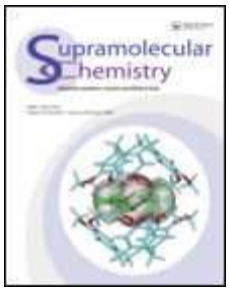
Cucurbituril Encapsulation of Fluorescent Dyes

Apurba L. Koner, Werner Nau



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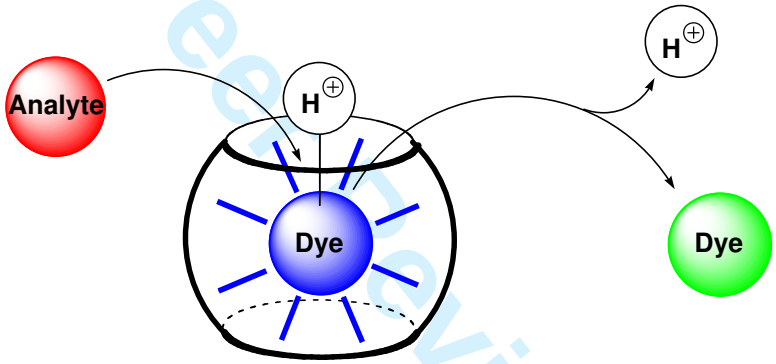
Graphical Abstract



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Running title: Cucurbituril and fluorescent dyes

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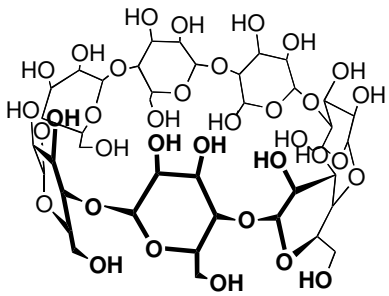
Abstract

The potential of cucurbiturils, water-soluble macrocyclic host molecules composed of glycoluril units, for tuning the properties of fluorescent dyes and advancing new applications is illustrated. Cucurbit[7]uril (CB7), which presents a particularly attractive derivative due to its intermediary size and high water solubility, has been shown to display a variety of advantageous effects on fluorescent dyes, which include increased fluorescence intensity and brightness, enhanced photostability, protection towards fluorescence quenchers, solubilization, and deaggregation. Particularly noteworthy is the prolongation of the fluorescence lifetimes of different dyes, which can be traced back to the low polarizability of the host cavity. In addition, the host serves as cation receptor, which causes a considerable shift of protonation equilibria and assists the protonation of fluorescent dyes. The latter effect can be exploited in the design of protolytic fluorophore displacement assays. The perspective of cucurbiturils as stabilizers for laser dyes, enhancement agents in time-resolved fluorescence (TRF) assays, contrast agents for fluorescence lifetime imaging (FLIM), and dyes for fluorescent collectors for solar cells is mentioned. Original experimental results for the effect of CB7 on the fluorescence properties of three dyes (Macrolex Yellow 10 GN, Dapoxyl, and 4-(dimethylamino)benzonitrile) are presented.

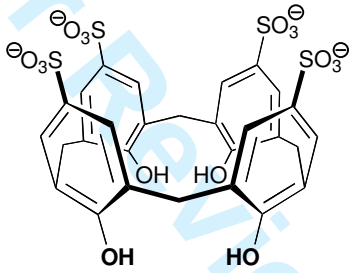
Introduction

Complexes of macrocyclic host molecules with smaller guest molecules present prototypal supramolecular architectures. The possibility to form complexes with chromophoric guest molecules[1] and thereby improve their fluorescence properties[2] remains an important application area of supramolecular photochemistry.[3] Owing to the importance of fluorescent probes and sensors for environmental and biological applications, it is usually desirable to "tune" fluorescent dyes in aqueous solution, which places the emphasis on the investigation of water-soluble hosts. The most common macrocyclic hosts, which have been studied in this context,[4] are cyclodextrins[5,6] and calix[*n*]arenes.[7-9]

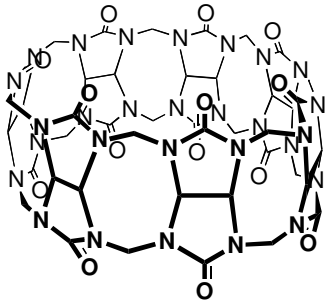
Scheme 1. Structures of water-soluble macrocyclic host molecules with comparable cavity size.



β-Cyclodextrin



p-Sulfonatocalix[4]arene



Cucurbit[7]uril

Cucurbiturils are another attractive class of water-soluble macrocyclic host molecules, which like cyclodextrins and calixarenes possess a concave interior capable of accommodating smaller guest molecules. They are composed of a different number of glycoluril units joined by pairs of methylene bridges. Cucurbit[6]uril, with 6 glycoluril units, represents the originally described condensation product by Behrend in 1905,[10] which was later revived by Mock.[11] The first practically relevant applications of this synthetic host were reported by Buschmann and Schollmeyer.[12,13] Cucurbit[*n*]urils (CBn) with different sizes (*n* = 5-8) have later been synthesized by Kim and coworkers[14] as well as Day and coworkers,[15] which has recently led to an unfolding of their supramolecular chemistry.[16-18] For example, sophisticated rotaxane structures have been constructed with cucurbiturils as macrocycles.[19-24]

Cucurbiturils are pumpkin shaped, highly symmetrical, and rigid macrocycles with an extremely non-polarizable (close to gas phase)[25] cavity. They are capable of forming strong complexes with positively charged (or neutral) molecules by coordination of cationic sites with their portals and/or immersing organic residues in their hydrophobic cavities.[11,16] The very small accessible inner volume of CB5 (< 50 Å^3 capacity) limits greatly its use in host-guest chemistry. CB6 is significantly larger (ca. 105 Å^3 capacity), and can include molecules with up to 7 heavy atoms,[16] but it is presumably too poorly water-soluble (20-30 μM)[16,26] to allow broad biologically or environmentally relevant utilization. CB7 is, in the present context, the most attractive host because it fulfills the requirement of sufficient water solubility (ca. 5 mM, if synthesized by our hands).[25,27] The inner CB7 has a favorable size (ca. 200 Å^3 capacity) to form 1:1 complexes with a large range of organic guest molecules and can accommodate at least 12 heavy atoms in its inner cavity.[14,25,28-32] CB8 suffers again from a very low water solubility (< 150 μM),[18,33] but its cavity is sufficiently large (ca. 300 Å^3 capacity) to encapsulate two guest molecules, which leads frequently to the formation of 1:2 host-guest complexes and interesting qualitative phenomena; the latter are in part reminiscent of those observed for the larger γ-cyclodextrin.[32-37] The isolation of guest-free CB10 and its host-guest chemistry has only recently been reported by Isaacs and coworkers;[38] CB10 is a very large host molecule which can itself encapsulate CB5[39] or other macrocycles like calix[4]arenes.[38]

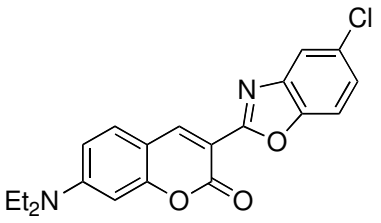
The effect of cucurbituril encapsulation on the photophysical and photochemical properties of guest molecules has been studied in comparably little detail. Complexation-induced changes of the absorption spectra have been previously used to monitor complexation and determine binding constants, e.g., for 4-methylbenzylammonium with CB6,[40] azobenzene with CB6,[41] azo dyes with CB6,[42] for methylviologen with CB7[43] and CB8,[44] and for 2,7-dimethyldiazapyrenium with CB7.[30] We have employed the solvatochromic shifts in the absorption spectra of 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) to study the microenvironment inside the cavity of CB7.[25] With respect to the use of fluorescent labels with cucurbiturils, some case studies have been reported for a

rotaxane-based molecular switch with a fluorescent string[20] and for a fluorescently tagged spermine with affinity to functionalized CB6.[45] The effect of cucurbituril encapsulation on the molecular fluorescence of organic dyes has also attracted more detailed interest.[29,46-49] Our own studies in this very field have focused on the manifold effects of CB7 on the fluorescence of neutral and cationic organic dyes.[25,27,28,50-54] The outcome of these investigations, which have revealed some conventional, many interesting, and also several very uncommon host properties, will be put into perspective in this concept paper to demonstrate the application potential of cucurbiturils for tuning fluorescent dyes.

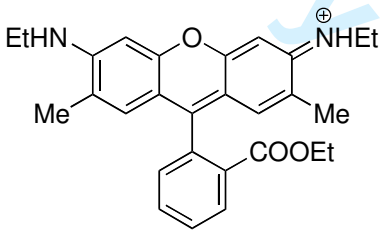
A. Solubilization and Deaggregation of Fluorescent Dyes with Cucurbituril

Macrocyclic host molecules have the potential to increase the solubility of poorly water-soluble or insoluble guest molecules by forming inclusion complexes. Numerous drug-related applications of cyclodextrins are based on this desirable supramolecular function. Cucurbiturils exhibit similar behavior for poorly water-soluble or insoluble fluorescent dyes. As one of several examples, we provide the fluorescence and UV absorption spectra of Macrolex Fl Yellow 10 GN in water, a fluorescent dye employed as fluorescent collector in solar cells (Figure 1a, this work).[55] This dye is entirely insoluble in neat water (no absorption and characteristic emission), but in the presence of CB7 (4 mM), the dye becomes sufficiently soluble to allow both, a sizable absorption as well as an emission, to appear.

Scheme 2. Structures of Macrolex Fl Yellow 10 GN, a dye used for fluorescent collectors, and rhodamine 6G, a dye used as laser dye and for referencing in FCS.



Macrolex Fl Yellow 10 GN



Rh6G

The solubilizing effect of CB7 suppresses also the adsorption of fluorescent dyes to material surfaces, e.g., of sample cuvettes and well plates, which has been representatively demonstrated for rhodamine 6G (Rh6G), *cf*. Figure 1b.[52] In the course of fluorescence correlation spectroscopy (FCS) experiments, for example, nearly 90% of the fluorescent dye were already lost during sample preparation (dissolution, pipetting, dilution, etc.), as can be seen from the reduced fluorescence intensity at the beginning of the experiment (t = 0 min, note the logarithmic intensity scale) for the sample without solubility-enhancing additive. In addition, the intensity depletes rapidly with time during the on-going experiment, which can be entirely prevented by the addition of CB7 (straight line in Figure 1b). Unspecific adsorption of Rh6G to the walls of the sample containers is presumed to be responsible for the observed rapid depletion of the dye, which is efficiently prevented by encapsulation into CB7; apparently, CB7 and its complexes have only a very low propensity to adsorb to glass and polymer materials.

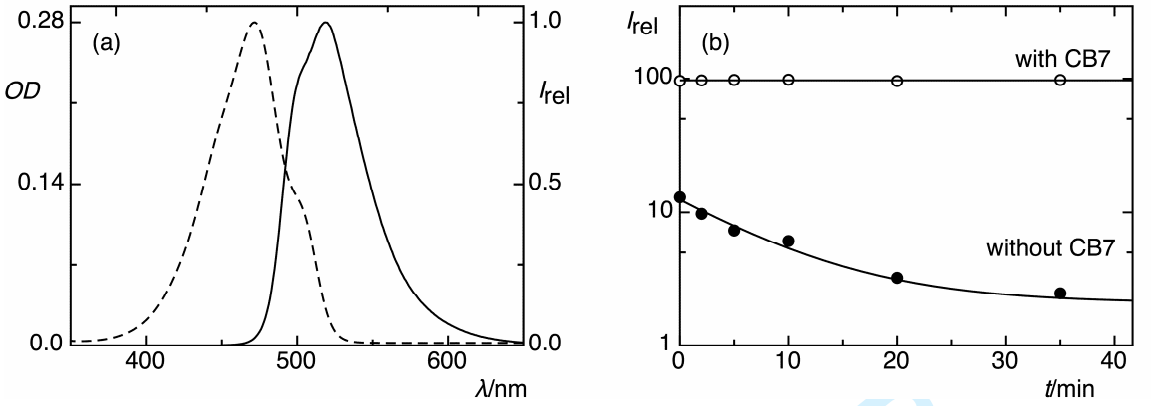


Figure 1. Effect of CB7 on the solubilization of fluorescent dyes. (a) Absorption (dashed line) and emission spectra (solid line) of Macrolex Fl Yellow 10 GN in water (ca. 5 μM) in the presence of CB7 (4 mM) at ambient temperature; note that this dye is insoluble in water in the absence of CB7 (no absorbance after treatment with ultrasound and subsequent filtration). (b) Dependence of the registered fluorescence count rate (I_{rel}) of a Rh6G solution (10 nM) in the time course of an extended FCS measurement in aerated water in the absence and presence of 1 mM cucurbit[7]uril, from ref. [52].

Closely related to the solubilizing properties are the deaggregating properties of water-soluble host molecules. In fact, dye aggregation can be remedied by CB7 as well.[28,52] This is in sharp contrast to CB8, which can accommodate two aromatic guest molecules and does therefore assist rather than suppress such aggregate formation. The latter is frequently undesirable from a photophysical point of view (fluorescence quenching), but in special cases it can also lead to interesting properties like regio- and diastereoselective [2+2] or [4+4] photocycloadditions.[32-37]

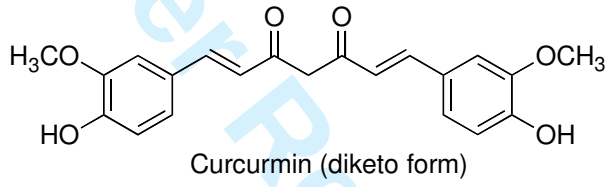
B. Fluorescence Enhancement of Dyes with Cucurbituril

Another area of interest is the fluorescence enhancement frequently observed when macrocyclic host molecules are added to aqueous solutions of fluorescent dyes. These are routinely traced back to the positioning of the dyes into a more hydrophobic environment. The fluorescence changes can be conveniently employed to determine the binding constants of the fluorescent complexes,[30,56,57] and they are most relevant for sensor applications, where the fluorescent dye may serve either itself as analyte, or, preferably, as probe to signal the binding of an analyte by an indicator displacement strategy.[58-60] Fluorescence enhancement upon encapsulation into cucurbiturils in solution was first observed and documented by Wagner and coworkers.[46-48] The authors observed an enhancement of 5 times for both curcumin and 2-anilinonaphthalene-6-sulfonate (2,6-ANS) with CB6 and ca. 25 times for 2,6-ANS with CB7. The enhancement for 1-anilinonaphthalene-8-sulfonate (1,8-ANS) with CB7 was larger (ca. 100), but different complexation stoichiometries applied.

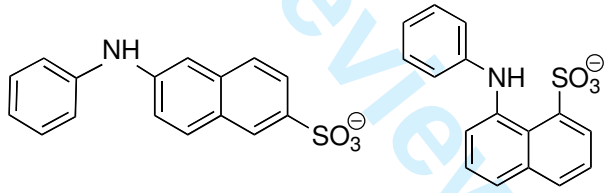
Fluorescence enhancements have been observed in our laboratory for several dyes as well. For example, we observed very large enhancements for two fluorescent intramolecular charge transfer (ICT) dyes:[61] Dapoxyl sulphonic acid sodium salt (Dapoxyl)[62-64] and 4-(dimethylamino)benzonitrile (DMABN).[61] Noteworthy is the differential response of the locally excited (LE) and the longer-wavelength charge transfer (CT) band of the Dapoxyl dye (Figure 2a, this work), for which the enhancement factors amount to > 200 and ca. 5, respectively. The fluorescence quantum yield increases

from 4% in water[63] to ca. 50 %, a value similar to that in alcoholic solvents.[63] This enhancement by about one order of magnitude is comparable to that observed for β-cyclodextrins as host, for which, however, no increase of the LE band of Dapoxyl was observed (this work). It should be noted in this context that the fluorescence enhancement of the Dapoxyl dye, which can be virtually monitored over the entire range of the visible spectrum, is ideally suited for the design of protolytic fluorophore displacement sensors and assays based on CB7 (see below). For DMABN, which has an extremely small fluorescence quantum yield of 0.07% in water,[65] the fluorescence enhancement of the CT band amounts to a factor of nearly 10 (Figure 2b, this work), comparable to the enhancement observed for DMABN with α-cyclodextrin.[61]

Scheme 3. Structures of fluorescent dyes previously investigated with CB6 and CB7

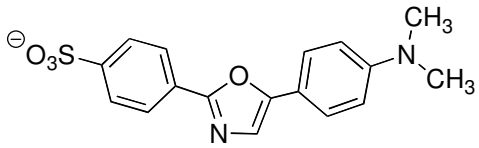


Curcurmin (diketo form)

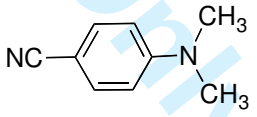


2,6-ANS 1,8-ANS

Scheme 4. Structures of fluorescent intramolecular charge transfer (ICT) dyes



Dapoxyl



DMABN

The photophysical effects of the inclusion complexation by cyclodextrins and calixarenes have been quite universally interpreted in terms of either the positioning of the fluorophore into the more hydrophobic environment of the host cavities ("polarity effect") or related to the geometrical confinement of the chromophore within the host ("confinement effect"). While a polarity effect (lower polarity inside cucurbiturils, see

below) and a confinement effect (decrease in nonradiative decay rates)[28,47,52] may well contribute to the observed fluorescence enhancements for cucurbiturils, charge-dipole interactions play unquestionably a dominant role in the interaction of fluorophores with cucurbiturils, which provides a contrast to the situation for cyclodextrins. These may lead to a host-assisted guest protonation, i.e., the dye becomes protonated once complexed (see below); this accounts for the observation of the LE band for Dapoxyl in Figure 2a, which actually corresponds to the emission of protonated Dapoxyl.

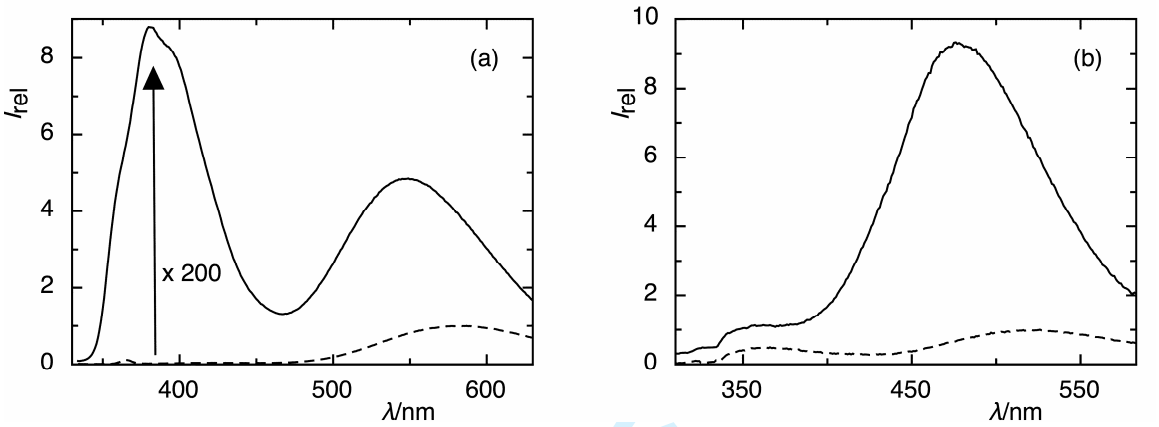


Figure 2. Fluorescence enhancement of ICT fluorescent dyes by CB7: (a) Fluorescence spectra of Dapoxyl (5 μM) in the absence (dashed line, normalized to 1 at maximum) and presence (solid line) of 1 mM CB7 in water at pH 5.5 (λ_{exc} = 282 nm, the far-UV isosbestic point during the titration with CB7). (b) Fluorescence spectra of DMABN (2.5 μM) in the absence (dashed line, normalized to 1 at maximum) and presence (solid line) of 0.1 mM CB7 in water (λ_{exc} = 282 nm, the apparent isosbestic point during the titration with CB7).

C. Solvatochromic Effects to Determine the Polarity of the Cucurbituril Cavity

Variations in the absorption and fluorescence spectra can be principally employed to estimate the polarity of the inner cavity which cannot be assessed by direct spectroscopic methods. Specifically, solvatochromic molecular probes are being employed, which show well-established trends of absorption or fluorescence properties

with the polarity of the microenvironment. Wagner and coworkers have employed curcumin to estimate the polarity of the CB6 cavity and found a value lower than water, but still substantially higher than ethanol.[48] The bathochromic shift of Rh6G observed upon addition of CB7 has also been interpreted in terms of a solvatochromic shift and provided in this case a polarity similar to that of *n*-octanol.[52] The environment experienced by fluorescent dyes inside cucurbiturils is therefore quite similar to that of alcohols or alcohol-water mixtures. Very similar polarities were previously determined for cyclodextrins or calixarenes by using 1,8-ANS as polarity-sensitive probe.[56,66,67] Presumably, the organic dyes are not completely immersed in the cavities of any host, such that the chromophores remain at least partially exposed to the surrounding water. The similarity of the polarities for the different macrocyclic host cavities reveals nicely, however, that the interior of cucurbiturils is nothing "special" from a polarity point of view. This contrasts the unique position of these glycoluril-based hosts with respect to the polarizability of the cavity (see below).

D. Solvatochromic Effects to Determine the Polarizability of the Cucurbituril Cavity

In addition to the polarity of the environment, another important parameter for chemical reactivity and photophysical properties is the polarizability/refractive index. We have introduced the use of DBO as a solvatochromic probe to determine the polarizability of solvents and supramolecular environments, including the cavities of macrocyclic host molecules (Table 1).[25,28,51,54,60] For this purpose, the oscillator strength of the near-UV absorption band of DBO or its radiative decay rate are measured, which are empirically or theoretically related to the polarizability or the refractive index of the immediate microenvironment, respectively.

Scheme 5. Structure of DBO, a polarizability-sensitive solvatochromic probe

DBO

The striking feature of CB7 as a macrocyclic host is its low polarizability, which falls below that of cyclodextrins, while aromatic hosts (calixarenes and hemicarcerands) display much higher polarizabilities (Table 1). In fact, the polarizability of the CB7 cavity falls even below that of perfluorohexane, the solvent commonly known to have the lowest polarizability. Guest molecules encapsulated by CB7 experience therefore an exceptionally low polarizability, close to the gas phase, which leads to novel and unprecedented chemical and photophysical properties. Incidentally, the observation of such an extreme physical property inside a macrocyclic host has then provided the long-sought-for spectroscopic evidence[25,54] for Cram's famous hypothesis, formulated for hemicarcerands, that the inner space of such "molecular container compounds" behaves as a new phase of matter.[68]

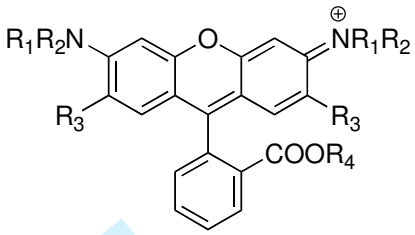
Table 1. Polarizability and refractive index inside macrocyclic host molecules, determined by using the DBO chromophore as a solvatochromic probe, relative to those in solvents and in the gas phase.

Environment	Polarizability (*P*) [a]	Refractive index (*n*) [b]
Gas phase	0.000	1.000
Cucurbit[7]uril[c]	0.12	1.19
Perfluorohexane	0.159	1.252
β-Cyclodextrin	0.20	1.33
H_2O	0.206	1.333
n-Hexane	0.229	1.375
DMe-β-CD (2:1 complex)[d]	0.24	1.39
p-Sulfonatocalix[4]arene[e]	0.25	1.41
Benzene	0.295	1.501
Diiodomethane	0.404	1.742
Hemicarcerand[f]	0.45	1.86

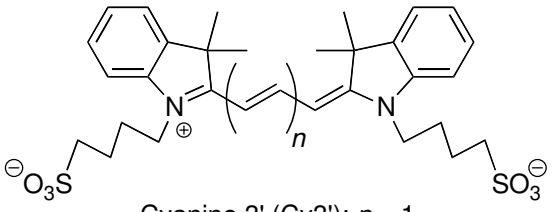
[a] Calculated from the refractive index using the formula $P = (n^2-1)/(n^2+2)$; for macrocyclic hosts, the polarizability was determined experimentally as described in ref. [25]. [b] From ref. [69]; for macrocyclic hosts, the refractive index was calculated from the polarizability using the formula $P = (n^2-1)/(n^2+2)$. [c] From ref. [25]. [d] From ref. [70] with heptakis-(2,6-di-*O*-methyl)-*β*-cyclodextrin as host. [e] From ref. [60]. [f] Value determined using biacetyl as solvatochromic probe, *cf*. ref.[25].

The low polarizability/refractive index of the cucurbituril cavity can be readily rationalized in terms of its chemical structural topology: The inner phase is very electron-deficient, because electron density is efficiently displaced to the carbonyl oxygens towards the upper and lower rim. All bonds accessible from the inside are strongly polarized, which is another feature of many molecules with low polarizability, e.g., perfluorohexane and water. In addition, there are no C–H bonds, π bonds, or lone pair orbitals, which point inwards and which could thereby enhance the polarizability. Note, in particular, that the concave nature displaces the electron density of the ureido nitrogen lone pairs towards the outside of the cavity.

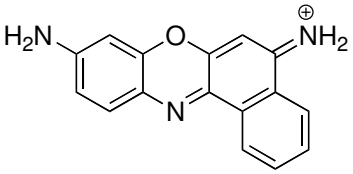
Scheme 6. Structure of common fluorescent dyes complexed by CB7



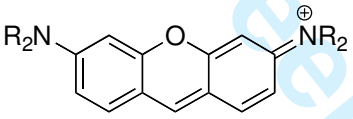
Rhodamine 6G (Rh6G)
(R_1 = H, R_2 = Et, R_3 = Me, R_4 = Et)
Tetramethylrhodamine (TMR)
(R_1 = R_2 = Me, R_3 = H, R_4 = H)
Rhodamine 123 (Rh123)
(R_1 = R_2 = H, R_3 = H, R_4 = Et)



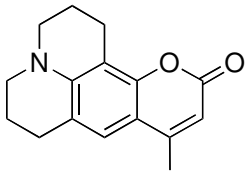
Cyanine 3' (Cy3'): n = 1
Cyanine 5' (Cy5'): n = 2



Cresyl violet (CV)



Pyronin Y (PyY): R = Me
Pyronin B (PyB): R = Et



Coumarin102 (C102)

Table 2. Photophysical parameters of fluorescent dyes (see Scheme 6) with and without 1 mM cucurbit[7]uril (CB7) in H_2O under air[a]

Fluorescent dye		λ_{abs}^{max} /nm	λ_{em}^{max} /nm	ε_{max} / (10^4 $M^{-1}cm^{-1}$)	ϕ_f	Brightness[b]	τ_f /ns
DBO[c]	without CB7	364	419	5.3×10^{-3}	0.26	1.4×10^{-3}	415
	with CB7	374	427	4.0×10^{-3}	0.19	7.6×10^{-4}	725
Rh6G	without CB7	526	552	8.02	0.89	7.14	4.08
	with CB7	535	555	9.24	0.89	8.22	4.76
TMR	without CB7	553	577	8.78	0.28	2.46	2.15
	with CB7	559	582	7.48	0.38	2.84	4.16
Rh123	without CB7	500	525	6.92	0.83	5.75	4.19
	with CB7	503	532	6.66	0.36	2.40	4.63
PyY	without CB7	546	565	13.2	0.47	6.20	1.69
	with CB7	544	568	13.1	0.63	8.25	3.44
PyB	without CB7	552	569	9.41	0.36	3.39	1.19
	with CB7	556	571	9.93	0.70	6.95	3.10
C102	without CB7	393	489	2.18	0.66	1.44	6.04
	with CB7	405	476	2.36	0.75	1.77	7.19
CV	without CB7	585	625	3.31	0.36	1.19	2.18
	with CB7	591	628	4.09	0.35	1.43	3.93
Cy3'	without CB7	545	560	12.0	0.04	0.48	0.46
	with CB7	559	571	10.7	0.03	0.32	0.58
Cy5'	without CB7	642	660	13.8	0.17	2.35	0.63
	with CB7	642	657	11.2	0.30	3.36	1.59

[a] From ref. [28]. [b] Calculated as $\varepsilon_{max}\phi_f/(10^4\ M^{-1}cm^{-1})$. [c] From ref. [25,51].

E. Prolongation of Fluorescence Lifetimes with Cucurbituril

The most characteristic consequence of the low polarizability experienced by guest molecules encapsulated inside cucurbiturils is a photophysical one, because the radiative decay rate (the ratio of fluorescence quantum yield and fluorescence lifetime) decreases. This is theoretically expected from the Strickler-Berg equation,[71] which predicts a dependence of the radiative decay rate on the square of the refractive index (as an alternative measure of polarizability). Literally speaking, this means that the fluorescence of dyes is emitted "slower" from cucurbiturils, and experimentally this results in an increase in fluorescence lifetime. This lifetime-prolonging effect of cucurbiturils has in the meantime been observed for numerous fluorescent dyes with CB7 (Table 2, last column) and we have referred to this method as "supramolecular radiative decay engineering",[54] borrowing a term introduced by Lakowicz for the (apparent) increase of radiative decay rates near metal surfaces and nanoparticles.[72]

The increase in fluorescence lifetimes could find practical applications in fluorescence lifetime imaging microscopy (FLIM), where a larger spread in fluorescence lifetimes would increase contrast. In addition, the complexation by CB7 results in the formation of particularly long-lived fluorescent dyes, which can be detected with superior sensitivity in time-resolved fluorescence (TRF) assays, e.g., to monitor enzymatic transformations.[27,73]

F. Photostabilization of Fluorescent Dyes with Cucurbituril

With respect to chemical reactivity, a low polarizability invariably reduces the rates of chemical reactions, while a high polarizability enhances them, e.g., for bond homolyses.[54,74] This combines with an intrinsically low chemical reactivity of cucurbiturils, e.g., towards oxidation,[75] to render the cucurbituril cavity a chemically inert reaction environment. Cyclodextrins and calixarenes, for comparison, have a higher polarizability and are also much more prone to undergo oxidation reactions, e.g., Ag(I) ions rapidly oxidize *p*-sulfonatocalix[4]arene, but form stable complexes with CB7

(unpublished results). The low polarity of the CB7 cavity (see above) and the efficient exclusion of water molecules further reduces the rates of ionizing reactions.[52]

In view of the chemical inertness of cucurbiturils, it is not surprising that CB7 efficiently suppresses photochemical reactions of fluorescent dyes and thereby their photodecomposition. This results in fluorescent dyes with improved photostability, which has been demonstrated, among others, for Rh6G. In detail, under high irradiance levels with 532-nm Nd-YAG laser excitation, the photostability of Rh6G increases by a factor of 30 in the presence of 1 mM CB7.[52] This photostabilization of CB7 combines with its favorable effects on the "thermal" stability of dyes (suppression of adsorption), and its deaggregating effect, to produce fluorescent dye solutions of unprecedented storage and working stability. These are high in demand, in particular, for dye laser applications and in confocal microscopy. The addition of CB7 to fluorescent dye solutions provides therefore a novel supramolecular approach to achieve photostabilization.[1,28,52,54] In addition, encapsulation by cucurbiturils should also enhance the chemical stability of dyes which are sensitive toward oxidation or hydrolysis; in fact, the stabilization of the dye Phenol Blue against hydrolytic decomposition presented one of the earliest application examples of CB6.[12]

G. Protection from External Fluorescence Quenchers by Cucurbituril

It is worth to mention, with respect to the use of cucurbiturils for optical applications, that these hosts are transparent in the visible and do not act as fluorescence quenchers at typically relevant concentrations (mM). On the opposite, encapsulation by cucurbiturils greatly reduces or completely suppresses the fluorescence quenching of dyes by external additives because it provides a protective shield. This protection was tested for DBO, which is efficiently quenched in its free form by a variety of electron and hydrogen atom donors, as well as singlet energy acceptors (Table 3). Strikingly, upon encapsulation into CB7, the fluorescence quenching is entirely shut off in most cases, because any intimate contact between excited probe and quencher is prevented by the separating walls of the supramolecular container. The observed protection is much more efficient, in fact, than

that found for cyclodextrins as alternative hosts (see value for ascorbate quenching in square brackets in Table 3),[76] and can be readily rationalized in terms of the rigid barrel-like shape of cucurbiturils. Solely the quenching by through-space (and therefore also "through-wall") mechanisms, like fluorescence resonance energy transfer for nitrotyrosine (critical transfer radius ca. 30 Å at pH 8), is still possible (although at a somewhat reduced rate). Expectedly, water causes also some residual quenching by accessing the complexed guest through the portals of cucurbituril; this can be eventually suppressed by sealing the portals with metal ions as "lids".[27]

Table 3. Fluorescence quenching rate constants (k_q) of DBO in its free and complexed (CB7•DBO) form, in H_2O, from ref. [27]

	$k_q/10^9$ $M^{-1}s^{-1}$	
Quencher	DBO	CB7•DBO[a]
Nitrotyrosine[b]	8.0	4.7
Biacetyl	6.1	< 0.05
Sodium nitrite	2.5	< 0.1
Oxygen	2.1	< 0.04
Ascorbic acid	2.1	< 0.006 [0.22] [c]
Tryptophan	2.1	< 0.1
Sodium iodide	1.6	< 0.005
dGMP[d]	0.5	< 0.005
H_2O[e]	4×10^{-5}	8×10^{-6}

[a] Measured under conditions of nearly quantitative complexation (>99 %, [DBO] = 2 mM, [CB7] = 3 mM), $K = 4 \times 10^5$ M^{-1}. [b] Measured in borate buffer at pH 8.0 with 30 μM DBO in the absence and presence of 100 μM CB7, respectively; A. Hennig, unpublished results. [c] Quenching rate constant for the β-cyclodextrin complex of DBO, from ref. [76]. [d] 2'-Deoxyguanosine-5'-monophosphate disodium salt. [e] This work; calculated from the fluorescence lifetimes in H_2O in the absence and presence of CB7 by

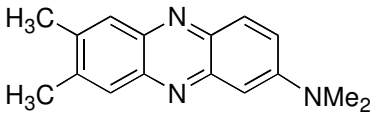
assuming an "unquenched" fluorescence lifetime of 1 μs, the one observed in the gas phase and in D_2O in the presence of capping metal ions, *cf*. ref. [27].

H. Increase in Brightness of Common Fluorescent Dyes with Cucurbituril

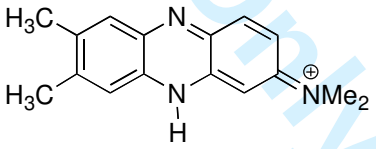
As stated above, encapsulation by cucurbituril, as well as by some other macrocyclic hosts, can enhance the fluorescence of dyes. Positive effects are most commonly observed for cationic dyes, which can interact with the carbonyl ureido portals of cucurbiturils on account of their cation receptor potential. Fortunately, many common dyes are positively charged, frequently due to cationic (mostly iminium) sites (Scheme 6), which expands the application potential of cucurbituril substantially.

The binding constants of several dyes were determined by means of UV-Vis or fluorescence titrations. They are generally very high (K = 10^4-10^5 M^{-1}), with few exceptions like Rh123 (K ca. 1000 M^{-1}).[25,28,52] For those fluorescent dyes which can occur in two prototropic forms, the binding constants of the different species need to be estimated from measurements at appropriate pH; they are typically two orders of magnitude higher for the protonated form when CB7 is used as host. An example are the binding constants for neutral red measured by fluorescence titrations: $K = 6 \times 10^5$ M^{-1} for the protonated form (NRH^+) at pH 2 and K = 6500 M^{-1} for the unprotonated form (NR) at pH 11.[53]

Scheme 7. Prototropic forms of neutral red



NR

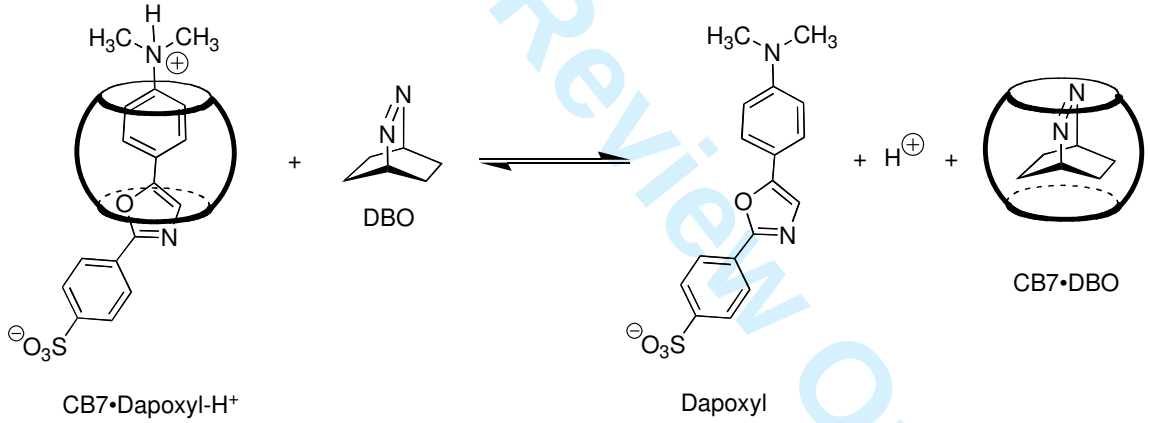


NRH^+

The most striking effects are observed for chromophores which have very low fluorescence quantum yields in water (Figure 2). However, to facilitate biological applications in particular, many fluorescent dyes have already been designed to display high fluorescence quantum yields in water, such that large enhancements factors due to additives are not expected. Nevertheless, in the case of several common fluorescent dyes

(Scheme 6), CB7 further increases the fluorescence (Table 2). The extinction coefficient (or absorption cross-section) presents an additional parameter to characterize the goodness of a fluorescent dye, and it is good practice to define a "brightness" as product of extinction coefficient (e.g., at the maximum) and fluorescence quantum yield. Complexation by CB7 leads quite universally in an increased brightness of chemically quite different fluorescent dyes, including rhodamines, pyronines, oxazines, coumarines, and selected cyanines (Table 2). Exceptions are the dyes Rh123 and Cy3', for which the brightness decreases as a consequence of a reduced fluorescence quantum yield (Table 2); presumably, the inclusion into CB7 promotes nonradiative decay of these two dyes.

Scheme 8. Tentative assignment of the structure of the CB7 complex with protonated Dapoxyl and displacement of the dye from CB7 by addition of DBO as a competitive guest; note that the release of Dapoxyl is accompanied by deprotonation (protolytic fluorophore displacement principle, see below).



It should be noted in this context that the mode of inclusion or association between fluorescent dye and CB7 is not accurately known in most cases, especially because the low dye concentrations commonly prevent detailed NMR characterization. Certainly, several dyes like Dapoxyl and Rh6G are too large to be entirely immersed inside the CB7 cavity. Nevertheless, there are several lines of evidence which suggest that the complexes with CB7 are of the (partial) inclusion and not of the association type. First, the solvatochromic shifts of the absorption and emission spectra are consistent with a less polar environment. Second, the increase in fluorescence lifetimes and the decrease

in radiative decay rates (see above) can only be reconciled in terms of a positioning into an extremely non-polarizable environment, i.e., inclusion into the cavity. Third, the translational diffusion coefficients of the dyes (measured by FCS) are reduced by a factor of 2-3 upon complexation with CB7,[28] which is also consistent with a tight complexation. Closely related, a decrease of the rotational diffusion coefficient by a factor of ca. 4 was also observed for the CB7 complex with neutral red by time-resolved fluorescence anisotropy.[53] Finally, the addition of DBO, which is sufficiently large to displace other organic residues from the cavity, yet sufficiently small not to interfere with binding to the outside walls or the portals of CB7, efficiently displaces several organic dyes like Dapoxyl and Rh6G from the complex (Scheme 8). This provides compelling evidence that an inclusion complex was actually formed, because it is firmly established that DBO itself forms a deep inclusion complex with CB7.[25]

I. Host-Assisted Protonation of Fluorescent Dyes with Cucurbituril

We have recently investigated in detail how the complexation of macrocyclic host molecules affects the protonation equilibria of guest molecules, and how the associated pK_a shifts can be analyzed.[77] *p*-Sulfonatocalix[4]arene, for example, stabilizes protonated guest molecules on account of charge-charge interactions, which was found to increase the pK_a values of azoalkanes as guest molecules by ca. 2 units.[77] CB6 stabilizes the protonated forms of guests by means of charge-dipole interactions, which was shown to increase the pK_a value of cyclohexylmethylammonium by 1.3 units.[78] More recently, we have also studied the complexation of the fluorescent dye neutral red (Scheme 7) with CB7 and found a pK_a shift by 2 units,[53] while Macartney and coworkers deduced a ground-state pK_a shift by 3.1 units for 2-ammoniumanthracene when complexed with CB7.[29] In the same study, a shift by up to 9 pK_a units was reported for the singlet-excited state, which is presumably in error, since it would exceed even the largest pK_a shifts observed in enzymes (5 units).[77]

From the combined studies, it becomes obvious that fluorescent dyes encapsulated in cucurbiturils should have a much higher propensity to become protonated

than the free dyes in aqueous solution. The protonation of dyes can be spectroscopically followed, as illustrated for the pH-dependent changes of the UV-Vis spectra for uncomplexed Dapoxyl (Figure 3a). In some cases, the protonated dye gives also rise to a distinct emission, e.g., the LE band in the case of protonated Dapoxyl (Figure 2a), which provides another means to directly follow the protonation process. Recall that the LE band of Dapoxyl at a particular pH is only observed in the presence of CB7, but not for the free dye; this is, in fact, a direct consequence of the selective stabilization of the protonated dye by this host, and not due to the more frequently implicated polarity or confinement effects (see above).

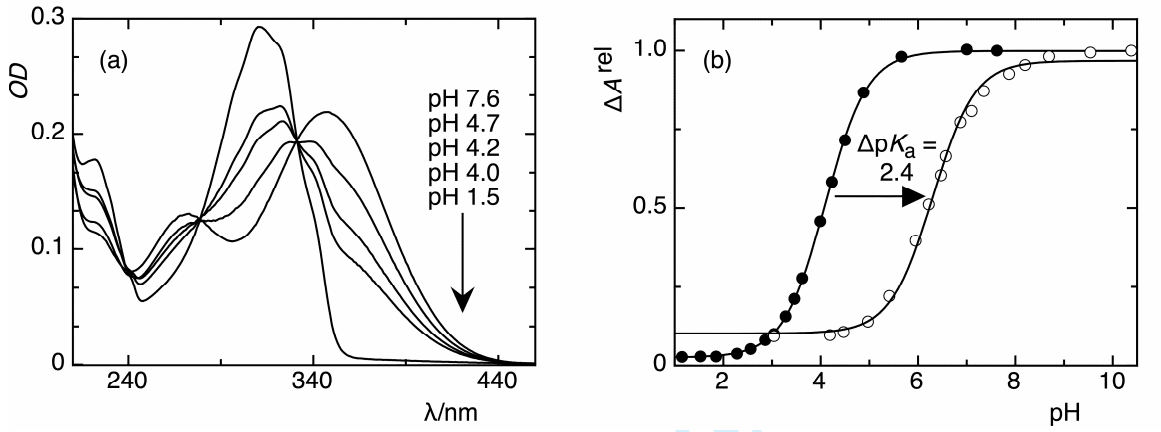


Figure 3. Analysis of protonation equilibria for Dapoxyl. (a) Change of the absorption spectra of uncomplexed Dapoxyl (9.1 μM) with pH. (b) pH titration for the UV-Vis absorbances of Dapoxyl (9.1 μM) in the absence (filled circles) and presence (open circles) of 1.7 mM CB7; note the large pK_a shift with CB7; the fitting of the titration curve in the presence of CB7 was performed according to a four-state equilibrium as described in ref [77].

The pK_a shift was quantitatively analyzed by UV-Vis pH-titrations for the uncomplexed and complexed Dapoxyl dye (Figure 3b); as can be seen, the pK_a value shifts by 2.4 units (this work). Of course, the pK_a shift should be directly reflected in the magnitude of the binding constants, e.g., a pK_a shift of 2 units corresponds to a 100 times larger binding constant for the protonated form of the guest.[77] The binding constants

determined for Dapoxyl by UV-Vis titrations (this work) amount indeed to $K = 2.2 \times 10^5$ M^{-1} for the protonated form (measured at pH 3) and only $K = 1500$ M^{-1} for the unprotonated form (at pH 9), which is in good agreement with the independently measured pK_a shift.

The protonation of fluorescent dyes in their cucurbituril complexes is a consequence of charge-dipole interactions, which assist the protonation of guest molecules in their ground state. In addition, they may also stabilize the singlet-excited states of cationic fluorescent dyes and thereby suppress an excited-state deprotonation otherwise observed for some dyes at comparable pH; this has been proposed to be the reason for the observation of the blue fluorescence of CB7-complexed 2-ammoniumanthracene in acidic solution.[29] Upon excitation to the singlet-excited state it remains protonated when complexed to CB7. In contrast, in its uncomplexed form, it tends to deprotonate rapidly due to a very low excited-state pK_a*, such that a green emission from the amine form is commonly observed. Finally, charge-dipole interactions could also stabilize charge-transfer separated states, especially when the positive charge is situated near the cation receptor sites (portals) of cucurbituril. This could favor fluorescence over nonradiative decay pathways of the charge-transfer state and may contribute to the enhancements observed for the CT emissions of both Dapoxyl and DMABN (Figure 2).

J. Protolytic Displacement Assays Using Cucurbituril

The pK_a shifts of fluorescent guests upon complexation by macrocyclic hosts have until now not been rationally exploited to increase the sensitivity of sensor applications. We suggest herewith a refined type of fluorophore displacement signaling, which exploits the different pK_a values of the complexed and uncomplexed form of the dye in such a way that the fluorescence of either the protonated or unprotonated form is only observed in its complexed or uncomplexed form; this boundary condition can be adjusted through pH, which should be chosen to lie in between the pK_a values of the complexed and uncomplexed forms. Addition of an external analyte leads then not only to a conventional

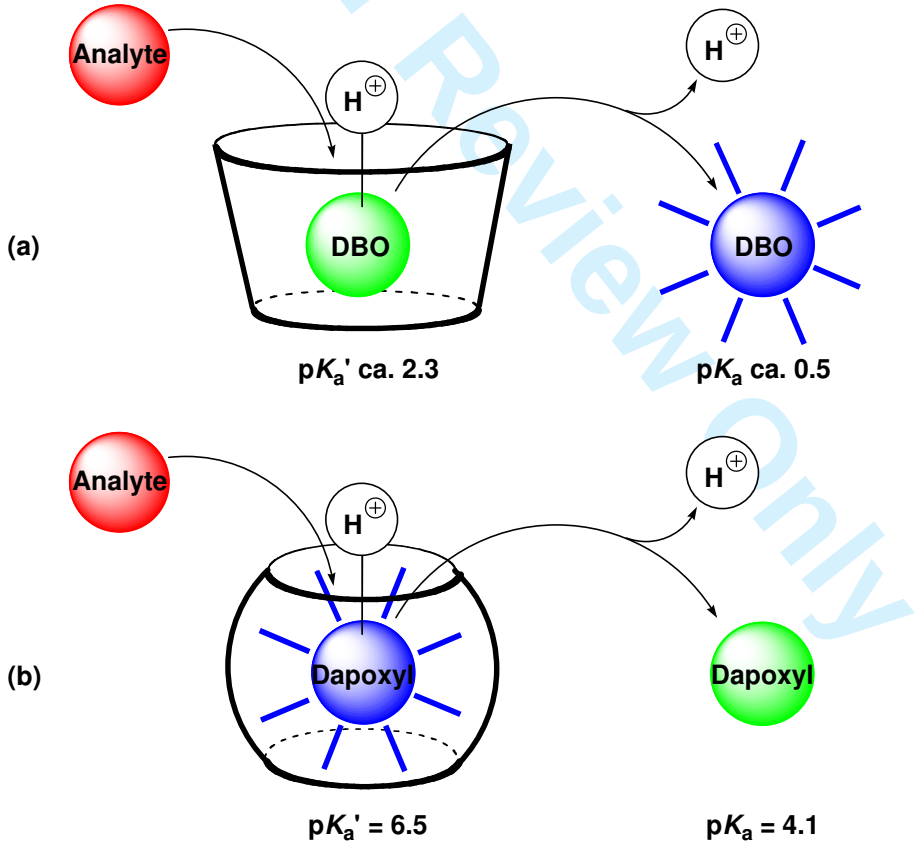
displacement of the fluorescent dye, but in addition it changes its protonation state as a consequence of the relocation into the aqueous bulk. Thus, while previously the variations in fluorescence upon addition of analyte were solely based on the differences in fluorescence of the complexed and uncomplexed form, changes in protonation state can have dramatic effects on the absorption and fluorescence spectra, which greatly increases the sensitivity toward analyte sensing. This sensing principle can be referred to as a "protolytic fluorophore displacement" and is best illustrated by providing two specific examples (Scheme 9).

The first protolytic fluorophore displacement system (Scheme 9a) employs indeed *p*-sulfonatocalix[4]arene as host,[59,60] which resembles cucurbiturils in their cation receptor properties and gives therefore rise to qualitatively similar host-assisted protonation effects, except that calixarenes lead frequently to fluorescence quenching rather than enhancement upon complexation. The system operates at pH 2.0 in water with the fluorescent guest DBO (K ca. 4500 M^{-1}), which has a pK_a value of ca. 0.5 in its free form and ca. 2.3 when complexed by *p*-sulfonatocalixarene;[77] under these conditions, the free form is mostly (> 95%) unprotonated, while the complexed form is significantly (> 50%) protonated. Addition of organic[60] or inorganic[59] cations releases the guest and thereby converts the protonated (nonfluorescent) form into the unprotonated (fluorescent) form. A marked increase in fluorescence intensity results, which has been previously noted,[59,60] however, without emphasizing the conceptual novelty of the sensor principle in acidic solution.

For environmental and biological applications, the pK_a values should preferably lie close to the physiological range, which encourages the use of different dyes, for example neutral red (pK_a = 6.8 when uncomplexed and pK_a' ≈ 8.8 when complexed)[53] or Dapoxyl (pK_a = 4.1 when uncomplexed and pK_a' = 6.5 when complexed, this work) in combination with CB7 (Scheme 9b). In the case of Dapoxyl at pH 5.5 in water, for example, the LE fluorescence appears exclusively in the complex, where the dye becomes protonated due to its 2.4 units higher pK_a value (see Figures 2a and 3). The addition of a competitive binder (like DBO, Scheme 8) leads then to a decrease in

fluorescence intensity, most pronounced for the LE emission. The protolytic fluorophore displacement principle can consequently be utilized for the design of conceptually novel, highly sensitive, and fully water compatible sensor applications based on CB7 over a large range of pH. These complement nicely the recently described sensor applications based on the formation of ternary complexes cooperatively held together by electron donor-acceptor interactions between two guests inside the larger cavity of CB8.[33,36,44]

Scheme 9. Protolytic fluorophore displacement principle (a) with fluorescence enhancement upon analyte binding ("on switching") using a sensor system based on *p*-sulfonatocalix[4]arene as host and DBO as guest in water at pH 2.0 and (b) with fluorescence decrease upon analyte binding ("off switching") using a sensor system based on CB7 as host and Dapoxyl as guest in water at pH 5.5.



Summary

The encapsulation of dyes by cucurbiturils is an emerging field with numerous applications. In comparison to cyclodextrins and calixarenes, cucurbiturils appear to be "better" water-soluble host molecules due to their desirable photophysical and photochemical effects on dye properties. This applies in particular to fluorescent dyes and the intermediary sized cucurbit[7]uril, for which the following benefits have been presently corroborated: solubilization, deaggregation, suppression of surface adsorption, fluorescence enhancement, increase in brightness, prolongation of fluorescence lifetimes, and photostabilization. The positive effects can be related to the low polarity of the cavity, which resembles that of alcohols, to the exceptionally low polarizability of the cavity, which falls in between that of perfluorohexane and the gas phase, to the spatial confinement and protection from the solvent, and to the low chemical and photochemical reactivity of these macrocyclic hosts. As a peculiarity, cucurbiturils can increase the pK_a values of included dyes on account of their cation receptor properties (host-assisted protonation). This can be employed to rationally alter photophysical properties and to design novel protolytic displacement assays, in which the fluorescence regeneration upon analyte binding is greatly exaggerated due to the shift in protonation equilibria of the fluorescent dye in its complexed and uncomplexed form.

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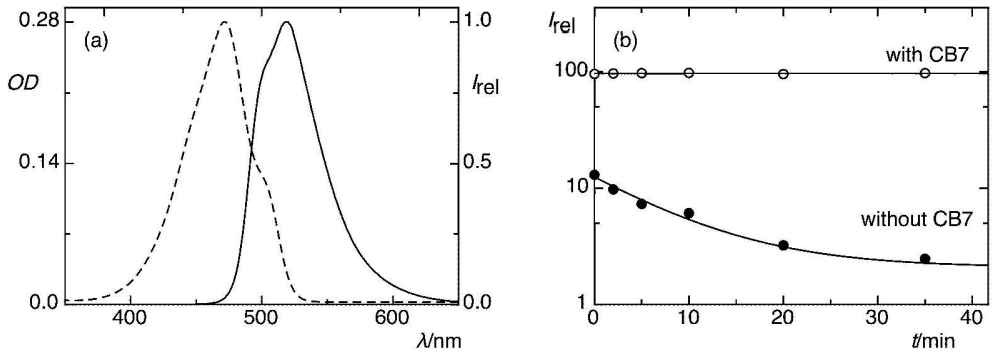

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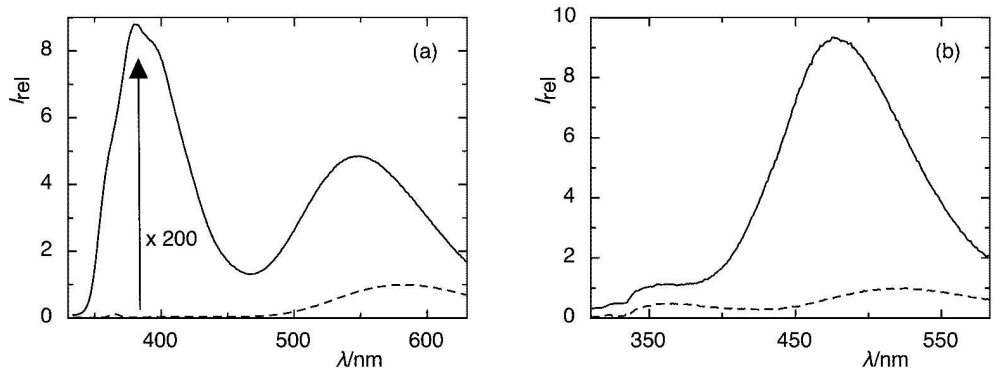

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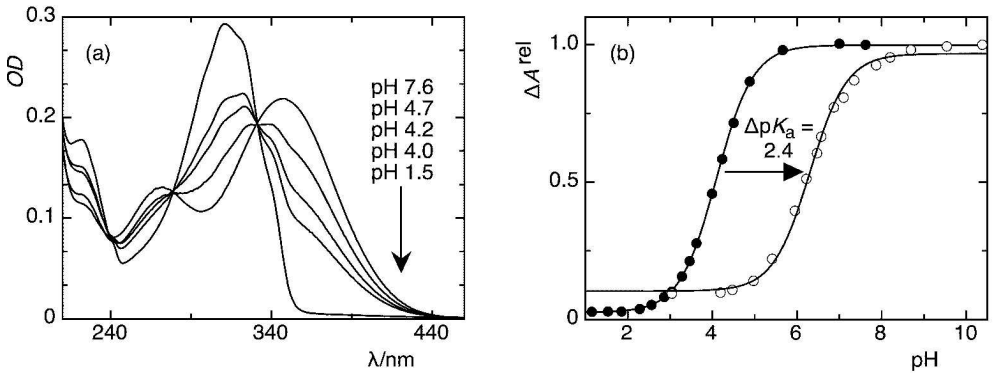

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