Host−Guest Complexation of Neutral Red with Macrocyclic Host Molecules: Contrasting pK_a Shifts and Binding Affinities for Cucurbit[7]uril and β-Cyclodextrin

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The photophysical properties of the phenazine-based dye neutral red were investigated in aqueous solution in the presence of the macrocyclic host molecule cucurbit[7]uril (CB7) using ground-state absorption as well as steady-state and time-resolved fluorescence measurements. The results are contrasted to those previously obtained for β-cyclodextrin (β-CD; Singh et al. *J. Phys. Chem. A* **2004**, *108*, 1465). Both the neutral (NR) and cationic (NRH^+) forms of the dye formed inclusion complexes with CB7, with the larger binding constant for the latter ($K = 6.5 \times 10^3\ M^{-1}$ versus $6.0 \times 10^5\ M^{-1}$). This result differed from that for β-CD, where only the neutral form of the dye was reported to undergo sizable inclusion complex formation. From the difference in binding constants and the pK_a value of protonated neutral red in the absence of CB7 (6.8), an increased pK_a value of the dye when complexed by CB7 was projected (~8.8). This shift differed again from the behavior of the dye with β-CD, where a decreased pK_a value (ca. 6.1) was reported. The photophysical properties of both NR and NRH^+ forms showed significant changes in the presence of CB7. Fluorescence anisotropy studies indicated that the inclusion complexes of both forms of the dye rotate as a whole, giving rotational relaxation times much larger than that expected for the free dye in aqueous solution. The thermodynamic parameters for the NRH^+·CB7 complex were investigated in temperature-dependent binding studies, suggesting an entropic driving force for complexation related to desolvation of the cation and the removal of high-energy water molecules from the CB7 cavity.

1. Introduction

Cucurbiturils (CBs) are macrocyclic container molecules composed of glycoluril monomers joined by pairs of methylene bridges.[1−5] Depending on the number of glycoluril units, homologues of different sizes are known, prominently CB5, CB6, CB7, and CB8. All CBs have a highly symmetrical, pumpkin-shaped structure with two identical portals and an interior hydrophobic cavity, akin to cyclodextrins and calixarenes in their cone conformation.[3,6] In recent years, CBs have been established as versatile and interesting host molecules, which form stable inclusion complexes with small guest molecules such as organic dyes,[7] metal cations,[8,9] and protonated alkyl and arylamines.[3,5,10−12] For example, cucurbit[7]uril (CB7, Chart 1) may have potential applications for drug delivery as it forms a stable 1:1 complex with the anticancer drug oxaliplatin.[3] Although the guest binding properties of cucurbituril in solution are being studied with increasing intensity, using NMR spectroscopy,[2,10,13−15] calorimetry,[16−18] and UV−vis absorption spectroscopy,[7] relatively little is known about the effect of CBs on the photophysical and photochemical behavior of fluorophores and in particular common fluorescent dyes.[4,19−26] We have now extended our study on the complexation behavior of fluorescent dyes with CBs since we expected fundamentally different photophysical and chemical behavior from two perspectives: First, dye molecules immersed in the cavity of CBs experience an exceptionally low polarizability (close to the gas phase),[21,22] which is expected to decrease the radiative decay rate constants, and second, CBs act as cation receptors and should have stronger interactions with cationic fluorescent dyes (the by far most abundant class) than cyclodextrins, in particular.

Herein, we report on the complexation behavior of CB7 with the fluorescent dye neutral red (3-amino-7-(dimethylamino)-2-methyl phenazine), which has been extensively used as a fluorescent probe and stain for biological systems.[27−30] In water, neutral red exists in two prototropic forms, one being neutral (NR) and the other one being protonated (NRH^+, nitrogen atom at 5-position of the phenazine ring is being protonated, cf. Chart 2);[31] the latter form is red and dominates in most biological applications, which accounts for the trivial name of this dye. Importantly, the pK_a value of neutral red lies in the physiologically most relevant region (6.8).[28] This has enabled numerous biological applications as a pH indicator,[30] because the dye shows characteristic color and fluorescence changes with pH, and has led to related applications in investigations of microheterogeneous and confined media such as micelles,[32] microemulsions,[33] and cyclodextrins.[34] In the case of β-cyclodextrin (β-CD, Chart 1), for example, only the neutral form of the dye undergoes complexation, while the protonated form prefers exposure to the aqueous bulk. In the present study, we have examined the interaction of CB7 with neutral red and the effect of complexation on the photophysical properties of the dye, to allow a comparison with the case documented for β-CD.[34]

2. Materials and Methods

Neutral red hydrochloride (NRH^+Cl^-) was obtained from Fluka, Switzerland, and further purified as reported.[35] CB7 was

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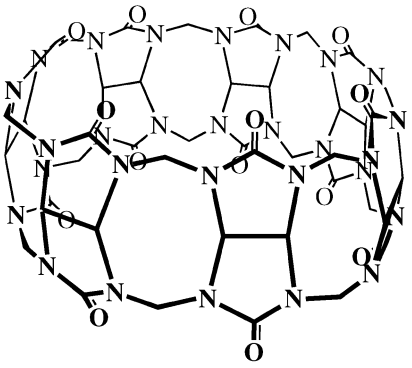
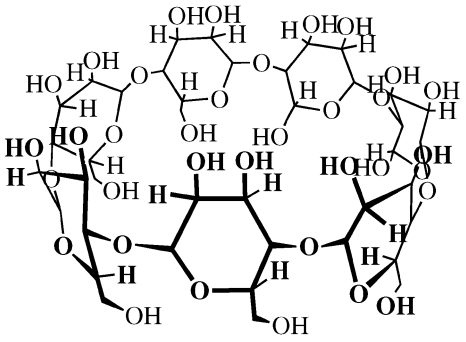
CHART 1

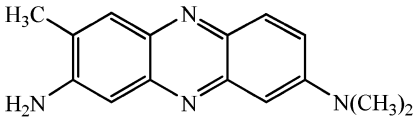


CB7

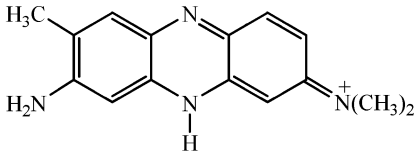


β-CD

CHART 2



NR



NRH^+

synthesized according to the reported modified procedure.[20] Nanopure water (Barnstead System, 0.1 μS cm^{-1} resistivity) was used throughout. The pH of the solution was adjusted by adding dilute perchloric acid or sodium hydroxide solution, and an ORION ionalyzer/901 was used for pH measurement. In the calculation for the CB7 concentration, a correction for water content (15 wt %) was made.

Absorption spectra were recorded with a Jasco V530 UV/VIS spectrophotometer (Tokyo, Japan). Steady-state fluorescence spectra were recorded using a Hitachi F-4010 spectrofluorometer (Tokyo, Japan). The samples were excited at the isosbestic point observed in the absorption spectra. The temperature of the solutions was controlled with a microprocessor-based Eurotherm (U.K.) temperature controller system, using a coldfinger arrangement. The time-resolved fluorescence measurements were carried out using a time-correlated single photon counting (TCSPC) setup from IBH (U.K.) described elsewhere.[36] In the present work, a 490 nm LED (~1.3 ns, 1 MHz repetition rate) was used for excitation. A reconvolution procedure was used to analyze the observed decays,[37] which could be satisfactorily fitted by mono- or biexponential decay functions.

For anisotropy measurements, samples were excited with a vertically polarized excitation beam and the vertically and horizontally polarized fluorescence decays were collected with a large spectral bandwidth of ~32 nm. With these polarized fluorescence decays, the anisotropy decay function, $r(t)$, was constructed as follows:[37]

$$r(t) = \frac{I_V(t) - GI_H(t)}{I_V(t) + 2GI_H(t)} \quad (1)$$

$I_V(t)$ and $I_H(t)$ are the vertically and horizontally polarized decays, respectively, and G is the correction factor for the polarization bias of the detection setup. The G factor was determined independently by using a horizontally polarized excitation beam and measuring the two perpendicularly polarized fluorescence decays;[37] measurements were repeated three times.

3. Results and Discussion

Complexation of fluorescent dyes by macrocyclic hosts is an area of considerable interest in supramolecular chemistry.[4] Macrocyclic hosts can improve the photophysical characteristics of dyes, most importantly enhance their fluorescence quantum yield or photostability and they can display a deaggregating effect and thereby improve water solubility.[26] The complexation of neutral red with β-CD has been previously examined,[34] and it is now interesting to compare the complexation behavior of CB7 as an alternative host. CB7 has an internal cavity with a diameter of ca. 7.3 Å and a portal diameter of ca. 5.4 Å,[3] and it is comparable in diameter to the β-CD cavity (6−7 Å).[34] In addition, CB7 has a moderate solubility in water (~20 mM),[3,21] which is comparable to that of β-CD (~16 mM).[3]

3.1. Absorption Spectral Characteristics in the Presence of Cucurbit[7]uril. The ground-state pK_a value of neutral red in water is 6.8.[28,31,35] In acidic aqueous solution the protonated form of the dye prevails (NRH^+), which has its absorption maximum at 535 nm; in alkaline media, the neutral form dominates with an absorption at 450 nm. Upon addition of CB7 (150 μM) to an aqueous solution of neutral red at pH 2 (NRH^+ form), the absorbance decreased slightly and the absorption peak showed a hypsochromic shift, from 535 to 529 nm, with an apparent isosbestic point at 520 nm (cf. Figure 1, inset). Interestingly, these spectral changes occurred already at con-

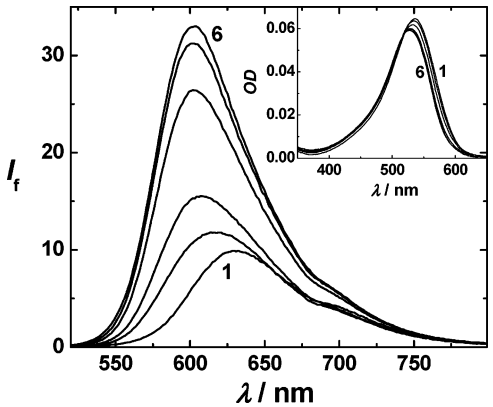


Figure 1. Steady-state fluorescence and ground-state absorption (inset) spectra of the NRH^+ form of neutral red (2.07×10^{-6} M) in aqueous solution at pH 2 at different concentrations of CB7. [CB7]/μM: (1) 0.0, (2) 0.8, (3) 1.5, (4) 3.9, (5) 14.0, and (6) 44.0.

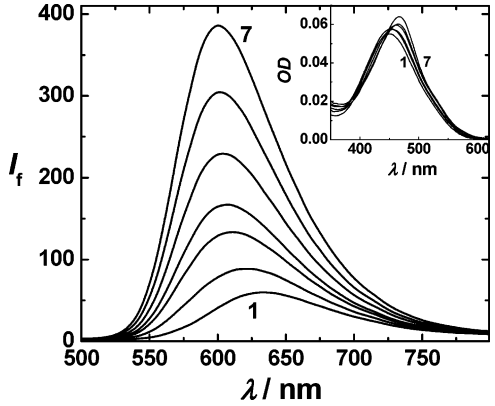


Figure 2. Steady-state fluorescence spectra and ground-state absorption spectra (inset) of the NR form of neutral red (3.8×10^{-6} M) in aqueous solution at pH ~11 at different concentrations of CB7. [CB7]/μM: (1) 0, (2) 14, (3) 35, (4) 50, (5) 75, (6) 112, and (7) 150.

centrations of a few μM CB7 and became effectively saturated at 20 μM, suggesting a very strong binding of the cationic NRH^+ form of the dye. In contrast, upon addition of CB7 to an aqueous solution of neutral red at pH 11 (NR form), the absorption peak showed a gradual increase in absorbance with increasing CB7 content and a bathochromic shift from 450 to 467 nm, with an isosbestic point at ~450 nm (cf. Figure 2, inset). These changes, however, took place with substantially higher CB7 concentration (~150 μM), which implies a weaker binding of the neutral NR form.[38]

3.2. Steady-State Fluorescence Characteristics in the Presence of Cucurbit[7]uril. Because the changes in the absorbance for the NR and NRH^+ forms of the dye upon addition of CB7 were relatively small, fluorescence titrations were preferred for the quantification of the binding constants. In fact, the steady-state fluorescence characteristics of both NR and NRH^+ forms of the dye showed dramatic changes upon addition of CB7, namely, a blue shift by 20−25 nm and a steep increase in fluorescence intensity (Figures 1 and 2). In the presence of 150 μM CB7, for example, the increase in fluorescence quantum yield (ϕ_f) for the NRH^+ and NR forms was 5 and 6, respectively. Such increased fluorescence intensities are frequently observed during the inclusion complexation by cyclodextrins and commonly attributed to the altered (less polar) microenvironment inside the macrocyclic host cavities and/or to a spatial confinement effect, which limits alternative deactivation pathways.[4] For neutral red, vibrational and rotational motions of the $N(CH_3)_2$ group of the dye are expected to be strongly coupled to the nonradiative de-excitation channel of the excited molecule, and a restriction of the motions of this group by confinement may be particularly effective in preventing radiationless deactivation. In the case of CB7, the exceptional chemical inertness of the host molecule and the low polarizability inside the cavity need also to be taken into account and may contribute to the fluorescence enhancement.[26] Regardless of the underlying reasons, the dramatic fluorescence enhancement provides strong evidence that inclusion complexes are indeed formed, a conclusion which is also supported by the fluorescence lifetimes and anisotropy measurements (see below).

The NR form of the dye shows dual solvatochromism and can emit either from a locally excited (LE) state or from the twisted intramolecular charge transfer (TICT) state, depending on the polarity of the medium; the TICT state of NR participates only in high polarity solvents.[31,34,35] In the present case, the emission for the NR·CB7 complex can be assigned to the LE state of the dye, which is consistent with the expectedly lower polarity and hydrophobicity inside the cucurbituril cavity.[39] For the NRH^+ form of the dye, TICT state formation is not possible such that the fluorescence of the NRH^+ form, also in its CB7 complex, can be assigned to the LE state as well.

The binding constants (K) for the neutral red·CB7 complexes were determined by fluorescence titration according to a 1:1 complexation model for both the NRH^+ and the NR form, assuming that only the protonated form of the dye needs to be considered at pH 2 (eq 2) and only the neutral form needs to be considered at pH 11 (eq 3).

$$NRH^+ + CB7 \overset{K(NRH^+)}{\rightleftharpoons} NRH^+\cdot CB7 \tag{2}$$

$$NR + CB7 \overset{K(NR)}{\rightleftharpoons} NR\cdot CB7 \tag{3}$$

Taking $[Dye]_0$ and $[CB7]_0$ as the total concentrations of dye and CB7, respectively, eq 4 applies for the concentration of free (uncomplexed) dye in equilibrium:

$$[Dye]_{eq} = [K_{eq}[Dye]_0 - K_{eq}[CB7]_0 - 1 + \{(K_{eq}[Dye]_0 + K_{eq}[CB7]_0 + 1)^2 - 4K_{eq}{}^2[Dye]_0[CB7]_0\}^{1/2}]/2K_{eq} \tag{4}$$

Exchange of the dye during its excited-state lifetime (<3 ns, see below), i.e., the conversion of the uncomplexed dye to the complexed one or vice versa, can be excluded since the corresponding guest exchange rate constants are very small for cucurbiturils,[5,10] much smaller than those found for cyclodextrins.[40] The fluorescence intensity can therefore be understood as a composite of the fluorescence intensity contributions from the complexed and uncomplexed forms according to eq 5

$$I_f = I^0_{Dye}\frac{[Dye]_{eq}}{[Dye]_0} + I^\infty_{Dye\cdot CB7}\frac{[Dye\cdot CB7]_{eq}}{[Dye]_0} \tag{5}$$

where I^0_{Dye} is the initial fluorescence intensity in the absence of CB7 and $I^\infty_{Dye\cdot CB7}$ corresponds to the fluorescence intensity if all the dye molecules in the solution were complexed by CB7. The change in fluorescence intensity (ΔI^λ_f) can be obtained by rearrangement (eq 6)[34,41]

$$\Delta I^\lambda_f = \left(1 - \frac{[Dye]_{eq}}{[Dye]_0}\right)(I^\infty_{Dye\cdot CB7} - I^0_{Dye}) \tag{6}$$

In the fluorescence titrations, we employed the integrated fluorescence intensity as experimental measure. The concentration of dye was kept constant at a particular pH (2 for NRH^+ or 11 for NR) and the concentration of CB7 was varied. The binding constants (K) obtained from the nonlinear fittings of the experimental data using eq 6 were $(6.0 \pm 1.0) \times 10^5$ M^{-1} for the NRH^+·CB7 complex and $(6.5 \pm 1.0) \times 10^3$ M^{-1} for the NR·CB7 complex. Typical titration curves are shown latter in section 3.6 in relation to the temperature effect on the binding constants. The point to note here is that the protonated form of neutral red binds more than 2 orders of magnitude tighter to CB7, but the binding of the neutral form with CB7 is also sizable.

To understand the stoichiometry of the dye−CB7 complexes, we also carried out the absorption measurements by varying mole fractions of dye and CB7 in the solution, keeping the sum of the dye and CB7 concentrations constant. The absorbances of the solutions thus obtained were correlated according to Job's plots. A typical plot for a NRH^+·CB7 complex is shown in Figure 3. A similar plot was also obtained for the NR·CB7

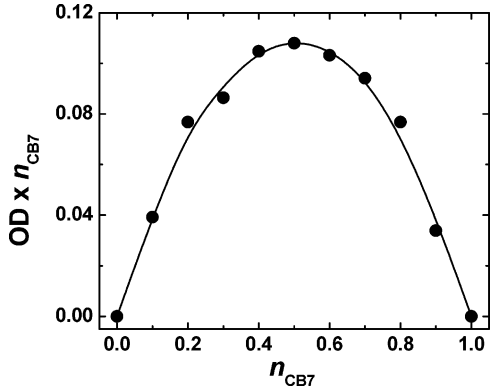
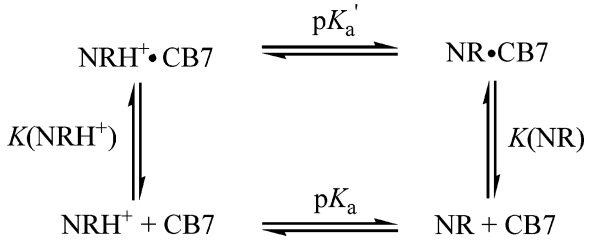


Figure 3. Job's plot for the NRH^+·CB7 complex. Symmetric plot with maximum at 0.5 mole fraction indicates the 1:1 inclusion complex formation in the present system.

SCHEME 1



complex. From these plots it is clearly indicated that the dye−CB7 inclusion complexes in the present systems are formed mainly with 1:1 stoichiometry. In the present context, we also tried to carry out the ^{1}H NMR spectra for both NRH^+·CB7 and NR·CB7 complexes to understand the binding modes of the two prototropic forms of the dye within the CB7 cavity. However, due to solubility limitations, it was not possible to get any conclusive results from the NMR study.

The increased binding of the protonated form can be readily rationalized by recalling that CBs have cation receptor properties, since their carbonyl rims can stabilize positive charges through ion−dipole interactions.[3] A closely related example has been previously documented for cyclohexylmethylamine as guest and CB6 as host;[10,40] while the binding of the amine form of the guest is very small (10 M^{-1}), its conjugate acid, the cyclohexylmethylammonium ion, has a much larger binding constant (170 M^{-1}) as a consequence of additional ion−dipole interactions.

3.3. Effect of CB7 Complexation on the Acidity Constant of the Dye. In the intermediary pH range around the pK_a value of neutral red, the prototropic equilibrium between NRH^+ and NR in the presence of CB7 should follow the four-state model in Scheme 1. Accordingly, complexation of the protonated and the neutral forms, as well as protonation of the uncomplexed and complexed neutral forms, need to be considered, and a thermodynamic cycle (eq 7) applies.[40] Accordingly, the pK_a' value of the dye·CB7 complex can be calculated from the independently determined binding constants for the neutral and protonated form and the known pK_a value of the uncomplexed dye. A pK_a' value of ca. 8.7 results, suggesting an exceptionally large pK_a shift by ∼2 units. This means that neutral red, once complexed by CB7, becomes a ∼100 times stronger base than in its free form, with the ion−dipole interactions offered by the CB7 macrocycle providing the additional driving force for complexation.

$$K_a' = K_a K_{eq}(NR)/K_{eq}(NRH^+) \qquad (7)$$

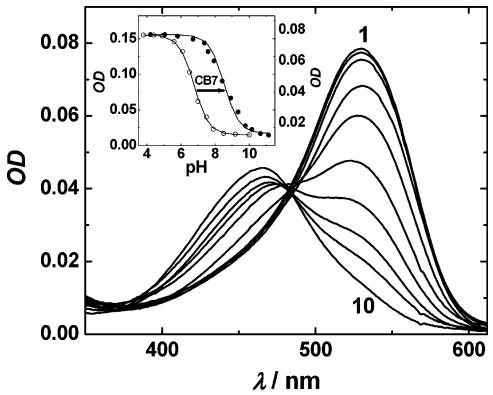


Figure 4. Absorption spectra of neutral red (2.9×10^{-6} M) in water containing 150 μM CB7 at different pH: (1) 3.54, (2) 4.20, (3) 5.76, (4) 7.26, (5) 7.85, (6) 8.33, (7) 8.82, (8) 9.24, (9) 9.66, and (10) 11.0. The inset shows the variation in absorbance with pH at 530 nm in the absence and presence of 150 μM CB7.

To allow an improved determination of the pK_a shift, we have carried out spectrophotometric titrations of neutral red in the absence and presence of 150 μM CB7 in dependence on pH (Figure 4). Unlike cases with simple acid−base equilibria, no well-defined isosbestic point is observed in Figure 4 since a four-state model applies (Scheme 1). The corresponding titrations (changes in the absorbance at 530 nm) revealed the previously documented[31] pK_a value around 6.8 for the free dye, but the pK_a' value of the complexed dye shifted by ca. 2 units to a value of 8.8 ± 0.1, applying a simple titration fit. This is in excellent agreement with the value projected from the relative binding constants (see above).

Shifts of pK_a values upon inclusion in different microenvironments are documented.[10,28,32−34] The pK_a value of neutral red, in particular, has been previously investigated in various media, including micelles, microemulsions, biomolecules, and cyclodextrins.[28,32−34] For example, Walz et al.[28] observed an increase in the pK_a value of the dye upon interaction with native and denatured DNA, suggesting that the NRH^+ form of the dye binds preferentially. The interesting aspect of the shift of the pK_a value of neutral red upon complexation by CB7 is its absolute magnitude (about 2 units) and the fact that it is opposite to that observed for β-CD (see below).[34]

3.4. Effect of CB7 Complexation on the Fluorescence Lifetime of the Dye. The fluorescence lifetimes of NR and NRH^+ forms are known to be highly sensitive to the solvent environment. In aqueous solution, the fluorescence lifetimes for both NR and NRH^+ forms ($\tau_f = 0.7$ ns and 0.3 ns, respectively) are in fact very short, which has been related to strong intramolecular hydrogen bonding and associated radiationless deactivation pathways.[31,35] In the present study, the fluorescence decays of neutral red in aqueous solution at pH 2 and pH 11 were investigated in dependence on CB7 concentration.

The fluorescence decays of both NR and NRH^+ were monoexponential in the absence of CB7. In the presence of CB7, the fluorescence decays of NRH^+ (at pH ∼2) showed biexponential behavior at lower CB7 concentrations, with the shorter lifetime component (∼0.3 ns) corresponding to uncomplexed NRH^+ and the longer lifetime component (∼0.72 ns) to the NRH^+·CB7 complex. At higher CB7 concentrations (>20 μM), the fluorescence decays became monoexponential again, indicating that the complexation of NRH^+ with CB7 was virtually quantitative under these conditions, as expected from the very large binding constant (see above).

As expected from the significantly lower binding constant of NR, only a fraction of the dye is complexed in the presence

of CB7. The fluorescence decays of NR (at pH 11) in the presence of CB7 were therefore biexponential even at the highest accessible CB7 concentration (150 μM). The lifetimes for the shorter component thus obtained (~0.7 ns) for all the decays at different CB7 concentrations were very similar to the lifetime of free NR in water and assigned accordingly. The longer-lived component (~2.8 ns) was attributed to the fluorescence from the NR·CB7 complex; its relative contribution increased gradually with CB7 concentration.

The substantial increase in the fluorescence lifetime of neutral form on complexation with CB7 can be qualitatively rationalized in terms of the polarity dependence of the fluorescence lifetimes of neutral red,[21,22] i.e., the dye is included in the CB7 cavity, where the polarity of the microenvironment is much lower than that in bulk water. However, a detailed analysis of the variations in the fluorescence lifetimes must differentiate between the effects on the radiative and radiationless decay rate constants. Guest molecules included inside cucurbiturils experience a microenvironment with a reduced polarizability, and this generally results in reduced radiative decay rate constants (calculated as ratio of quantum yield and fluorescence lifetime).[42] Interestingly, the expected trend is not observed for neutral red. For NRH^+, the radiative decay constant increases strongly (from 1.0×10^7 s^{-1} in H_2O to 2.1×10^7 s^{-1} in CB7), while for NR the radiative decay rate increases slightly (from 0.6×10^7 s^{-1} to 0.9×10^7 s^{-1}). The deviation from the established relationship is presumably due to the exceptional behavior of neutral red in water, which affects its photophysical properties in a peculiar, unprecedented way.[31,34] The observed decrease of the radiationless decay rates (from 3.3×10^9 s^{-1} in H_2O to 1.4×10^9 s^{-1} in CB7 for NRH^+ and from 1.42×10^9 s^{-1} in H_2O to 0.35×10^9 s^{-1} in CB7 for NR) may be related to a confinement effect and the partial rupture of the hydrogen bonding network with water;[31] both factors are expected to slow radiationless decay.[31]

3.5. Fluorescence Anisotropy Studies. Time-resolved fluorescence anisotropy measurements were carried out in aqueous solutions of the dye in the presence of 150 μM CB7 at pH 2 and pH 11, respectively. The fluorescence anisotropy decays thus obtained for NRH^+ and NR forms are shown in parts A and B of Figure 5, respectively. In both cases the anisotropy decays appeared to be monoexponential in nature. A biexponential decay behavior (for the complexed and uncomplexed forms) is principally expected, but the rotational correlation times (τ_r) for both uncomplexed NRH^+ and NR forms are too short (108 ps in H_2O at 25 °C)[34] to be detected with the employed setup. In the presence of CB7, the anisotropy decays for NRH^+ and NR were sufficiently slow, however, and gave τ_r values of about 360 ± 40 and 470 ± 40 ps, respectively. The initial anisotropy (r_0) was also estimated to be quite high in the presence of CB7 (0.32 for NR and 0.23 for NRH^+). The reduced rotational diffusion times are fully consistent with the formation of tight inclusion complexes, for which the diffusion of the dye is slowed by the encapsulating host.

The rotational correlation time (τ_r) for the complex can be related to its rotational diffusion coefficient and the viscosity of the environment according to the Stokes−Einstein relationship[43]

$$\tau_r = 1/(6D_r) \quad \text{where} \quad D_r = \frac{RT}{6V\eta} \tag{8}$$

Here, V is the hydrodynamic molecular volume of the complex, η is the viscocity of the medium, and T is the absolute temperature. For the 1:1 inclusion complex between neutral red and CB7, a rough estimation of the hydrodynamic molecular volume can be obtained by considering the complex as an effective sphere and the hydrodynamic diameter similar to the outer diameter of the CB7 molecule (16 Å).[3] Thus, the volume V can be roughly estimated as 1292 cm^3mol^{-1}. The η value can be taken to be that of water at 25 °C, since the outer walls of the host experience friction with this medium. Thus, by using eq 8, an estimated rotational correlation time of 465 ps results, which is in excellent agreement with the experimental result for the NR·CB7 complex. Noteworthy, the rotational correlation time for the NRH^+·CB7 complex is distinctly lower than that of the NR·CB7 complex; this is possibly related to the more tight binding of the protonated NRH^+ form with CB7. Regardless of the differences in the τ_r values for NRH^+·CB7 and NR·CB complexes, the fluorescence anisotropy behavior of the dye in the presence of CB7 corroborates the formation of inclusion complexes, which appear to rotate as a whole in aqueous solution; i.e., the independent rotation of the dye inside the CB7 cavity is highly restricted.

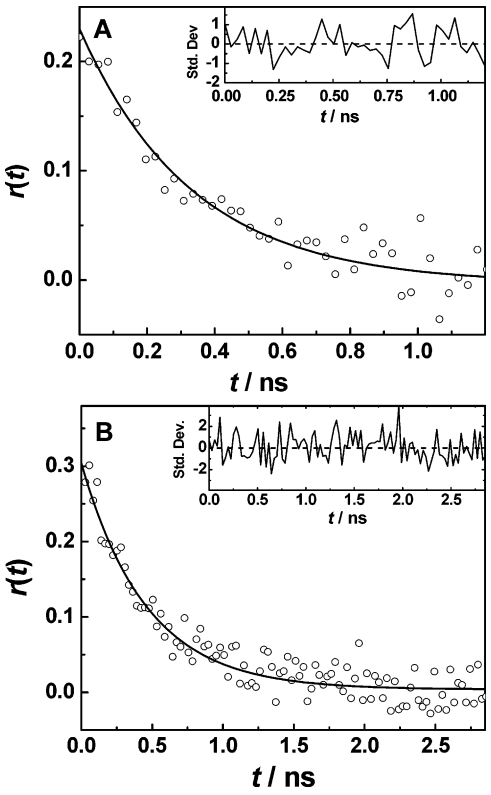


Figure 5. Time-resolved anisotropy decay curves for (A) the NRH^+·CB7 complex at pH 2 and (B) the NR·CB7 complex at pH 11, both in water at 25 °C.

3.6. Thermodynamic Parameters of the CB7 Inclusion Complexes. The binding constants of the CB7 inclusion complexes for the two prototropic forms of neutral red dye have been determined using spectrofluorimetric titrations at different temperatures, ranging from 30 to 60 °C with 10 °C intervals, to establish the nature of binding of the complex. Figure 6 shows the changes in the integrated fluorescence intensity as a function of CB7 concentration for the NRH^+ form of the dye at different temperatures. From these plots, K values were obtained by fitting the data according to eqs 4 and 6. In the case of the NRH^+·CB7 complex, the binding constant decreased gradually with temperature. The thermodynamic parameters for complex formation were estimated using the van't Hoff equation ($\ln K = -\Delta H/RT + \Delta S/R$).[6] From the resulting linear correlation, see inset of Figure 6, the complexation enthalpy ($\Delta H = -1.8 \pm$

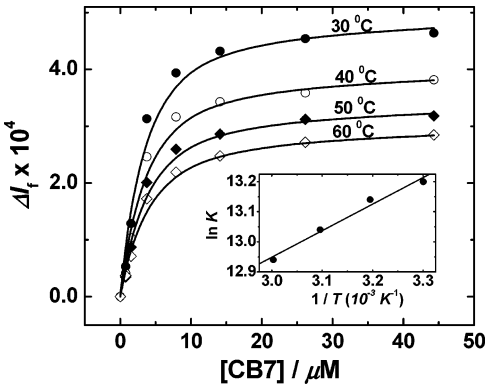


Figure 6. Changes in the integrated fluorescence intensity (ΔI_f) versus [CB7] for the NRH^+·CB7 complex at different temperatures. The inset shows the plot of ln K vs $1/T$.

0.2 kcal mol^{-1}) and the entropy term ($T\Delta S = 6 \pm 1$ kcal mol^{-1}) were obtained, suggesting that the complexation process has a small enthalpic, but dominant, entropic driving force. This is not unusual for cation receptors, e.g., similar observations were made in the complexation of organic cations with cucurbit[6]uril[16−18] and *p*-sulfonatocalixarenes.[44−47] The entropy gain is associated with the removal of high-energy water molecules from the host cavity and the desolvation of the cation,[6,46,47] which is frequently found to offset the entropically unfavorable effect related to confinement effects.[6,44,48,49] The fluorescence intensity of the uncomplexed and complexed unprotonated dye (NR) was also investigated in dependence on temperature. As a peculiarity, the fluorescence intensity of the NR form is known to increase with temperature when uncomplexed;[31] this has been tentatively attributed to the rupture of the intermolecular hydrogen bonding framework between the dye and water molecules at elevated temperatures. In the presence of CB7, however, the fluorescence intensity decreased with temperature. This is possibly due to the partial exclusion of water from the inclusion complex, which reduces hydrogen bonding and therefore the associated temperature effects related to cleavage of these hydrogen bonds. Unfortunately, the binding constants extracted from the temperature-dependent fluorescence titrations showed a large scatter, owing among others to the weaker binding and the limited host concentration range (<150 μM), which prevented reliable thermodynamic data from being obtained for the NR form.

3.7. Comparison of the Photophysical Properties upon Complexation with CB7 and β-CD. Although both macrocyclic hosts, CB7 and β-CD, possess a hydrophobic cavity,[6,50] the topology of the upper and lower rim of both macrocycles is fundamentally different (see Chart 1). CB7 possesses two equivalent ureido carbonyl rims, which confer cation binding properties, while β-CD has an upper, wider rim laced with secondary hydroxyl groups and a lower, tighter one with primary hydroxyl groups; these promote binding mainly by hydrogen bonding and less through ion−dipole interactions. In general, hydrophobic interactions are more important for cyclodextrins than for cucurbiturils.[2,3] These differences account directly for the differential binding of both host molecules with neutral red. Complexation by CB7 is greatly favored for the protonated NRH^+ form due to the additional ion−dipole interactions, thereby accounting for the large difference in binding constants ($(6.5 \pm 1.0) \times 10^3$ M^{-1} for NR vs $(6.0 \pm 1.0) \times 10^5$ M^{-1} for NHR^+) as well as the pronounced pK_a shift toward *higher* values (from 6.8 to 8.8), which reflects the increased stability of the protonated dye in the complex. Complexation by β-CD, on the other hand, is stronger for the neutral form ($K = 4.11 \times 10^2$ M^{-1}),[34] because the protonated form is more hydrophilic and has a better water solubility and a lower tendency to be immersed in a hydrophobic environment. In fact, binding of the NRH^+ form with β-CD is apparently too weak to allow detection.[34] The reduced binding of the protonated form accounts similarly for the sizable pK_a shift toward *lower* values (from 6.8 to 6.1), reflecting a lower stability of the protonated form in its β-CD complex.

Generally speaking, CB7 forms at least 10 times stronger inclusion complexes with neutral red than β-CD. For potential practical applications, it is important to note that a virtually quantitative complexation (>95%) of the protonated dye can be readily achieved even with small amounts of CB7 around neutral pH. This is due to the high binding constants and the preferential inclusion of the protonated form. From a photophysical point of view, the addition of both host molecules results in a fluorescence enhancement of neutral red, but for CB7 this effect is more universal, and not limited to the neutral form of the dye. Pragmatically speaking, CB7 is a much "better" host for neutral red, especially for its protonated form.

4. Conclusions

The results obtained from the ground-state absorption and steady-state as well as time-resolved fluorescence and anisotropy studies indicate that both the cationic and neutral form of neutral red form inclusion complexes with CB7, particularly strong for the protonated NRH^+·CB7 complex ($K > 10^5$ M^{-1}). The ground-state pK_a value of neutral red in the presence of CB7 has been estimated using a four-state kinetic model, revealing an exceptionally large pK_a shift by ∼2 units. The increase in pK_a value of the protonated dye reflects the strong affinity of CB7 to the protonated form, which is stabilized in the complex by additional ion−dipole interactions with the carbonyl ureido rim of the macrocyclic host. The fluorescence lifetimes and quantum yields of both prototropic forms of neutral red increase substantially upon complexation with CB7. As revealed by the observation of monoexponential fluorescence anisotropy decays, the complexes appear to rotate as a whole in solution, which is in line with the presumed formation of tightly bound inclusion complexes. The thermodynamical parameters for the NRH^+·CB7 complex formation were also determined, suggesting a dominant entropic driving force. The comparison of the complexation behavior of neutral red toward CB7 with that toward β-CD reveals that although both hosts have a hydrophobic cavity, additional recognition elements such as cation receptor sites at the portals of the cavity are quintessential to promote a strong and selective binding of fluorescent dyes. This emerges as an important design criterion for the optimization of fluorescent dye properties through supramolecular association.

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