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Photophysical Properties and Rotational Relaxation Dynamics of Neutral Red Bound to β-Cyclodextrin

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Photophysical properties and the rotational relaxation dynamics of a phenazine based dye, neutral red, have been investigated in aqueous solutions in the presence of β-cyclodextrin (β-CD) using ground-state absorption and steady-state and time-resolved fluorescence measurements. It has been observed that while the neutral form (NR) of the dye forms an inclusion complex with β-CD, its protonated form (NRH$^+$) does not. In the presence of β-CD (10 mM), the pK_a value of the dye is estimated to be about 6.06 ± 0.05, much lower than the pK_a value of 6.81 ± 0.05 measured in aqueous solution. Both photophysical properties and the rotational relaxation dynamics of NR undergo substantial changes in the presence of β-CD. Steady-state and time-resolved fluorescence studies suggest simultaneous formation of 1:1 and 1:2 complexes of NR/β-CD in aqueous solutions. Time-resolved fluorescence anisotropy measurements confirm that only NR forms an inclusion complex with β-CD and the NR/β-CD complex rotates as a whole with a relaxation time much longer than that observed for free NR in aqueous solution.

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides composed of α-1,4-glycoside linkages.[1–4] The most common members of this family are the α-, β-, and γ-CDs, which are made up of six, seven, and eight d-glucose residues. In general, CDs have good solubility in water. By shape, the CDs are torus, having an inner hydrophobic cavity and the outer hydrophilic surface consisting of edge hydroxyl groups. The most remarkable property of the CDs is their ability to form inclusion complexes with a variety of organic molecules.[1–7] Many hydrophobic substrates of appropriate size and shape are known to form supramolecular host–guest complexes with specific CDs and can thus be solubilized in aqueous solutions. This unique property of the CDs has led to widespread utilization of these materials in pharmaceuticals, food technology, chemical industries, and many other applied areas.[8]

In the last few decades a number of spectroscopic techniques have been used to understand the nature of the interaction of CDs with many guest molecules. Fluorescence spectroscopy has been found to be an excellent technique for characterizing the binding of organic molecules in the CD cavities. Other techniques such as NMR spectroscopy, circular dichroism, and electrochemical methods have also been used extensively to understand the nature of the host–guest interactions in such supramolecular complexes.[2–5]

The presence of the hydrophobic environment inside the CD cavity and the restricted movement of the guest molecule inside these cavities often markedly influence the photophysical and photochemical properties of the latter.[1–7] In recent years many investigators have employed such properties of the CD inclusion complexes to understand the mechanistic details of many processes such as isomerization reactions, twisted intramolecular charge transfer (TICT) process, etc. In relation to the effect of CD complexation on the TICT process in the guest molecules, different groups have proposed various arguments that are controversial and contradictory.[9–15] Recently we have investigated the photophysical and photochemical properties of neutral red (NR: 3-amino-7-(dimethylamino)-2-methylphenazine) in both aqueous and organic solvents.[16,17] These studies have shown interesting dual solvatochromism of the neutral form of the dye, NR, due to the existence of the closely spaced locally excited (LE) and TICT states of the excited NR molecule. In low-polarity solvents, the fluorescence of NR arises mainly from the LE state, whereas in high polarity solvents the observed fluorescence is mainly due to the TICT state. Interestingly it is seen that unlike NR, the cationic/protonated form of the dye, NRH$^+$, does not undergo LE to TICT conversion even in a strongly polar solvent. In the present study, the effect of β-cyclodextrin (β-CD) complexation on the photophysical properties and the rotational relaxation dynamics of NR has been investigated in aqueous solutions using ground-state absorption and steady-state and time-resolved fluorescence measurements. The aim of this study is to know how the neutral and cationic forms of neutral red interact with β-CD and what the effect of the microenvironment and the restricted motion inside the β-CD cavity is on the photophysical properties and the rotational relaxation dynamics of the probe molecule.

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2. Materials and Methods

Materials. Neutral red in its hydrogen chloride form (NR·HCl) was purchased from Fluka, Switzerland. The neutral form of the dye, NR was obtained on neutralizing a concentrated solution of NR·HCl with a strong base. The NR thus obtained was purified by preparative TLC as described elsewhere.[16,17] β-CD was purchased from Fluka and used as received. Nanopure water, having a conductivity of 0.1 μS cm^{-1}, obtained by passing distilled water through a Barnstead Nanopure water system to remove all the ionic and organic impurities was used for all the solution preparations. Most of the studies for the neutral form (NR) of the dye were performed at pH $\sim$9 and that for the cationic/protonated form (NRH$^+$) at pH $\sim$4.

Methods. Ground-state absorption spectra were recorded with a Shimadzu (model 160-A) UV–visible spectrophotometer. Steady-state fluorescence measurements were carried out with a Hitachi (model F-4010) spectrofluorimeter. The pHs of the solutions were adjusted by adding HClO$_4$ and NaOH to the solutions and measured by using an Orion Ionalyzer (model 901) fitted with a combination electrode (Cat. No. 91-2). For all these studies the concentration of NR was kept in the range 5–10 μM.

The time-resolved fluorescence measurements were carried out using a picosecond fluorescence spectrometer, working on the principle of time-correlated single photon counting (TC-SPC).[18] The details of this experimental setup have been described elsewhere.[19,20] In these experiments, the samples were excited by vertically polarized laser light at 444 nm, using frequency-doubled output of a picosecond Ti:saphire laser. The fluorescence emission was collected at magic angle (54.7°) and analyzed by a reconvolution procedure using an iterative nonlinear least-squares fitting method.[18] The instrument response functions required in the fitting procedure were obtained by substituting the sample with a light scatterer. The fluorescence decays $I(t)$ were analyzed using an exponential function as

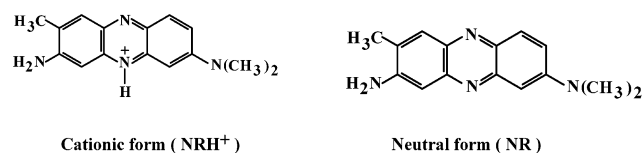$$I(t) = \sum_i B_i \exp\left(\frac{-t}{\tau_i}\right) \tag{1}$$

where B_i and τ_i are the preexponential factor and the lifetime for the ith component of the fluorescence decay. The goodness of the fit and consequently the single, double, or triple exponential nature of the decays were judged by statistical parameters such as reduced χ-square (χ^2) values and the distribution of the weighted residuals (r_w) among the data channels. For all good fits, the χ^2 values were close to unity and the r_w were randomly distributed among the data channels.[18] Unless otherwise stated, the absorption and fluorescence measurements were carried out at 25 °C. The temperature of the solutions was controlled with a microprocessor based Eurotherm (U.K.) temperature controller system.

The anisotropic fluorescence decays $I_\parallel(t)$ and $I_\perp(t)$ were obtained by keeping the emission polarizer parallel for the former and perpendicular for the latter with respect to the vertically polarized excitation beam. Simultaneous fit for both $I_\parallel(t)$ and $I_\perp(t)$ curves was obtained by using a nonlinear least-squares routine that uses a Marquardt algorithm as described by Bevington.[21] The functional forms of $I_\parallel(t)$ and $I_\perp(t)$ used in the fitting were

$$I_\parallel(t) = \left(\frac{1}{3}\right) I(t) \{1 + 2r(t)\} \tag{2}$$

$$I_\perp(t) = \left(\frac{1}{3}\right) I(t) \{1 - r(t)\} \tag{3}$$

CHART 1

Cationic form (NRH$^+$)   Neutral form (NR)

where $I(t)$ represents the isotropic fluorescence decay (cf. eq 1) and $r(t)$ represents the time-dependent anisotropy of the probe molecule.[22] The time-resolved anisotropy $r(t)$ can be considered as a multiexponential function

$$r(t) = \sum_j r_{0j} \exp\left(\frac{-t}{\tau_{rj}}\right) \tag{4}$$

where r_{0j} is the zero-time or limiting anisotropy and τ_{rj} are the individual rotational relaxation times. The same values of the parameters namely, r_0, τ_r, and $I(t)$ were used for both $I_\parallel(t)$ and $I_\perp(t)$ decays during the analysis. A monoexponetial function was found to be sufficient to describe the anisotropy decay in the absence and presence of high β-CD concentration. However, in the lower β-CD concentration range the anisotropy decay fits better to a biexponential function.

3. Results and Discussion

3.1. Ground-State Absorption Measurements. In aqueous solution, neutral red exists in two different prototropic forms, namely the cationic/protonated (NRH$^+$) and the neutral (NR), depending on the pH of the solution.[17,23] The structures of the two forms of the dye are given below (Chart 1).

The absorption spectra of the two forms of the dye are drastically different. The absorption peak of NRH$^+$ appears at $\sim$535 nm and that of the NR appears at $\sim$452 nm in aqueous solution. From the pH-dependent absorption studies, the ground-state pK_a value of NRH$^+$ has been estimated to be 6.81 $\pm$0.05.[17,23]

$$[\text{NRH}^+] \overset{\text{p}K_a}{\rightleftharpoons} \text{NR} + \text{H}^+ \tag{5}$$
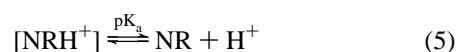
In the presence of β-CD at pH $\sim$9, the absorption spectra of NR is seen to undergo a marginal blue shift along with a gradual reduction in the peak absorbance. As shown in Figure 1A, the absorbance at the longest wavelength absorption maxima ($\sim$452 nm) of NR with the increasing concentration of β-CD at pH $\sim$9 initially decreases and then attens a limiting value at a β-CD concentration of $\geq$5 mM, indicating almost complete complexation of NR with β-CD. The absorption maxima (λ_{abs}^{max}) shows a blue shift of $\sim$8 nm in the presence of 10 mM concentration of β-CD. It has also been observed that while the absorbance of NR in the 450-nm region reduces with increasing concentration of β-CD, a small increase in absorbance is seen in the 350-nm region. Since the changes in the absorption spectra of NR with increasing concentration of β-CD are relatively small, it has not been used to evaluate the association constant for the complexation of NR with β-CD. At pH $\sim$4, where neutral red exists in its cationic form (NRH$^+$), the absorption spectra does not show any change even in the presence of the highest concentration of β-CD used (10 mM), indicating that NRH$^+$ does not form an inclusion complex with β-CD. This observation is further supported by the results obtained in the SS and TR fluorescence studies and the fluorescence anisotropy measurements discussed later.

3.2. Steady-State Fluorescence Studies. The fluorescence characteristics of NR in aqueous solutions at pH $\sim$9 are seen
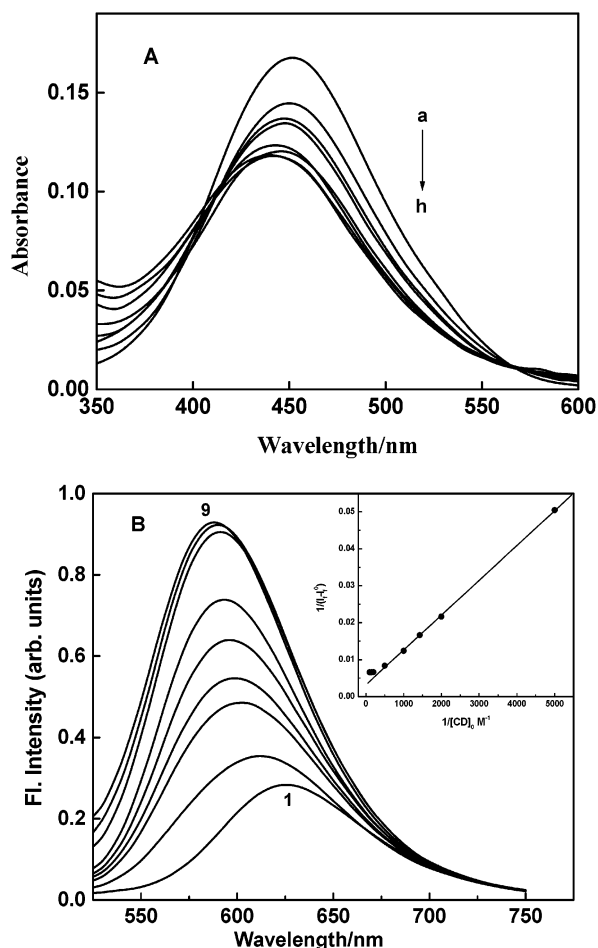
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Figure 1. (A) Absorption spectra of NR (1.17×10^{-5} M) in aqueous solutions containing varying concentrations of β-CD at pH $\sim$9. Concentrations of β-CD: (a) 0, (b) 0.2, (c) 0.7, (d) 1.0, (e) 2.0, (f) 5.0, (g) 7.0, and (h) 10 mM. (B) Fluorescence spectra of aqueous NR (1.17×10^{-5} M) at pH $\sim$9 containing (1) 0, (2) 0.2, (3) 0.5, (4) 0.7, (5) 1.0, (6) 2.0, (7) 5.0, (8) 7.0, and (9) 10 mM β-CD. Inset: Double reciprocal plot of NR/β-CD complex, $1/(I_f - I_f^0)$ vs $1/[\text{CD}]_0$.

to undergo drastic changes in the presence of β-CD. The fluorescence spectra undergo a gradual blue shift on increasing the β-CD concentration. In the presence of $\sim$10 mM β-CD, the fluorescence peak of NR ($\lambda_{\text{em}}^{\text{max}} \sim$588 nm) is blue-shifted by $\sim$37 nm as compared to that observed in the absence of β-CD ($\lambda_{\text{em}}^{\text{max}} \sim$ 625 nm). The fluorescence intensity of NR is also seen to increase substantially in the presence of β-CD. Figure 1B shows the effect of increasing β-CD concentration on the fluorescence spectra of NR, whereas Figure 2 shows the plot of observed changes in the fluorescence intensity with increasing concentration of β-CD. It is seen from this plot that the fluorescence intensity of NR initially increases with increasing β-CD concentration and then saturates to a limiting value at a β-CD concentration of $\geq$5 mM, indicating the incorporation of almost all the NR molecules in the β-CD cavity. These results correlate well with those observed in the ground-state absorption studies.

The association constant for the NR/β-CD complex formation has been determined by analyzing the changes in the intensity of fluorescence maxima with the β-CD concentration. In the case of a 1:1 complex formed between NR and β-CD the equilibrium can be written as

$$\text{NR} + \text{CD} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{NR:CD} \tag{6}$$
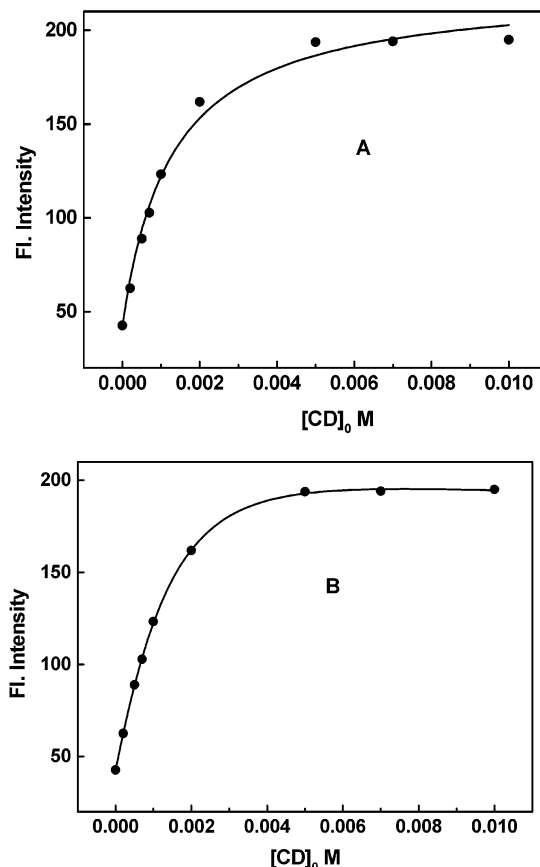

Figure 2. Plot of fluorescence intensity I_f versus $[\beta\text{-CD}]_0$ of NR complexed to β-CD. (A) The line represents the nonlinear regression fit to the experimental data considering a 1:1 NR/β-CD complex using eq 12. (B) The line represents the nonlinear regression fit to the experimental data considering simultaneous formation of 1:1 and 1:2 NR/β-CD complex using eq 14.

where CD represents β-CD. The equilibrium constant K_1 for the above reaction can be expressed as

$$K_1 = \frac{[\text{NR:CD}]_{\text{eq}}}{[\text{NR}]_{\text{eq}}[\text{CD}]_{\text{eq}}} \tag{7}$$

where $[\text{NR:CD}]_{\text{eq}}$ is the equilibrium concentration of the 1:1 complex for a given β-CD concentration. If $[\text{NR}]_0$ and $[\text{CD}]_0$ are the initial concentrations of NR and β-CD, respectively, in the present study where $[\text{CD}]_0 \gg [\text{NR:CD}]_{\text{eq}}$ eq 7 reduces to

$$K_1 = \frac{[\text{NR:CD}]_{\text{eq}}}{([\text{NR}]_0 - [\text{NR:CD}]_{\text{eq}})[\text{CD}]_0} \tag{8}$$

At any stage the observed fluorescence intensity I_f is the sum of the fluorescence intensities arising from both free NR and NR:CD, respectively. These fluorescence intensities are proportional to their respective concentrations present in the solution. Therefore, one can write

$$I_f = I_f^0 \frac{[\text{NR}]_{\text{eq}}}{[\text{NR}]_0} + I_{\text{NR:CD}} \frac{[\text{NR:CD}]_{\text{eq}}}{[\text{NR}]_0} \tag{9}$$

where I_f^0 is the fluorescence intensity in the absence of β-CD and $I_{\text{NR:CD}}$ is fluorescence intensity when all NR molecules are complexed with β-CD forming 1:1 NR/β-CD complex. Since

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[NR]$_{eq}$ is equal to ([NR]$_0$ – [NR:CD]$_{eq}$) eq 9 can be further rearranged as

$$\frac{[NR:CD]eq}{[NR]_0} = \frac{I_f - I_f^0}{I_{NR:CD} - I_f^0} \quad (10)$$

From eqs 8 and 10 one gets eq 11, which is a modified Benesi-Hildebrand relation.[24]

$$\frac{1}{I_f - I_f^0} = \frac{1}{I_{NR:CD} - I_f^0} + \frac{1}{I_{NR:CD} - I_f^0}\left(\frac{1}{K_1[CD]_0}\right) \quad (11)$$

For a 1:1 NR/β-CD complex formation the double reciprocal plot of $(1/I_f - I_f^0)$ vs $1/[CD]_0$ (inset of Figure 1B) should yield a straight line. From the slope and intercept of this plot the K_1 value has been estimated to be 490 M^{-1}. However, the fit is not very good especially in the higher β-CD concentration range, where the data points deviate from a straight line.

Nonlinear least-squares regression is an alternative approach of data analysis, which is used to fit the data directly into the relevant equations. For such analysis eq 11 can be rearranged as

$$I_f = \frac{I_f^0 + I_{NR:CD}K_1[CD]_0}{1 + K_1[CD]_0} \quad (12)$$

The changes in I_f with β-CD concentration are plotted in Figure 2. Using eq 12 a nonlinear least-squares fitting of the data using the Origin 5.0 program is shown in Figure 2A. The nonlinear least-squares regression analyzed data clearly show a large deviation of data points and fitting is not very good at higher β-CD concentrations. These results thus suggest that the complexation of NR with β-CD is not entirely of 1:1 type. There are a number of reports where complexation of the substrate with cyclodextrins have been suggested to be of 1:2, 2:1, and 2:2 type in addition to 1:1 complexes.[25−30] Considering the dimensions of NR (14.8:6.9:3.8 Å^3)[19,31] and the β-CD cavity (length ∼8 Å, width ∼6−7 Å), it is quite likely that 1:2 (NR-(β-CD)$_2$) complexes are also formed in the present systems. Fluorescence lifetime and fluorescence depolarization studies (discussed later) also support the simultaneous formation of both 1:1 and 1:2 complexes between NR and β-CD in aqueous medium.

For a successive 1:2 complex formation between NR and β-CD the second step of the equilibrium can be written as

$$NR:CD + CD \overset{K_2}{\rightleftharpoons} NR:(CD)_2 \quad (13)$$

For this equilibrium similar to eq 12 one can write,[25,27]

$$I_f = \frac{I_f^0 + I_{NR:CD}K_1[CD]_0 + I_{NR:(CD)_2}K_1K_2[CD]_0^2}{1 + K_1[CD]_0 + K_1K_2[CD]_0^2} \quad (14)$$

Using eq 14 experimental data points fit nonlinear least-squares regression analysis nicely with a dependency of 0.99 as shown in Figure 2B. From such analysis two equilibrium constants K_1 and K_2 were estimated to be 411 ± 25 and 420 ± 99 M^{-1} respectively.

3.3. pH Effects and Acidity Constants. To know the effect of β-CD on the prototropic equilibrium between NRH$^+$ and NR (eq 5), the pH-dependent changes in the absorption spectra of the dye in aqueous solution containing β-CD have been recorded and are shown in Figure 3. Following pH-dependent changes
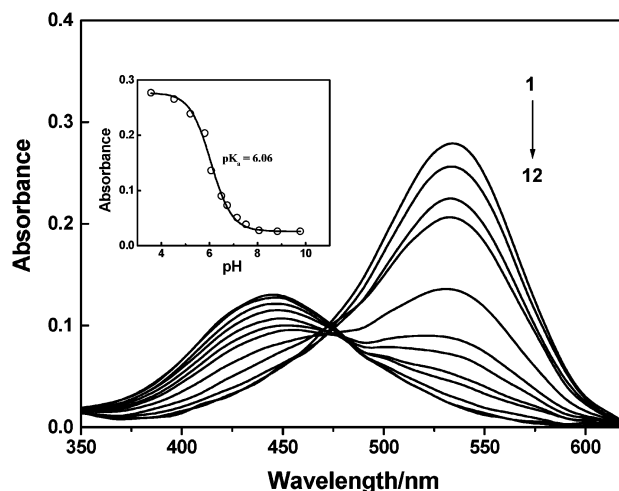
Figure 3. Absorption spectra of neutral red (1 × 10^{-5} M) in water containing 10 mM β-CD at different pHs. (1) 3.56, (2) 4.53, (3) 5.2, (4) 5.8, (5) 6.07, (6) 6.5, (7) 6.74, (8) 7.15, (9) 7.52, (10) 8.07, (11) 8.83, and (12) 9.78. Inset: Variation in absorbance with pH at 535 nm.

in the absorbance at 535 nm (inset of Figure 3), the ground-state pK_a of the dye in the presence of β-CD (10 mM) has been determined to be 6.06 ±0.05, which is much lower than the value of 6.81 ±0.05 measured in the absence of β-CD.

In supramolecular complexes, depending upon relative affinity of the substrate for the host, pK_a is known to change as a result of complexation.[23,32−35] Walz et al.[23] have studied the binding of neutral red with DNA and have reported a value of pK_a of the NR−DNA complexes as 8.46 for native DNA and 7.72 for denatured DNA. The authors have established that DNA binds preferentially to the protonated form of the dye. As the NRH$^+$ form complexes with DNA, the equilibrium 5 effectively shifts toward the left, resulting in an increase in the apparent pK_a value. Drummond et al.[33] and Moulik et al.[34] have also investigated the effect of micelles and microemulsions on the acid−base equilibrium of neutral red. These authors have observed either an increase or a decrease in the pK_a value of NRH$^+$ depending upon the affinity of a prototropic form of the dye with the host. In the present study, it is observed that the neutral form of the dye (NR) preferentially binds to β-CD to form the inclusion complex, whereas its cationic form, NRH$^+$, does not interact with β-CD. The complexation of NR with β-CD causes equilibrium 5 to shift in the forward direction. Therefore, a lowering in the pK_a value of NRH$^+$ in the presence of β-CD is as expected.

At this point it is interesting to compare the present results with those reported by Yuan et al.[35] on the inclusion complex formation of neutral red with β-CD. The authors have investigated the interaction of neutral red with β-CD using the techniques of pulse polarography and spectrophotometry. During spectrophotometric measurements the authors have observed that the absorbance at the 450-nm band increases and that at the 535-nm band decreases with an increase in the β-CD concentration in aqueous solution at neutral pH. These changes in the absorption characteristics of the neutral red solution in the presence of β-CD have been assigned to the formation of an inclusion complex between NRH$^+$ and β-CD. The authors have determined the formation constant (K) for the NRH$^+$/β-CD inclusion complexes as 984 dm^3 mol^{-1} from the changes in the absorbance and 952 dm^3 mol^{-1} by differential pulse polarography for a 1:1 inclusion complex. As these studies were carried out at neutral pH and since the pK_a of the dye is 6.81 ±0.05,[17,23]

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TABLE 1: Fluorescence Lifetimes Estimated from the Analysis of the Fluorescence Decays of NR in Aqueous Solution at pH $\sim$9 in the Presence of Different Concentrations of β-CD[a]

solvent	τ_1, ns	a_1	Φ_1	τ_2, ns	a_2	Φ_2	τ_3, ns	a_3	Φ_3	χ_2
water	0.7 ± 0.1	1.0	1.0							1.1
				Biexponential Analysis						
0.5 mM β-CD	0.81 ± 0.02	0.78 ± 0.02	0.44	4.03 ± 0.01	0.23 ± 0.02	0.56				1.17
1 mM β-CD	0.90 ± 0.02	0.72 ± 0.02	0.33	4.08 ± 0.01	0.28 ± 0.02	0.67				1.19
2 mM β-CD	1.01 ± 0.02	0.60 ± 0.03	0.26	4.11 ± 0.02	0.40 ± 0.02	0.74				1.20
5 mM β-CD	1.22 ± 0.02	0.50 ± 0.02	0.22	4.20 ± 0.03	0.50 ± 0.02	0.78				1.01
7 mM β-CD	1.29 ± 0.01	0.47 ± 0.02	0.21	4.24 ± 0.01	0.53 ± 0.02	0.79				0.97
10 mM β-CD	1.35 ± 0.01	0.44 ± 0.02	0.20	4.25 ± 0.01	0.56 ± 0.02	0.80				1.12
				Triexponential Analysis						
0.5 mM β-CD	0.7 (fx)	0.72 ± 0.01	0.37	1.9 ± 0.10	0.14 ± 0.02	0.19	4.4 ± 0.18	0.14 ± 0.02	0.44	1.11
1 mM β-CD	0.7	0.57 ± 0.01	0.23	1.9 ± 0.03	0.20 ± 0.01	0.21	4.4 ± 0.02	0.23 ± 0.01	0.56	1.07
2 mM β-CD	0.7	0.43 ± 0.02	0.14	1.9 ± 0.11	0.26 ± 0.02	0.23	4.4 ± 0.10	0.32 ± 0.04	0.63	1.00
5 mM β-CD	0.7	0.24 ± 0.01	0.06	1.9 ± 0.04	0.32 ± 0.02	0.24	4.4 ± 0.03	0.43 ± 0.02	0.70	0.97
7 mM β-CD	0.7	0.20 ± 0.02	0.04	1.9 ± 0.08	0.36 ± 0.02	0.24	4.4 ± 0.04	0.44 ± 0.02	0.72	0.90
10 mM β-CD	0.7	0.20 ± 0.02	0.04	1.9 ± 0.06	0.33 ± 0.02	0.23	4.4 ± 0.02	0.46 ± 0.02	0.73	1.11
				Global Triexponential Analysis						
0.5 mM β-CD	0.7^b	0.7	0.34	1.9^b	0.16	0.22	4.4^b	0.14	0.44	1.19
1 mM β-CD		0.55	0.21		0.23	0.24		0.22	0.55	1.06
2 mM β-CD		0.41	0.13		0.29	0.25		0.30	0.62	1.02
5 mM β-CD		0.24	0.06		0.35	0.25		0.41	0.69	0.96
7 mM β-CD		0.19	0.05		0.36	0.24		0.45	0.71	0.88
10 mM β-CD		0.17	0.04		0.37	0.25		0.46	0.71	1.12

[a] The samples were excited at $\lambda_{exc} = 444$ nm and emission were detected at $\lambda_{em} = 625$ nm. The relative contributions are defined as $\Phi_i = a_i\tau_i/\sum a_i\tau_i$ where a_i is the preexponential factor. [b] Fluorescence lifetimes obtained on global triexponential analysis, linking the lifetimes together with overall $\chi^2 = 1.02$.

both NR and NRH$^+$ forms coexist in the solution. Thus, at neutral pH the absorption spectrum of neutral red shows both the absorption peaks due to the neutral (NR) and protonated (NRH$^+$) forms of the dye at 450 and 535 nm, respectively. The present studies clearly indicated that only the NR form of the dye forms inclusion complex with β-CD but not the NRH$^+$. Further, due to the complex formation the pK_a value of NRH$^+$ decreases in comparison to that in aqueous solution. Thus, it is evident that both the increase in the absorbance at 450 nm and a decrease in the absorbance at 535 nm with an increase in the β-CD concentration at the neutral pH are due to the enhanced deprotonation of the NRH$^+$ in the presence of β-CD.

To correlate pK_a changes with the nature of complex formed (1:1 or 1:2) measurements are performed at varying β-CD concentrations. We measured the pK_a of NRH$^+$ in the presence of 1, 2, and 5 mM β-CD concentrations also. The β-CD concentrations are selected considering the fact that transition in the stoichiometry of NR/β-CD complex from 1:1 to 1:2 should take place in this range. The measured pK_a values do not show significant variation in the selected β-CD concentration range, 1 mM (6.1 $\pm$ 0.04), 2 mM (6.0 $\pm$ 0.05), and 5 mM (6.05 $\pm$ 0.04), respectively. From these studies it is inferred that the changes in pK_a are associated with 1:1 complex and the preferential binding of the NR, which affects equilibrium 5. The successive binding of the 1:1 complex by a second β-CD does not have any impact on pK_a. Further, even in a 1:1 complex the site of first protonation in NR is well protected from the bulk. It is thus inferred that the dimethylamino moiety of NR enters the β-CD cavity first to form a 1:1 complex.

3.4. Fluorescence Lifetime Measurements. We observed earlier that the fluorescence lifetime of NR is highly sensitive to the solvent environment.[16] In aqueous solution the fluorescence lifetime of NR is very short (0.8 ns) in comparison to that ($\sim$3$-$4 ns) in other solvents. This unusually short fluorescence lifetime of NR in water was attributed to strong intermolecular hydrogen bonding between NR and water molecules, resulting in an increase in the nonradiative decay rate of the NR excited state. As a result of complexation with

CDs, an increase in substrate lifetime has been reported in a number of cases.[27,36-38] In the present study, the fluorescence decay characteristics of NR in water at pH $\sim$9 were investigated both in the presence and absence of β-CD. It is seen that whereas the fluorescence decay of NR in water fits reasonably well with a single-exponential function, those in the presence of β-CD show distinct biexponential behavior. It is seen that the fluorescence lifetime of NR gradually becomes longer as the β-CD concentration is increased. The fluorescence decay parameters for NR in aqueous solutions containing different concentrations of β-CD obtained from biexponential analysis of the decay curves are listed in Table 1. The two lifetimes estimated increase gradually in the range of 0.8$-$1.4 ns for the shorter and 4.05$-$4.3 ns for the longer lifetime component and none of the two lifetimes matches with the lifetime of free NR (0.7 ns) in aqueous solution. It was also observed during the least-squares fitting of the time-resolved fluorescence data that at least in the lower β-CD concentration range the distribution of residuals was not very good. This means that there must be three different species present in the aqueous solution of NR containing β-CD. Therefore, lifetime data were further analyzed for triexponential decay, keeping the fastest component (0.7 ns, lifetime of free NR) fixed. However, under such condition global analysis of lifetime data, linking the lifetimes together is a better way of analysis.[27] We attempted a global analysis of all the lifetime data collected with varying concentrations of β-CD linking the lifetimes together. Similar results were obtained from both the methods for triexponential analysis of the decay curves, giving three different lifetimes with varying preexponential factors. Results obtained from triexponential analysis of decay curves from both the methods are also listed in Table 1. These results confirm that there are three species present in the aqueous solution of NR/β-CD having lifetimes 0.7, 1.9, and 4.4 ns, where the two longer components represent the lifetime of the NR/β-CD inclusion complexes.

Previous reports from different groups suggest that polarity of the β-CD cavity is similar to that of alcoholic solvents.[39,40] The longest component of lifetime (4.4 ns) for one of the NR/

β-CD complexes matches very well with that of NR in ethanol.[16] Therefore, in one of the NR/β-CD complexes the dye molecule is completely protected from the bulk aqueous phase facing an environment similar to that of ethanol. Considering the dimensions of NR (14.8:6.9:3.8 Å[3] [19,31]) and the β-CD cavity (length ~8 Å, cavity diameter ~6–7 Å) it is clear that in a 1:1 inclusion complex about 45% of the probe molecule will remain extended outside the cavity in aqueous medium. An environment similar to ethanol is possible only when NR is sandwiched between two β-CD molecules having a barrel-type structure. Therefore, it is inferred that one of the NR/β-CD complexes is of 1:2 type. A decrease of local polarity reduces the nonradiative decay rate (through TICT path), as a result the lifetime of NR in a 1:2 complex increases to 4.4 ns. For a 1:1 complex, in addition to an increase in the effective polarity, the hydrogen bonding interaction with water molecules will further reduce the lifetime in comparison to that of the 1:2 complex. Thus, the lifetime component of 1.9 ns is assigned to a 1:1 NR/β-CD complex. Lucia Flamigni[38] obtained similar results from the time-resolved fluorescence study of the inclusion complexes between fluorescein and its halogenated derivatives with CDs. The author has suggested that the two lifetimes of inclusion complexes are due to two different types of complexes. Lucia Flamigni[38] proposed a hypothesis that two different orientations of the substrate inside the CD cavity are responsible for the two distinct lifetimes of the inclusion complexes. In the present study, β-CD concentration dependent pK_a measurements suggest that the 1:1 complex prefers an orientation with the dimethylamino moiety of NR trapped inside the β-CD cavity. Therefore, formation of a 1:2 NR/β-CD complex in addition to 1:1 seems to be more logical. Temperature-dependent lifetime and time-resolved fluorescence depolarization studies were performed on the NR/β-CD complex to verify this fact. It should be noted that in the high β-CD concentration range (5–10 mM) the relative fraction of 1:1 and 1:2 NR/β-CD complex does not appear to change on increasing the β-CD concentration (cf. Table 1). This could be attributed to the similar values of association constants K_1 and K_2 (eqs 6 and 13) for 1:1 and 1:2 NR/β-CD complex formation and the limitation of solubility for β-CD (up to 10 mM) in aqueous solutions. As a result, when most of the NR molecules get complexed the relative fraction of the two species does not show a significant change on increasing β-CD concentration in this range.

At this stage we would like to discuss the interpretations, claims, and discrepancies reported by different groups in relation to the polarity inside the β-CD cavity. In his review, Connors[4] has discussed the polarity of the β-CD cavity in view of the inferences and claims made by different groups. A number of parameters influence the characteristics of the probe molecule, therefore the claims about the polarity inside the β-CD cavity has been found to be highly dependent on the molecule used to probe it. Local polarity, motional freedom, partial encapsulation of the probe, the number of water molecule trapped inside the cavity, and the hydrogen bonding sensitivity of the probe are the factors that can cause a large difference in the estimated polarity for β-CD cavity. In the case of 1:2 NR/β-CD complexes, it is expected that the NR molecule will be completely encapsulated by the two β-CD cavities and experience a polarity very much similar to that of the β-CD cavity. For 1:2 probe/β-CD complexes, using pyrene as the probe, Xu et al.[29] and Yang and Bohne[30] have suggested that the polarity of β-CD cavity is similar to that of hexane. In contrast, Nigam and Durocher,[25] using 3H-indole derivatives as probe, estimated the polarity of the β-CD cavity to be similar to that of a 80:20
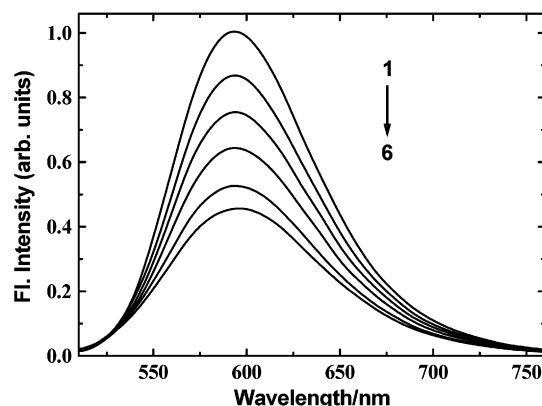

Figure 4. Fluorescence spectra of NR in aqueous solution containing 10 mM β-CD at temperatures (1) 25 °C, (2) 35 °C, (3) 45 °C, (4) 55 °C, (5) 65 °C, and (6) 75 °C, respectively.

methanol–water mixture. Our interpretation about the polarity of β-CD cavity is based on the SS and TR fluorescence results. The observed longest lifetime component of 4.4 ns matches well with the fluorescence lifetime of NR in ethanol solution (4.41 ns).[16] This is further supported by the fact that the fluorescence peak (588 nm) for NR/β-CD system at ~10 mM β-CD concentration is also very close to the fluorescence peak (585 nm) for NR observed in ethanol solution.[16] Therefore, in a 1:2 NR/β-CD complex the probe experiences a polarity similar to that of ethanol.

From the SS and TR fluorescence studies on NRH$^+$ in aqueous solution at pH ~4, both in the presence and absence of β-CD, it is seen that the fluorescence intensity, fluorescence spectra, and fluorescence lifetime of NRH$^+$ remains more or less same even in the presence of the highest concentration (10 mM) of β-CD used. These results are thus in accordance with the observations made from the ground-state absorption studies and clearly indicate that the cationic form of the dye does not form an inclusion complex with β-CD. This inference is further supported by the results obtained in the fluorescence anisotropy measurements, discussed latter in section 3.6.

3.5. Effect of Temperature on Fluorescence Behavior. In our earlier studies on NR in different organic solvents,[16] it was observed that the fluorescence intensity decreases with an increase in the temperature, a normal behavior observed for most of the fluorescent molecules. In aqueous solutions, however, it was observed that the fluorescence intensity of NR increases with an increase in the temperature of the solution. This unusual behavior was attributed to strong intermolecular hydrogen bond formation between NR and water molecules.[16] Such interactions increase the nonradiative deactivation process of the excited molecule, due to fast energy dissipation through the vibrations associated with the hydrogen bonds. With an increase in the temperature, some of these hydrogen bonds are broken, resulting in an increase in the number of free NR molecules thus increasing the fluorescence intensity. For the NR/β-CD complex it is interesting to see how the fluorescence intensity changes with temperature. Temperature-dependent changes in the fluorescence emission spectra of NR in aqueous solution in the presence of β-CD (10 mM) are shown in Figure 4. It is seen that in the presence of β-CD the fluorescence intensity of NR decreases with an increase in the temperature. Thus for the NR/β-CD complex, the temperature effect on its fluorescence behavior is similar to that observed in most of the organic solvents; however, the trend is just opposite to that observed for NR in the aqueous solutions in the absence of β-CD. From fluorescence lifetime studies we know that, in the presence of

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TABLE 2: Fluorescence Lifetime of NR Containing 10 mM β-CD at Various Temperatures[a]

solvent	25 °C	35 °C	45 °C	55 °C	65 °C	75 °C
				temp		
water	0.7	0.72	0.74	0.76	0.78	0.8
ethanol	4.1	4.04	3.95	3.9	3.8	3.73
10 mM β-CD[b]						
τ_1	0.7 (0.06)	0.72 (0.08)	0.74 (0.09)	0.76 (0.11)	0.78 (0.12)	0.80 (0.17)
τ_2	1.9 (0.23)	1.86 (0.24)	1.74 (0.24)	1.64 (0.25)	1.61 (0.26)	1.57 (0.32)
τ_3	4.4 (0.71)	4.00 (0.68)	3.67 (0.67)	3.40 (0.64)	3.08 (0.62)	2.88 (0.51)

[a] Relative contributions coming from different species are shown in brackets. Temperature-dependent fluorescence lifetime of NR in aqueous solution and in ethanol is also given for comparison. [b] For triexponential analysis of fluorescence decays the fastest component corresponding to free NR was kept constant.

10 mM β-CD, ~71% of the emission is contributed by the 1:2 NR/β-CD complex, where the NR molecule is completely shielded from bulk water, fluorescence contribution from the 1:1 complex is relatively small, and that of free NR is almost negligible (cf. Table 1). Further, in a 1:1 NR/β-CD complex also the NR is not completely exposed to form intermolecular hydrogen bonding with the water molecules in bulk. Therefore, changes in the fluorescence intensity of NR with temperature in the presence of β-CD in aqueous solution is as expected.

As inferred earlier, in an aqueous solution of NR containing β-CD both 1:1 and 1:2 inclusion complexes are formed simultaneously. If the attractive force to form 1:2 complexes was not strong enough, it is possible that with an increase in temperature the 1:2 complex may start breaking and at a certain temperature there will only be 1:1 complex in the solution. Temperature-dependent changes in the fluorescence lifetime of NR in the presence of 10 mM β-CD in aqueous solution were investigated and the lifetime changes are listed in Table 2. Temperature effect on the fluorescence lifetime of free NR in aqueous solution and in ethanol was also studied and the lifetimes are listed in Table 2 along with those of NR/β-CD for comparison. For triexponential analysis of fluorescence decays the fastest component corresponding to free NR was kept constant. It is evident from these results that the lifetime of both the 1:1 and the 1:2 complex decreases whereas that of free NR increases with an increase in temperature. For free NR in aqueous solution on increasing temperature some of the hydrogen bonds with water molecules are broken, as a result the lifetime increases. However, for the 1:1 complex the fluorescence lifetime decreases on increasing temperature, thus in a 1:1 complex the NR is not completely exposed to bulk water to form strong intermolecular hydrogen bonds. For a 1:2 NR/β-CD complex it is observed that the complex is quite stable in the temperature range of 25 °C to 75 °C, used in present investigation. However, there was a substantial decrease in the lifetime of 1:2 complex from 4.4 ns at 25 °C to 2.9 ns at 75 °C. Such drastic change in the fluorescence lifetime was not observed for free NR in ethanol solution (cf. Table 2). These observations suggest that on increasing temperature packing of the two β-CD molecules of the 1:2 complex becomes loose to some extent allowing some of the water molecules to come closer to the NR, resulting in an increase in the nonradiative deexcitation process. Further, the preexponential factors (percentage yield) for free NR and the 1:1 complex increase gradually whereas that of the 1:2 complex reduces drastically (cf. Table 2) on increasing the temperature. It is thus inferred that the equilibrium (eq 13) shifts in favor of the 1:1 complex with an increase of experimental temperature.

3.6. Studies on the Rotational Relaxation Dynamics. Time-resolved fluorescence anisotropy measurements were carried out to investigate the rotational relaxation dynamics of NR in aqueous solutions in the absence and presence of β-CD at pH

~9 and at pH ~4, respectively. To understand the rotational relaxation dynamics, it is important to have an estimate of the frictional force experienced by the probe molecules. According to the Stokes−Einstein−Debye (SED) hydrodynamic theory,[41] the rotational reorientation time τ_r, for a probe molecule is given by

$$\tau_r = \frac{\eta V}{k_B T}(fC) \tag{15}$$

where V is the molecular volume of the probe, η is the viscosity of the solvent, k_B is the Boltzmann constant, T is the absolute temperature, f is the shape factor, and C is a constant depending on the boundary condition. Depending on the probe and solvent system, the boundary condition can be either of stick or slip type.[41] It has been observed that when the size of the rotating probe is much bigger than the solvent molecules, the value of $C = 1$, which corresponds to the stick boundary condition. For molecules much smaller than the solvent molecules, the value of C could be close to zero, when the boundary condition is considered to be perfectly slip. For probe molecules having size comparable to that of the solvent molecules, the value of C can be within $0 \leq C \leq 1$, which are the intermediate cases between stick and slip boundary conditions. The value of C mostly depends on the axial ratio of the nonspherical rotating probe.[42]

To calculate the rotational relaxation time τ_r of the non-spherical probe NR, its dimensions has been estimated as 14.8:6.9:3.8 $Å^3$ using the Corey-Pauling-Koltum space filling model. The van der Waals' volume of NR has been calculated to be 234 $Å^3$ using Edward's volume increment method.[43] Since for a NR molecule all three dimensions are of different lengths, it has been modeled as an asymmetric ellipsoid. For such an ellipsoidal rotor the rotational relaxation time τ_r is given by the following expression.[44]

$$\tau_r = \frac{1}{12}\left[\frac{4D_1 + D_2 + D_3}{D_1 D_2 + D_2 D_3 + D_3 D_1}\right] \tag{16}$$

where D_1, D_2, and D_3 are the diffusion coefficients along the long, short in-plane and short out-of-plane axes, respectively. The diffusion coefficients along the three axes were calculated from the friction coefficients tabulated for the stick and slip boundary conditions, given by Small and Isenberg[45] and Sension and Hochstrasser.[46] It was assumed that the transition dipole for NR is along the long axis of the molecule. The rotational reorientation time at unit viscosity, τ_r/η, for NR was thus calculated as 135 ps/mPa s using stick boundary condition and 51 ps/mPa s using slip boundary condition at 25 °C.

Though the molecular dimensions of β-CD have been reported by many workers,[3,6,7] one can find small variations in these results reported by different groups. Considering the dimensions of NR (14.8:6.9:3.8 $Å^3$)[19,31] and the β-CD cavity
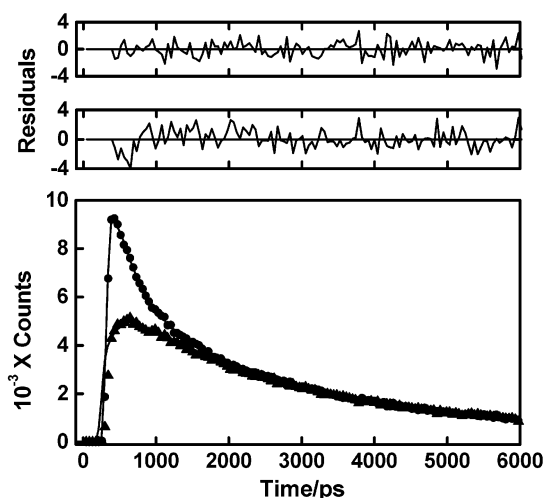
Figure 5. Time-resolved anisotropy decays of the aqueous solution of NR containing 10 mM β-CD at 25 °C. The experimentally measured $I_{\text{II}}(t)$ and $I_{\perp}(t)$ curves are represented by circle (●) and triangles (▲), respectively. The lines passing through them are the fitted ones following a single-exponential function ($\tau_r = 520$ ps, $\chi^2 = 1.02$). The instrument response function has a full width at half-maximum of 80 ps. The lower and upper panels represent the distribution of the weighted residuals for $I_{\text{II}}(t)$ and $I_{\perp}(t)$, respectively.

(length $\sim$8 Å), it is expected that even when NR goes deeper inside the β-CD cavity in a 1:1 complex about 45% of the NR molecule will still remain exposed outside the cavity. The volume of a 1:1 NR/β-CD complex have been estimated to be 1424 Å^3 from the dimensions of NR and β-CD. The rotational reorientation time at unit viscosity, τ_r/η, for a 1:1 NR/β-CD complex was thus calculated as 350 ps/mPa s using stick boundary condition at 25 °C. Similarly for a 1:2 NR/β-CD complex τ_r/η was estimated to be 646 ps/mPa s using stick boundary condition.

Rotational correlation time τ_r of NR in the absence of β-CD has been measured as 108 ± 7 ps at ambient temperature (25 °C). Although water is highly polar, for a given solute the contribution of the dielectric friction toward the rotational dynamics is almost negligible in water.[47] Therefore, the observed reorientation time of NR in water can be considered to be mainly due to hydrodynamic friction. Since at 25 °C the viscosity of water is about 0.89 mPa s,[48] the measured τ_r/η for NR will be 121 ps/mPa s, which is in very good agreement with the τ_r/η value of 135 ps/mPa s calculated using eq 16 for the stick boundary condition. Since the size of NR is much bigger than that of water molecules, it is expected that the probe in aqueous solution will follow the stick boundary condition,[41] as indicated by the experimental and calculated τ_r/η values.

In the presence of 10 mM β-CD, it is seen that the τ_r of NR at 25 °C increases drastically to 540 ± 35 ps. The fluorescence anisotropy decay of NR in water in the presence of 10 mM β-CD fits reasonably well with a monoexponential function. The typical $I_{\text{II}}(t)$ and $I_{\perp}(t)$ curves obtained in aqueous solution at ambient temperature in the presence of 10 mM concentration of β-CD are shown in Figure 5. The fittings of the $I_{\text{II}}(t)$ and $I_{\perp}(t)$ curves following the single-exponential anisotropy decay for NR/β-CD complex are also shown in the same figure as continuous lines along with the distribution of the weighted residuals among the data channels, indicating goodness of the fit. Rotational dynamics studies have been performed at various β-CD concentrations. In the lower β-CD concentration range the rotational dynamics data fits nicely to a biexponential function with a shorter component matching with the τ_r of free NR and a longer component which increases gradually with an

increase of β-CD concentration. For example at 1 mM β-CD concentration we measured $\tau_{r1} = 99 \pm 16$ ps and $\tau_{r2} = 460 \pm 55$ ps, respectively. In the lower β-CD concentration range, emission from all the three species, free NR, 1:1 complex, and 1:2 complex, contribute significantly to the total emission. However, in the high β-CD concentration range fluorescence is mainly dominated by the NR complexed to β-CD; therefore, fluorescence anisotropy decay fits reasonably well to a monoexponential function.

In the presence of 10 mM β-CD the reorientation time has been measured to be 540 ± 35 ps at 25 °C (i.e., $\tau_r/\eta = 607$ ps/mPa s), which is close to the theoretically calculated τ_r/η value for a 1:2 NR/β-CD complex (646 ps/mPa s) at 25 °C. A small decrease in experimentally measured τ_r/η value can be attributed to the contribution from a 1:1 complex. From lifetime studies it is evident that under the condition used $\sim$71% of the total emission is contributed by a 1:2 complex, whereas $\sim$25% emission comes from a 1:1 complex. The theoretically calculated τ_r/η for a 1:1 NR/β-CD complex for stick boundary condition is 350 ps/mPa s. In general, the least-squares method of data analysis does not differentiate between the two species unless their time constants differ by 3–4 times. That is why the time-resolved anisotropy fits reasonably well to a single-exponential decay.

Balabai et al.[47] have measured rotation relaxation times of different probe molecules encapsulated in β-CD using a time-resolved fluorescence depolarization technique. The volume of a single molecule of β-CD molecule has been estimated by Balabai et al.[49] as 1147 Å^3. Using this value the authors have calculated the rotational correlation time of the β-CD molecule as 280 ps and that of a 1:1 inclusion complex as 350 ps for stick boundary condition.[49] The rotational correlation time of CD complexes in aqueous solution is reported in the range 200–300 ps.[49–51] It has also been observed that the rotational relaxation time for CDs often increases as a result of formation of inclusion complexes with probe molecules.[52] If we examine these results, the increase in the relaxation time of probe/CD inclusion complexes appears to be mainly due to an increase in the effective volume and dimension of CDs as a result of the partial inclusion of a guest in the CD cavity. This is so because in several systems studied, the size of the probe is bigger than the CD cavity size. Thus it is obvious that a small portion of the probe molecule remains extended out of the CD cavity resulting in an increase in the effective volume and dimension of the probe/CD complex. Consequently the latter will experience an increased friction during rotation in comparison to the free CD molecule.

In the work of Balabai et al.,[49] the rotational relaxation times were measured in aqueous medium for three different probe molecules, namely, resorufin, oxazine-118, and oxazine-725, encapsulated in a β-CD cavity. The authors observed that the rotational relaxation dynamics of resorufin/β-CD (59 and 301 ps) and oxazine-118/β-CD (56 and 281 ps) complexes follow biexponential decay, whereas that of the oxazine-725/β-CD (406 ps) complex follows a single-exponential decay. From these results the authors inferred that the long time components for all these complexes were due to the overall motion of the host–guest complex and the short time components observed in resorufin/β-CD and oxazine-118/β-CD complexes were due to independent internal motion of the probe molecules inside the β-CD cavity. According to Balabai et al.,[49] due to larger size of oxazine-725 the probe cannot rotate independently inside the β-CD cavity. Considering the diameter of the β-CD cavity (6–7 Å) and the dimension of NR molecule (14.8:6.9:3.8 Å^3),

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it seems that the NR molecule fits tightly inside the β-CD cavity. As a result, the independent rotation of NR inside the β-CD cavity is restricted for the NR/β-CD complex. Thus, in the high β-CD concentration range, rotational relaxation of the NR/β-CD complex follows a single-exponential decay with a relatively high τ_r value corresponding to the overall rotation of the complex.

The measured rotational relaxation times for the cationic form NRH$^+$ of the dye in aqueous solution (at pH ∼4) in the presence and absence of β-CD remains the same (112 ± 8 ps) even in the presence of the highest concentration (10 mM) of β-CD used. It is thus confirmed that unlike NR the NRH$^+$ does not form any inclusion complex with the β-CD. These results are thus in good agreement with those obtained from ground-state absorption and SS and TR fluorescence measurements.

4. Conclusions

The results obtained from the ground-state absorption and steady-state (SS) and time-resolved (TR) fluorescence measurements indicate that only the neutral form (NR) of the dye, neutral red, forms an inclusion complex with β-CD. The cationic form (NRH$^+$) of the dye does not form any complex with β-CD. Following the pH-dependent changes in the absorbance at 535 nm, the ground-state pK_a values of the dye have been estimated in the presence of varying concentrations of β-CD. The measured pK_a of 6.06 ±0.05 in the presence of 10 mM β-CD is much lower than the pK_a value of 6.81 ±0.05 measured in the absence of β-CD. The pK_a values of the dye remain same (6.06 ± 0.05) within experimental error in the β-CD concentration range 1−10 mM, where transition from a 1:1 to a 1:2 complex is expected. Therefore, lowering of the pK_a is due to the first step of complexation between NR and β-CD, which causes the equilibrium NRH$^+$ ⇌ NR + H$^+$ to shift in the forward direction. Successive binding of the 1:1 complex by a second β-CD does not have any impact on pK_a. Further, even in a 1:1 complex the site of first protonation in NR is well-protected from the bulk, hence the dimethylamino moiety of NR enters the β-CD cavity first to form a 1:1 complex.

The fluorescence decay of the aqueous solution of NR in the presence of β-CD is triexponential with lifetimes 0.7, 1.9, and 4.4 ns respectively, corresponding to free NR molecule and the 1:1 and 1:2 NR/β-CD complexes. Fluorescence lifetime measurements further support the formation of 1:1 and 1:2 NR/β-CD inclusion complexes simultaneously. In a 1:2 NR/β-CD complex it is expected that the probe molecule will be shielded completely from the bulk and should face an environment similar to that of β-CD cavity. From the SS and TR results obtained on the 1:2 NR/β-CD complex the effective polarity experienced by the probe inside the β-CD cavity is similar to that of ethanol. However, in a 1:1 NR/β-CD complex a large portion of NR remains extended outside the cavity and experiences an environment similar to an alcohol−water mixture. For a 1:1 complex in addition to a change in the polarity, hydrogen bonding with water molecules reduces the lifetime further due to an increase in the nonradiative decay rate of the excited NR molecules.

From the TR fluorescence anisotropy measurements it is confirmed that only the neutral form of the dye NR forms an inclusion complex with β-CD. In the lower β-CD concentration range the anisotropy decay fits a biexponential function where the faster component corresponds to free dye and the slower component is due to NR/β-CD complex. It should be noted that the slower component of rotational reorientation time arises due to both the 1:1 and 1:2 NR/β-CD complex (unresolved).

Therefore, it has been observed that the time constant for the slower component increases gradually with an increase of β-CD concentration. In the high β-CD concentration range (5−10 mM) emission from free dye is negligible and fluorescence emission is mostly coming from the 1:2 NR/β-CD complex, hence the rotational relaxation dynamics data fit well with a single-exponential function. This indicates that the NR/β-CD complex rotates as a whole and the independent rotation of NR inside the β-CD cavity is highly restricted.

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