring residue have been constructed.[13,22,23] The anchoring group is then included with a predictable affinity into the cavity, while the actual chromophore remains immersed in the aqueous phase; consequently, any microenvironmental effects on the fluorescence are greatly reduced as compared to a direct immersion of the dye (factor 2 or less),[2,3,13,23] and in some cases, the fluorescence properties remain unaltered which may be desirable for different applications.[22]

We aimed at developing an improved system with a reliable *relay mechanism* through which the fluorescence of the dye undergoes a large and predictable change upon complexation of the anchor. In particular, complexation-induced pK_a shifts, which have recently been conceptualized, appeared to be attractive.[16,24−29] Accordingly, a host−dye pair can be selected such that the dye remains unprotonated in its uncomplexed form but becomes protonated, accompanied by pronounced photophysical effects, upon complexation (host-assisted guest protonation).[24] Such supramolecular pK_a shifts range from 1−5 units.[16,24−28] Thus, when working in a pH region between the pK_a values of the uncomplexed and complexed dye, large fluorescence changes are expected upon addition of host or upon addition of a competitive binder to the preformed host−guest complex. Our idea was to design the protonation site of an anchoring group such that it would be conjugated to the aromatic system of the chromophore and thereby ensure a pronounced fluorescence response upon protonation as induced by complexation of the anchor. The novel working principle of our system is depicted in the TOC graphics. Because the actual chromophore does not have to be included in the host cavity, this strategy allows for a large variablity in the choice of the fluorescent dye with respect to size, charge, and binding affinity. To document our idea, we present here a carbazole dye[30,31] derivatized with a diamino-anchor (**1**) with a high affinity to cucurbiturils.[22,32−37]



3-Amino-9-ethylcarbazole was reacted with 4-(BOC-amino)butyric acid in a DCC-mediated coupling to produce the derivatized dye **1** after reduction with $LiAlH_4$ (Scheme 1). Dye **1** can be viewed as a carbazole fluorophore linked with a 1,4-diaminoalkyl functionality. 1,4-Diaminobutane (putrescine) is known to have a high binding affinity to cucurbit[6]uril (CB6) ($K = 2.0 \times 10^7\ M^{-1}$)[37] as well as other cucurbiturils, e.g., cucurbit[7]uril.[8] The charge status of compound **1** differs from that of putrescine in that only the

Scheme 1. Synthesis of **1**



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terminal aliphatic amino group (pK_a ~ 10)[38] is protonated in water near neutral pH because the anilino type nitrogen in **1** has a pK_a of 5.3 (see below). Compared to the parent 3-amino-9-ethylcarbazole, the derivative **1** shows virtually the same UV absorption properties (λ_{max} = 364 nm; $\varepsilon = 1.8 \times 10^3$ M^{-1} cm^{-1}). The fluorescence of **1** shows a small red shift (from 452 nm for the parent to 458 nm), accompanied by a small increase in quantum yield (from 0.63 for the parent to 0.76, both at pH 7.5).

Expectedly, the fluorescence of **1** is pH-sensitive. Above neutral pH (≥7), a strong fluorescence band with a maximum near 458 nm is observed which is attributed to the charge-transfer type electronic transition of the unprotonated anilino nitrogen form. At acidic pH (e.g., 3), the weaker locally excited band (λ_{max} = 375 nm) predominates (Figure 1a). The

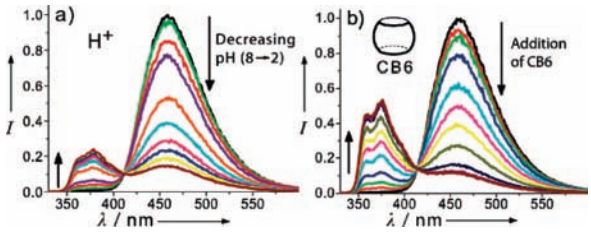

Figure 1. Fluorescence titrations of **1** (47 μM, λ_{exc} = 311 nm) (a) upon lowering the pH in water and (b) upon successive addition of CB6 (up to 50 μM) in 10 mM NH_4OAc buffer, pH 7.

longer-wavelength fluorescence band is strongly decreased at acidic pH but does not vanish, suggesting a competitive deprotonation within the excited-state lifetime, resulting in emission from both protonated and unprotonated forms (Förster cycle).[39,40] The pH titrations of the fluorescence bands (Figure 2), which showed a decrease or increase in intensity depending on the monitoring wavelength, provided a pK_a value of 5.3 when fitted according to a two-state model.[25,29] We assign this pK_a to the protonation of the anilino-type nitrogen of **1**, which, as projected, causes a pronounced effect on the photophysical properties.

We anticipated that the complexation by a cation-receptor macrocycle would promote the protonation of the same nitrogen. Indeed, upon successive addition of CB6 (up to 50 μM), the long-wavelength fluorescence band showed a systematic decrease (Figure 1b). In contrast, the short-wavelength band increased by a factor of 100 to reach a plateau value at high cucurbituril concentration. The complexation of the dye can consequently be followed by a dual fluorescence response of either the switch-on or switch-off type. The binding constant of **1** to CB6 was determined by ITC as (2.22 ± 0.16) × 10^7 M^{-1} in 10 mM NH_4OAc buffer at pH 7 (assuming a 1:1 complexation model). The fluorescence behavior upon addition of the macrocycle is assigned to the selective formation of an inclusion complex with the aminoalkyl anchor of the derivatized dye **1**, which has a substantially higher affinity than the carbazole chromophore itself.[41] The variations in the fluorescence spectra are fully consistent with the idea that the complexation of the macrocycle causes a concomitant protonation of the anilino nitrogen of **1**; that is, the fluorescence of the protonated form increases at the expense of the fluorescence characteristics of the unprotonated form. Strikingly, the net effect of addition of CB6 resembles the effect of addition of acid (compare Figure 1a,b) because complexation exposes the dye to an apparently more acidic microenvironment. The pH titration of the complexed dye (**1**·CB6) obtained under conditions of virtually quantitative complexation (47 μM **1**, 50 μM CB6, corresponding to >99% dye complexation, Figure 2) allowed

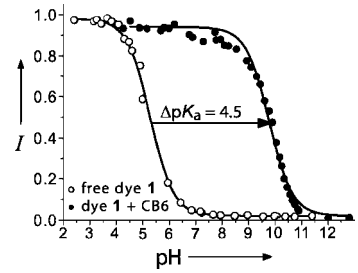

Figure 2. pK_a shift of **1** upon complexation by cucurbit[6]uril monitored through the fluorescence change upon pH variation for the free dye **1** (open circles) and the **1**·CB6 complex (solid circles) (λ_{exc} = 311 nm, λ_{em} = 375 nm).

a direct estimate of the pK_a' value of the complex which was found to be 9.8. This corresponds to a supramolecular pK_a shift of 4.5 units, one of the largest pK_a shifts yet reported.[24−28]

The strong fluorescence response (up to a factor of 100 increase) of the **1**·CB6 system allows its direct implementation for indicator displacement.[1−7] Moreover, the distinct fluorescence response of the two spectrally well-separated fluorescence bands allows for the first time a *ratiometric* detection of the displacement process from a cation-receptor macrocycle, which is well-known to have several advantages.[42] A typical displacement titration is depicted in Figure 3 where the addition of 1,5-diamino pentane (cadaverine), a well-known high-affinity guest toward several cucurbiturils,[33,35] fully reverts the fluorescence changes originally caused by the addition of the macrocycle. This fluorescence response, namely, a decrease of the short-wavelength band with a parallel increase of the long-wavelength fluorescence

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(41) The inclusion pattern was independently confirmed through characteristic ^{1}H NMR shifts, which revealed an upfield shift of the aliphatic protons of the anchor and downfield shifts of the aromatic protons (see Supporting Information). Moreover, the fluorescence response of the parent dye (3-amino-9-ethylcarbazole) upon addition of CB6 was markedly different from that obtained for the derivative **1** (small fluorescence increase for both fluorescence bands).

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band, is fully consistent with the notion that the added diamine, present in its diammonium form at pH 7, displaces efficiently the protonated dye from the complex, such that the original fluorescence properties of the unprotonated dye are restored (compare Figure 3 with Figure 1). The competitive titration was used to recover the binding constant of the competitor,[6,8] providing a value of $(9.5 \pm 1.1) \times 10^9$ M^{-1} in 10 mM NH_4OAc buffer. This is consistent with the literature value of 1.5×10^9 M^{-1},[37] if one considers the higher salt concentration in the latter study (50 mM NaCl).

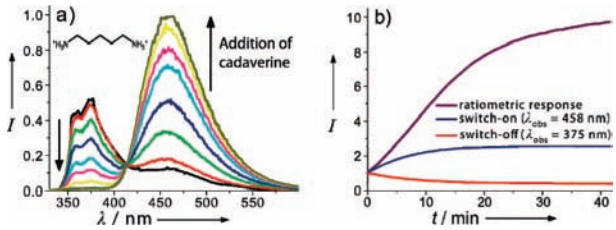

Figure 3. (a) Fluorescence titration for the competitive displacement of **1** (47 μM) from CB6 (50 μM) by cadaverine (up to 50 μM) in 10 mM NH_4OAc buffer at pH 7. (b) Supramolecular tandem assay for lysine decarboxylase (in 10 mM NH_4OAc buffer at pH 7) with the **1**•CB6 reporter pair (40 μg/mL of enzyme, 50 μM lysine, 10 μM **1**, 10 μM CB6, $\lambda_{exc} = 311$ nm), cf. refs 8 and 9, with continuous monitoring of the fluorescence intensity at two different wavelengths and the associated ratiometric signal (see Supporting Information).

According to Figure 2, the largest fluorescence response associated with the host-assisted pK_a shift of the dye is expected in the region between the pK_a values of the uncomplexed dye and its complexed form, i.e., between pH 5 and 9.5. In this pH range, a large fraction of the uncomplexed dye is deprotonated and converted, upon complexation, into a protonated fluorophore with different photophysical properties. The new displacement system is consequently ideally suited and designed to operate in the physiologically relevant neutral pH region (7 ± 2) and can be directly implemented into our recently developed supramolecular tandem assays for monitoring enzymatic activity.[8,9] We have tested this conjecture by using the **1**•CB6 complex as a new reporter pair to monitor the enzymatic decarboxylation of lysine, which converts the amino acid L-lysine into cadaverine. The binding constant of the substrate (e.g., 870 M^{-1} for cucurbit[7]uril)[8] is too low to displace the more strongly bound fluorescent dye (2.22×10^7 M^{-1}), while the product cadaverine competes very efficiently ($K_c = 9.5 \times 10^9$ M^{-1}) (Figure 3a). Indeed, when the enzymatic conversion of 50 μM substrate by lysine decarboxylase (40 μg/ml) was monitored in neutral buffer solution in the presence of 10 μM **1** and 10 μM CB6, we were able to monitor the enzyme kinetics readily by fluorescence (Figure 3b). As a particular advantage, we could not only select the monitoring wavelength at will to observe either a fluorescence decrease or increase (where the latter switch-on response is frequently more desirable) but also increase the sensitivity by recording a ratiometric response.

In conclusion, we have introduced a general supramolecular concept for designing high-affinity dyes which show a large and predictable fluorescence on−off as well as off−on switching upon complexation with cation-receptor macrocycles.[16,24,25,29] Not only can the affinity of the dye be tuned through the structure of a suitable anchor but also the charge, size, and hydrophobicity of the actual chromophore can be varied over a large range because it remains positioned outside the cavity. The fluorescence response is solely ensured through a host-assisted protonation of an aryl amino group, which mimics a pH jump by 4 units or more. The approach should be transferable to aliphatic amino groups, which have been abundantly used in the construction of photoelectron-transfer-based sensors, and it will be of interest for the design of new optical devices such as logic gates.[43−45] Most importantly, we have optimized an indicator-displacement strategy to conveniently monitor and quantify analyte binding to cucurbit[6]uril (CB6), which is too small to include most conventional dyes and whose analyte binding needed to be previously investigated by alternative techniques, particularly by ^{1}H NMR[33,36] or isothermal titration calorimetry.[37]

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Supporting Information Available: Experimental details, NMR spectra, titrations with CB7 and CB8, and photophysical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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