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Study of γ-Cyclodextrin Host−Guest Complex and Nanotube Aggregate by Fluorescence Correlation Spectroscopy

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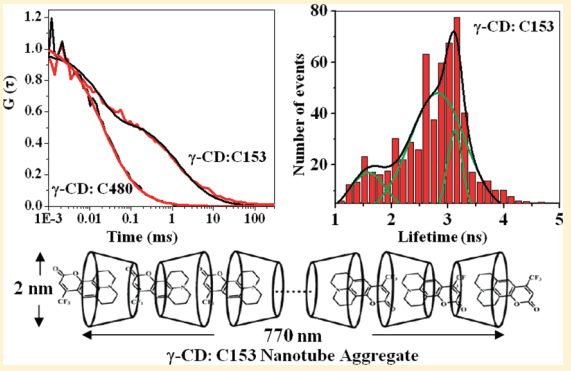
ABSTRACT: Fluorescence correlation spectroscopy (FCS) has been used to study the formation of large nanotube aggregates involving γ-cyclodextrin (γ-CD) and coumarin 153 (C153). It is observed that the length of a γ-CD:C153 nanotube aggregate is ∼770 nm. This is ∼480 times larger than the length of a 1:1 γ-CD:C480 complex (∼1.6 nm) and ∼950 times that of a γ-CD. This implies that 950 γ-CD units are noncovalently attached in the γ-CD:C153 aggregate. Binding constants (K_b) of both the dyes to γ-CD were obtained from the fluctuation in fluorescence intensity. The rate of association and dissociation are obtained from the inverse of τ_{off} and τ_{on}, respectively. The binding constant for the 1:1 γ-CD:C480 complex is ∼1000 M^{-1}. The burst integrated fluorescence lifetime (BIFL) histogram reveals presence of three distinct lifetime 1.8 ns (18%), 2.8 ns (69%), 3.2 ns (13%). These three lifetimes correspond to C153 present in bulk water and at the end and middle of the γ-CD:C153 nanotube aggregate, respectively. The lifetime of C480 in the 1:1 γ-CD:C480 complex is found to be 3.7 ns.



1. INTRODUCTION

Fluorescence correlation spectroscopy (FCS) has been widely used as a powerful technique to study diffusion in complex systems.[1−7] From the diffusion coefficients the size of the particle is obtained using the Stokes−Einstein relation:

$$r_h = \frac{k_B T}{6\pi\eta_0 D_t} \quad (1)$$

Using this method size of many systems has been determined with the help of the FCS technique. This includes micelle,[1a] polymer−surfactant aggregate,[1b] proteins in the denatured state[2] and cyclodextrin host−guest complexes.[3] Most recently, diffusion coefficients in different regions of a P123 gel[4a] and vesicles[4b,c] have been obtained using probes of varying hydrophobicity. Diffusion coefficients in a vesicle are multivalued and exhibit a distribution.[4b,c] This suggests that individual vesicles differ because of shape fluctuations.[4b,c] Thus a single-molecule study reveals that each individual system is different. A solution study gives an ensemble average of the Avogadro number of molecules. Similar variations of rate in individual enzyme molecules have been obtained by single-molecule studies.[5]

If the fluorescence quantum yield of a probe in free state in bulk water is different from that of the probe bound to a host, association and dissociation of the probe lead to a fluctuation of the fluorescence intensity. The rate constant of association is obtained from the inverse of τ_{off}, and the rate constant of dissociation is obtained from inverse of τ_{on}.[6,7] This strategy has been applied to calculate the binding constant of fluorescence probes to lipids,[6] proteins,[7a−c] DNA,[7d] and so forth.

In this work, we focus our attention on two kinds of supramolecular assembly involving γ-cyclodextrin (γ-CD): a 1:1 host−guest complex and a nanotube aggregate. Many fluorescent probes are known to form 1:1 host−guest complexes with γ-CD.[8a] The binding constant of this is obtained from steady state absorption and fluorescence spectroscopy (e.g., for γ-CD:C480 (C480 = coumarin 480)).[8a] For γ-CD:C153[8,9] (C153 = coumarin 153; Scheme 1A) and several other cyclodextrin assemblies,[10,11] the fluorescence anisotropy decay exhibits a very long component (>20 ns). This corresponds to a length >40 nm, i.e., >50 cyclodextrin units "stitched together" by the guest (C153).[8b] Most recently, the formation of such cyclodextrin nanotube aggregates has been detected by atomic force microscopy (AFM) and transmission electron microscopy (TEM).[11]

The formation of such nanotube aggregates involving cyclodextrin has not been studied before by FCS or in the solution phase by any microscopic technique. In order to accurately determine the size of the γ-CD:C153 nanotube aggregate in the solution phase, we applied FCS. It may be recalled that Seidel and co-workers[3a] used FCS to study the structure, dynamics, and binding equilibrium of cyclodextrin host−guest complexes. However, they did not detect any nanotube aggregate.

2. EXPERIMENTAL SECTION

Laser-grade dyes C153 (Scheme 1A) and C480 (Scheme 1B) were purchased from Exciton Inc. and used without further

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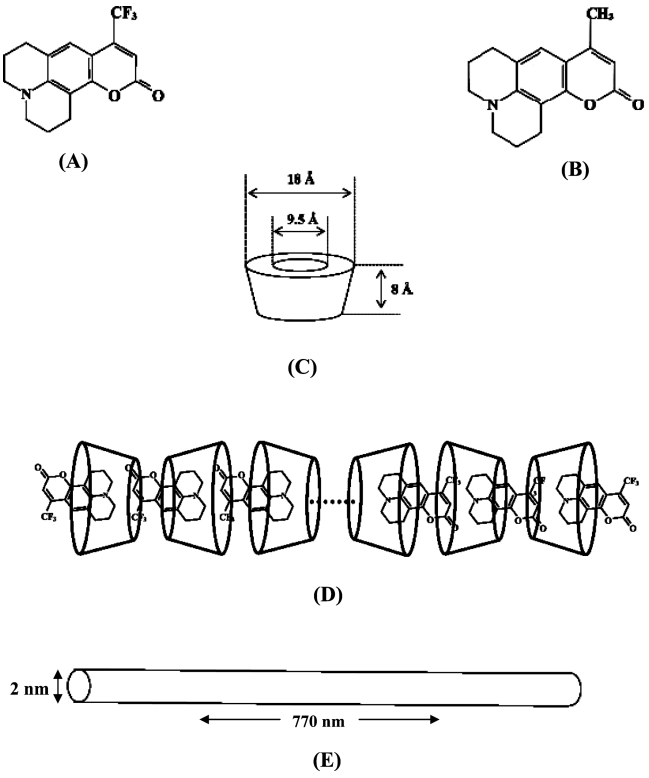
Scheme 1. Schematic Representation of (A) C153 and (B) C480, Model of (C) γ-CD and (D) γ-CD:C153 Linear Nanotube Aggregate, and (E) Rod-like Shape of a Nanotube Aggregate



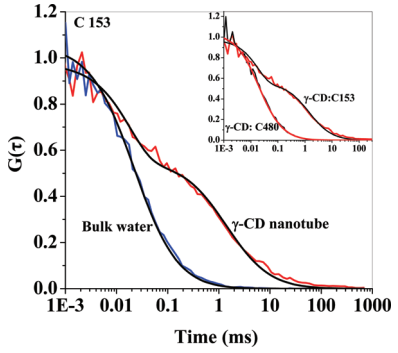


Figure 1. (a) Normalized FCS curves for C153 in bulk water (blue line) and γ-CD: C153 nanotube aggregate (red line). The black lines denote the best fit to the correlation curves. In the inset, comparison between the correlation curves of a γ-CD:C153 nanotube aggregate and a 1:1 γ-CD:C480 complex is shown. The black lines denote the best fit to the correlation curves.

purification. The γ-CD (Aldrich, Scheme 1C) was used as received. We prepared 50 μM γ-CD in aqueous solution. The concentration of C153 and C480 was maintained at 10 nM concentration in both γ-CD:C153 and γ-CD:C480 solution.

The samples were studied by FCS using a confocal microscope (Pico Quant, MicroTime 200) with an inverted optical microscope (Olympus IX-71). A water immersion objective (60× 1.2 NA) was used to focus the excitation light (405 nm) from a pulsed diode laser on the sample placed on a coverslip. After collecting the fluorescence via the same objective, it was allowed to pass through a dichroic mirror and appropriate band-pass filters. We used an HQ430LP filter to block the exciting light (405 nm). To detect the fluorescence, suitable band-pass filter (HQ480/40M) was used. The fluorescence was then focused through a pinhole (50 μm) and eventually on a nonpolarizing beam splitter prior to entering the two single-photon counting avalanche photodiodes (SPADs). The fluorescence cross-correlation signal was recorded using two detectors (SPADs). The signal was subsequently processed by the PicoHarp-300 time correlated single photon counting card (PicoQuant) to generate the time correlation function of the fluorescence $G(\tau)$,[2b] where

$$G(\tau) = \frac{1}{N}\left[\frac{f_f}{\left(1+\frac{\tau}{\tau_f}\right)\sqrt{1+\frac{\tau}{\tau_f\cdot(r_0/z_0)^2}}} + \frac{f_b}{\left(1+\frac{\tau}{\tau_b}\right)\sqrt{1+\frac{\tau}{\tau_b\cdot(r_0/z_0)^2}}}\right] \quad (2)$$

Here f_f and f_b are the fractions of free and bound probe molecules, respectively. τ_f and τ_b are the diffusion times of the free and bound species. N is the total average number of molecules in the sampling volume, and r_0 and z_0 are the dimensions of the sampling volume in the $x-y$ plane and the z direction, respectively. The diffusion coefficient of free and bound species is given by

$$D_{f,b} = \frac{r_0^2}{4\tau_{f,b}} \quad (3)$$

Data analysis of individual correlation curves was performed using the SymPhoTime software supplied by Pico-Quant.

2.1. Measurement of On and Off Time. Fluctuations in the fluorescence intensity at the individual binding sites were measured using the image processing software (Sympho time, PicoQuant). The time constants for association and dissociation of C153 and C480 dyes were estimated as follows. As the fluorescence dye (C153 and C480) binds to the γ-CD, its fluorescence intensity is markedly altered from that of the free dye in bulk water. Thus each period of association and dissociation was measured as an on- and off-time, respectively.

2.2. Kinetic Analysis. Interactions between the probe and the γ-CD were analyzed assuming multiple exponential kinetics. Each of the on- and off-time cumulative histograms was fitted to a biexponential function. Rate constants for binding (association) were measured from the inverse of the time component obtained from the off-time (τ_{off}) histograms. In contrast, rate constants of unbinding (dissociation) were obtained from the inverse of the time component of on-time (τ_{on}) histograms.

2.3. Burst Integrated Fluorescent Lifetime (BIFL) Analysis. The lifetime distribution for both the dyes was fitted to a Gaussian function.

$$y = y_0 + A\exp\left[-\left(\frac{x-x_0}{\text{width}}\right)^2\right] \quad (4)$$

3. RESULTS AND DISCUSSION

3.1. D_t and Size of γ-CD:C153 Nanotube Aggregate and 1:1 γ-CD:C480. The autocorrelation curves in Figures 1 and 2

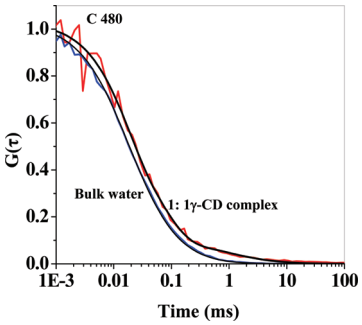


Figure 2. (a) Normalized FCS curves for C480 in bulk water (blue line) and in 1:1 γ-CD:C480 complex (red line). The black lines denote the best fit to the correlation curves.

Table 1. Diffusion Coefficient (D_t) of the Dyes in Different Systems

system	$D_{t,1}$ ($\mu m^2/s$)	length (L) (nm)	$D_{t,2}$ ($\mu m^2/s$)	length (L) (nm)
C153−water			550 (100%)	0.76
γ-CD:C153−water	10 (98%)	770	550 (2%)	0.76
C480−water			600 (100%)	0.70
γ-CD:C480−water	270 (10%)	1.6	600 (90%)	0.70

describe the diffusion of γ-CD assemblies for C153 nanotube aggregates and C480 complexes, respectively. Table 1 summarizes the diffusion coefficients (D_t) in different media. Figures 1 and 2 also illustrate the diffusion of the dyes in bulk water. The inset of Figure 1 shows the difference between a γ-CD:C153 nanotube aggregate and a 1:1 γ-CD:C480 complex.

For the γ-CD complex and nanotube aggregate, the data were fitted to two diffusion components: one for free dye in bulk water, and the other for the dye bound to γ-CD complex. The diffusion coefficient (D_t) of C153 in bulk water is 550 $\mu m^2\ s^{-1}$ (Table 1). For the γ-CD:C153 nanotube aggregate, it is observed that there is a very major (98%) component of diffusion constant (10 $\mu m^2\ s^{-1}$), which is 55-fold slower than that in bulk water. A very small fraction (2%) of the dyes displays a bulk water-like component of 550 $\mu m^2\ s^{-1}$. The ultraslow component of diffusion (10 $\mu m^2\ s^{-1}$) indicates very large volume of the cyclodextrin nanotube aggregate.

The γ-CD:C153 nanotube aggregate resembles a needle-like structure. For such a structure, the Stokes−Einstein formula (eq 1) is not valid. Bagchi and co-workers[12] proposed that for such a system the diffusion is anisotropic with a component ($D_\parallel$) parallel to the long axis and another ($D_\perp$) perpendicular to the long axis,

$$\left.\begin{aligned} D_\parallel &= \frac{k_B T \ln(L/b)}{2\pi\eta L} \\ D_\perp &= \frac{k_B T \ln(L/b)}{4\pi\eta L} \end{aligned}\right\} \quad (5)$$

The overall diffusion coefficient is $D_{total} = D_\parallel + 2D_\perp$.

In the present case of a γ-CD:C153 nanotube aggregate, D_{total} = 10 $\mu m^2\ s^{-1}$. Also, for this system, b is the outer diameter of the cyclodextrin cavity (∼ 2 nm), k_B is the Boltzmann constant, T is the temperature (293 K), η is the viscosity of the medium, and L is the length of the aggregate. Equation 5 was solved by the Newton−Raphson procedure, and length L was found to be 770 nm. This means that in the γ-CD:C153 nanotube aggregate, around 770/0.8∼950 cyclodextrin units are noncovalently attached. In this aggregate, $D_\parallel$ and $D_\perp$ are found to be 5 $\mu m^2\ s^{-1}$ and 2.5 $\mu m^2\ s^{-1}$, respectively.

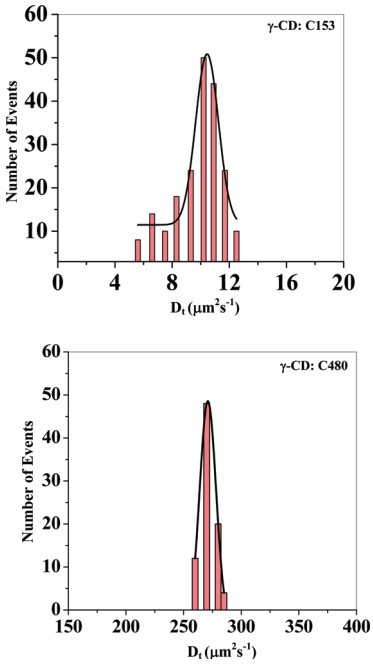


Figure 3. Distribution of D_t values of the γ-CD:C153 nanotube aggregate and the 1:1 γ-CD:C480 complex obtained by analyzing the data several times. The mean value of D_t was determined by fitting the distribution to a Gaussian function.

The diffusion coefficient (D_t) of C480 in bulk water is 600 $\mu m^2\ s^{-1}$ (Table 1). This is similar to that of C153 because of the similarity in size. For the 1:1 γ-CD:C480 complex, it is observed that the minor (10%) component of diffusion (270 $\mu m^2\ s^{-1}$) is 2.2 fold slower than that in bulk water. In this case, we found around ∼90% of the dye displays bulk water-like component of 600 $\mu m^2\ s^{-1}$, i.e., 90% of the dye remains in free. The γ-CD:C480 complex is nearly a sphere of diameter ∼1.6 nm. For this we used the Stokes−Einstein formula (eq 1). The height of one cyclodextrin unit is 0.8 nm,[8b] and the length of C480 is 1.2 nm. Thus a length of 1.6 nm implies that 0.4 nm of C480 is inside the cyclodextrin cavity in the 1:1 complex.

Figure 3 describes the distribution of the D_t−s for the γ-CD:C153 nanotube aggregate and the 1:1 γ-CD:C480 complex. It is readily seen that the distribution is quite narrow. This implies an almost uniform size distribution for both the γ-CD:C153 nanotube aggregate and the 1:1 γ-CD:C480 complex.

In summary, the FCS and diffusion data indicate that the γ-CD:C153 nanotube aggregate has a rod (or prolate)-like structure with a length of 770 nm (Scheme 1E). It is ∼480 times larger than the 1:1 γ-CD:C480 complex and ∼950 times larger than that of a γ-CD cavity.

3.2. Association and Dissociation Kinetics. The kinetics of binding of the dyes to γ-CD was studied from the fluctuation in fluorescence intensities of the probe (Figure 4). In this case, the fluctuation arises because the quantum yield of the dye inside the cyclodextrin cavity (nonpolar) is higher than that in bulk water.[13a−c]

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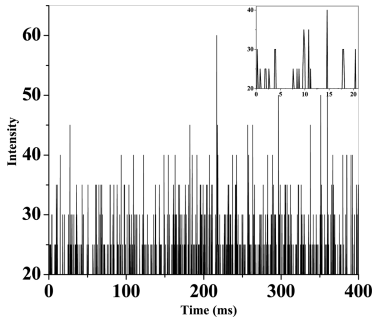

Figure 4. Fluorescence intensity−time trace of ~10 nM C153 in γ-CD. Inset shows the intensity−time trace in expanded time scale.

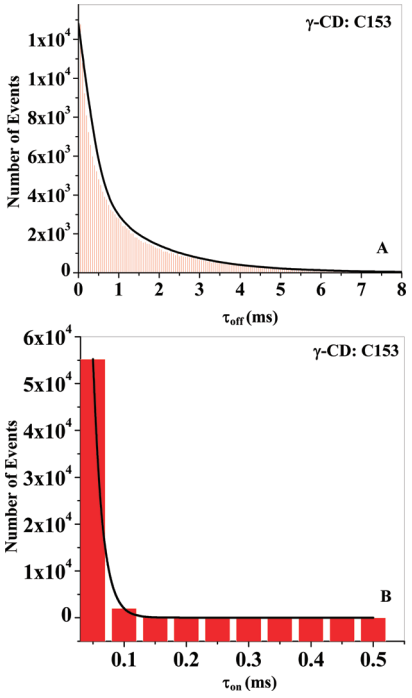

Figure 5. Association and dissociation kinetics between C153 and γ-CD. The figures indicate cumulative histograms of the (A) off time (τ_{off}) and (B) on time (τ_{on}).

From the τ_{off} histogram (Figures 5A and 6A) obtained from the MCS time trace, we have studied the kinetics of association (binding) of C153 and C480 to γ-CD. The kinetics of dissociation (unbinding) was monitored from the τ_{on} histogram. τ_{off} and τ_{on} are measured by fitting the histogram with a two exponential decay function.

Table 2 summarizes the off and on time for C153 and C480 in the γ-CD system. It is readily seen that C153 binds to two sites of the cyclodextrin aggregate with a major (65%) contribution to one site and a minor (35%) contribution to the other. In the γ-CD:C153 system, the τ_{off} value corresponding to the major (65%) binding site 1 is 275 μs. This is nearly 6 times faster than the corresponding τ_{off} value (1580 μs) for the minor (35%) binding site 2 (Table 2). The rate constants of association (inverse of τ_{off}) for C153 at the major and minor binding site were found to be 365 $nM^{-1}s^{-1}$ (65%) and 65 $nM^{-1}s^{-1}$ (35%), respectively.

By fitting the τ_{on} histogram (Figure 5B) to a two exponential, we obtained two rate constants for dissociation (unbinding) of the C153. Following the discussion in the previous section, we

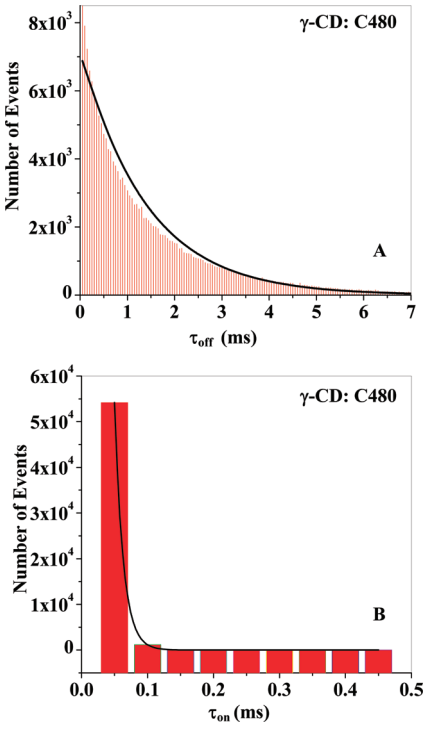

Figure 6. Association and dissociation kinetics between C480 and γ-CD. The figures indicate cumulative histograms of the (A) off time (τ_{off}) and (B) on time (τ_{on}).

Table 2. Association and Dissociation Parameters for Different Systems

system	τ_{off} (ms)	τ_{on} (ms)
γ-CD:C153−water	0.275 (0.65) 1.580 (0.35)	0.006 (0.54) 0.033 (0.46)
γ-CD:C480−water	1.400	0.013

assign the major component of dissociation (τ_{on}= 6 μs, 54%) to site 1 and the minor component to site 2 (τ_{on} = 33 μs, 46%) (Table 2). The rate constants of dissociation (inverse of τ_{on}) for C153 at the major and minor binding sites were found to be 166 ms^{-1} (54%) and 30 ms^{-1} (46%), respectively.

From the structure of the nanotube aggregate (Scheme 1D), it is obvious that the major site corresponds to the C153 interacting to two ends of nanotube aggregate and the minor site corresponds to C153 connecting two γ-CDs, i.e., present in between the nanotube aggregate.

For γ-CD:C480 system only one τ_{off} value (1400 μs) are obtained, which indicates only one binding mode of C480 to γ-CD. The corresponding τ_{on} value (13 μs), for the γ-CD:C480 system indicates only one binding site of C480 to γ-CD in the 1:1 complex (Figure 6B).

3.3. Binding Constant of the Dyes to the 1:1 Complex and Nanotube Aggregate. The binding constants of the dyes to γ-CD were studied using the following equation, which was used earlier by Seidel and co-workers:[3a]

$$\tau_D = \frac{\tau_f(1 + K[CD]}{1 + \frac{\tau_f}{\tau_b}K[CD]} \quad (6)$$

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Table 3. Binding Constant and Diffusion Time Constants in Different Systems

system	γ-CD:C153−water	γ-CD:C480−water
K_b [M^{-1}]	10^5	10^3
τ_f [ms]	0.022	0.020
τ_b [ms]	1.210	0.045

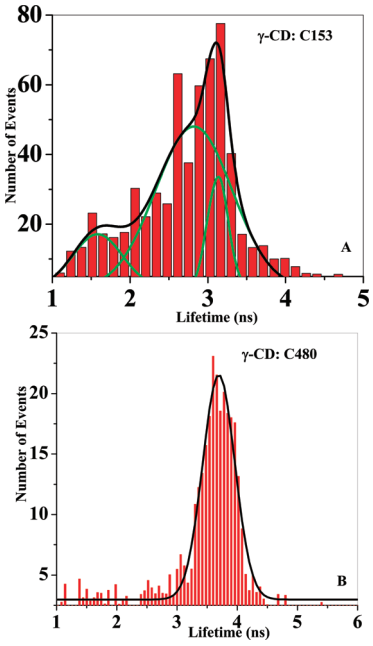

Figure 7. (A) Lifetime histogram of C153 in γ-CD, defining the distribution of lifetime at three binding sites of γ-CD chain aggregation. (B) Lifetime histogram of C480 in γ-CD, only one lifetime was obtained in this case.

In eq 6, τ_f and τ_b are the diffusion times of the free and bound dyes, respectively. K is the binding constant, [CD] indicates total host concentration, i.e., of cyclodextrin in this case. The mean diffusion time, τ_D, depends on the diffusion coefficient ($D_{f,b}$), and is obtained from the diffusion coefficient of the free (D_f) and bound (D_b) dyes from the relation[3a]

$$\tau_D = \frac{r_0^2}{4D_{f,b}}, \quad D_{f,b} = X_f D_f + X_b D_b \tag{7}$$

X_f and X_b are the number fractions of the free and bound dyes, respectively. For the 1:1 γ-CD:C480 complex, the binding (equilibrium) constant (K_1) is expressed as

$$K_1 = \frac{[D:CD]}{[D][CD]} \tag{8}$$

[D:CD] is the concentration of the complex, and [D] and [CD] are the concentrations of the free dye and free cyclodextrin, respectively. For the γ-CD:C153 nanotube aggregate, we considered the nth order binding constant (K_n), which is expressed as

$$K_n = \frac{[D_n: CD_{n+1}]}{[D][D_nCD_n]} \tag{9}$$

[D_n:CD_{n+1}] is the concentration of the nth order complex (nanotube aggregate), and [D] and [CD] are the concentrations of free dye and free cyclodextrin, respectively.

Table 4. Analysis of Lifetime Histograms for Different Systems

system	lifetime (τ_1), (ns)	fraction, %	lifetime (τ_2), (ns)	fraction, %	lifetime (τ_3), (ns)	fraction, %
γ-CD:C153−water	1.80	18	2.8	69	3.2	13
γ-CD:C480−water	3.7	100				

Using the above eq 6 we found that the binding constant of the C153 in the γ-CD:C153 nanotube aggregate is on the order of ~10^5 M^{-1}, whereas for the 1:1 γ-CD:C480 complex, the observed binding constant is ~10^3 M^{-1} (Table 3). From the FCS analysis we have observed that only 2% of the dye (C153) remains free in bulk water in the case of the γ-CD: C153 nanotube aggregate, whereas for the 1:1 γ-CD:C480 complex, the contribution of the free dye is 90%. From eqs 8 and 9 we have seen that the concentration of the free dye remains in the denominator in the expression of the binding constant. This may be the major reason for the higher binding constant for C153 to the γ-CD:C153 nanotube aggregate.

3.4. Lifetime Analysis. The BIFL histogram for C153 dye displays a distribution of lifetime with three maxima (Figure 7A). This suggests that the lifetimes of the dyes bound to different sites are different from each other.

The lifetime of C153 in water is ~1.8 ns,[13d] and it increases with decrease in polarity.[13a−c] In the case of the γ-CD:C153 nanotube aggregate (Figure 7A), it is evident that C153 bound to the γ-CD nanotube exhibits three lifetimes of 1.8, 2.8, and 3.2 ns (Table 4). In this case, the observed long components 2.8 ns (69%) and 3.2 ns (13%) correspond to the C153 in relatively nonpolar medium. The two values may respectively arise from the two environments: one with C153 at the end (2.8 ns) and the other within the middle (3.2 ns) of the nanotube. The minor component (1.8 ns, 18%) with bulk water-like lifetime corresponds to an exposed site and may be partially due to the small amount (2%) of free dye and some very loosely bound dye. Therefore our lifetime histograms of the γ-CD:C153 nanotube aggregate suggest the presence of three distinguished environments sensed by C153- at the end and middle of the aggregate and in bulk water.

For the 1:1 γ-CD: C480 complex from the BIFL study we found only one maxima at 3.7 ns (Figure 7B). Therefore it shows that almost all of the C480 molecule remains bound to the 1:1 γ-CD:C480 complex. We hardly see any presence of subpopulation in the distribution of lifetime histogram for C480 present in the 1:1 γ-CD:C480 complex; the single lifetime suggests that C480 dyes are in a uniform and similar environment in the 1:1 γ-CD: C480 complex.

4. CONCLUSIONS

The main findings are the following: Using FCS, we could determine the size of 1:1 γ-CD:C480 and γ-CD:C153 nanotube aggregates in the solution phase. Nanotube aggregates were found to be ~480 times larger than the 1:1 γ-CD:C480 complex and corresponds to ~950 cyclodextrin units. In the nanotube aggregate, the different lifetime component clearly reveals the presence of different environments. The binding constant in the γ-CD:C153 nanotube aggregate is ~100 times larger than that for the 1:1 γ-CD:C480 complex.

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