THE BINDING OF FLUOROPHORES TO β-CYCLODEXTRIN DERIVATIVES

By

Kellie J. Forrestall

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ABSTRACT

β-cyclodextrin (β-CD) is a seven-unit cyclic sugar molecule with a torroidal shape, a hydrophilic exterior and a hydrophobic cavity. It has been suggested that pyrene (P) forms a 2:1 ($P(\beta\text{-CD})_2$) complex with β-CD, where pyrene lies in-between the two wider rims of the β-CD. We examined this hypothesis by comparing the spectral properties of pyrene in the presence of substituted β-CDs. These properties include the shape and lifetime of pyrene fluorescence and the effect on pyrene fluorescence of added iodide quencher and alcohol. The derivatives studied included those with substituents only on the narrow rim, and those with substitution on both the narrow and wide rims. Our evidence indicates that a 2:1 complex is formed when there is no substitution on the wider rim of the β-CD molecule. The complexing patterns of β-CDs with two other fluorophores have also been examined: 2-acetylnaphthalene (2-AN) and N-phenyl-2-naphthylamine (NP-2-NA). The analysis using 2-AN utilized only the methylated β-CDs, while the NP-2-NA analysis only examined the β-CD monomer.

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INTRODUCTION

β-Cyclodextrin (β-CD) is a seven-unit cyclic sugar consisting of seven α-1,4-linked glucose monomers, in the shape of a ring or torus, with a hydrophilic exterior and hydrophobic cavity.[1] The hydrophobic interior is due to the glycosidic oxygen bridges connecting the units, which allows for the association with nonpolar organic molecules or portions of organic molecules forming inclusion complexes. The hydrophilicity of the exterior is due to the twenty-one hydroxyl groups lining the edges of the torus.[2] The dimensions for β-CD are 7.8 Angstroms (Å) in height, 6.5-7.8 Å in diameter of the narrower rim and 15.4 Å in diameter for the wider rim (Figure 1).[2,3]

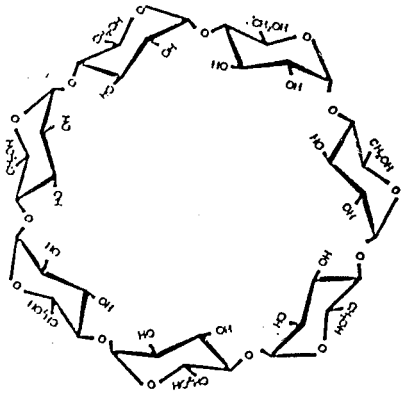
Pyrene is an attractive fluorophore for this work because its fluorescence intensity, lifetime, and the shape of its emission spectrum all can change when pyrene binds in a CD cavity.[4,5] For example, the fluorescence spectrum of pyrene has five vibronic bands. The relative hydrophobicity of pyrene's environment can be determined by calculating the ratio of the first (I) to the third (III) band (I / III ratio). The lower the I / III ratio the more hydrophobic the environment.[6] These properties that pyrene possesses allow for an analysis based on the shape and lifetime of pyrene fluorescence and on the effect on pyrene fluorescence of added iodide quencher and alcohol.

It has been suggested that pyrene is too large to fit completely into a single β-CD binding site (Figure 2) so it forms a 2:1 β-CD / pyrene complex in which the pyrene is "double capped" by two β-CD hosts in a "clam shell" arrangement (Figure 3).[7,8,9] A second suggestion has been reported where the complex takes on the "sandwich" form (Figure 3).[9] In this arrangement, which is based on a crystal structure, two CDs literally sandwich the fluorophore between them.

Figure 1: Structure and Properties of β-CD.

β-Cyclodextrin

Molecular Weight	**1135**
Glucose Units	**7**
Cavity Volume (mL/g)	**0.14**
H_2O Molecules in Solution	**11**
Height	**7.8 Å**
Narrow Rim Diameter	**6.5 – 7.8 Å**
Wide Rim Diameter	**15.4 Å**



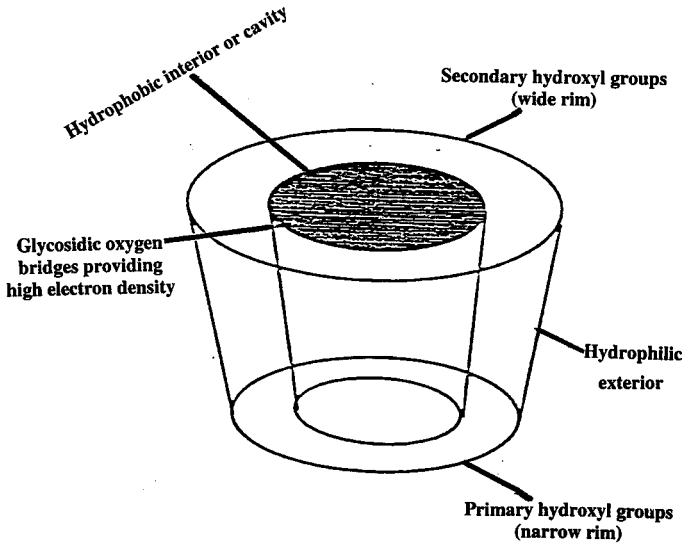
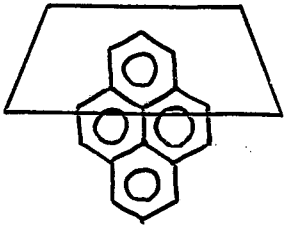
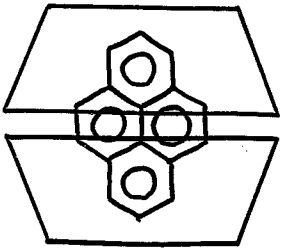


Figure 2: β-CD / Pyrene Complexing Patterns.



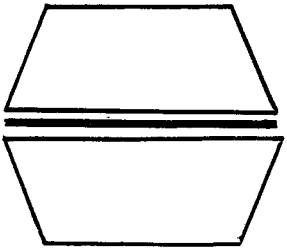
Scheme 1

1:1 β-CD / Pyrene Complex



Scheme 2

2:1 β-CD / Pyrene Complex
"clam shell"



Scheme 3

2:1 β-CD / Pyrene Complex
"sandwich"

We attempted to confirm that a 2:1 β-CD / pyrene complex does exist. This interaction was examined by comparing the spectral properties of pyrene in the presence of substituted β-CDs with those of pyrene bound to β-CD. These properties include the spectral shape and lifetime of pyrene fluorescence and the effect on pyrene fluorescence of added iodide quencher and alcohol in the presence of several β-CD derivatives.

The derivatives that were studied through the course of this research were those with substituents only on the narrow rim (6-O-Maltosyl and 6-O-α-D-Glucosyl) and those with substitution on both the narrow and wide rims (Randomly-Methylated, Methylated, Heptakis-(2,6-di-o-methyl), Hydroxypropyl, P125 Product, and β-CD Polymer). Each of these nine β-CD derivatives differs in substitution and in molecular weight. These data can be found in Tables 1, 2, and 3 for each of the nine derivatives. A description of these compounds is given below.

- β-CD

  Monomer, no substitution.

- 6-O-α-D-Glucosyl

  One glucosyl unit per β-CD molecule. Substitution occurs on the narrow rim of the β-CD.

- 6-O-Maltosyl

  This is a Japanese preparation with two glucopyranosyl residues attached to β-CD by an α-glycosidic bond. Substitution occurs on the narrow rim of the β-CD at the 6-position.[10]

- Hydroxypropyl

  Average substitution, on both the narrow and wide rims, of 6.2 $-CH_2CH_2OH$ groups per β-CD molecule.

- Randomly-Methylated

  A preparation from Wacker with an average substitution of 12.32 methyls per molecule. Substitution occurs on both the narrow and wide rims of the β-CD.[10]

- Methylated

  Average substitution, on both the narrow and wide rims, of 13 methyls per β-CD molecule.

- Heptakis-(2,6-di-o-methyl)

  Average substitution, on both the narrow and wide rims, of 12-14 methyls per β-CD molecule.

- P125

  Prepared by Josef Pitha; a product of the reaction of β-CD with epichlorohydrin. Nearly all of the species present have just one cyclodextrin moiety per molecule. Substituents are either 2,3-dihydroxypropyl or $-CH_2-CH(CH_2OH)$ units crosslinking the 2° and 3° hydroxyls. Substitution is on both the narrow and wide rims of the β-CD.[10]

- β-CD Polymer

  Substitution occurs on both the narrow and wide rims. The general formula for the polymer is: $[CD-(CH_2-CHOH-CH_2-O)_nX]_p$ where CD is the β-CD; X is H or CD; p is > 1, but < 6-8 and n is >1, but < 21. The percent of β-CD in the polymer is 55%.[11]

Table 1: Molecular Weights of β-CD Derivatives.

Type of β-CD	Source	Molecular Weight (g / mol)
β-CD	Cerestar	1135
6-O-α-D-Glucosyl	TCI	1297
6-O-Maltosyl	Pitha	1818
Hydroxypropyl	Amaizo	1408*
Randomly-Methylated	Pitha	1308*
Methylated	Cerestar	1317*
Heptakis-(2,6-di-o-methyl)	Aldrich	1331*
P125 Product	Pitha	**
β-CD Polymer	Cyclolab	~2000***

* Average molecular weight from ESI-MS data

** No data available

*** Value reported by Werner et al.[7]

Table 2: β-CD Derivatives Studied.

Substituent on β-CD	Average # of Subst. / CD	Substituents on Wide Rim
glucosyl	1	No
maltosyl	2	No
hydroxypropyl	6.2	Yes
randomly-methylated[1]	12.9*	Yes
methylated[2]	12.9*	Yes
2,6-di-o-methyl	14.9*	Yes
2,3-dihydroxypropyl or	2	Yes
$-CH_2-CH(CH_2OH)$		
polymer	< 6-8	Yes

[1] Gift from Dr. Josef Pitha

[2] Gift from Cerestar U.S.A, Inc.

* Based on ESI-MS data from the laboratory of Prof. Laszlo Prokai, University of Florida

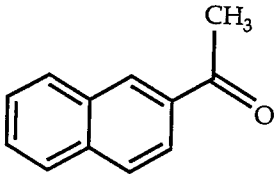
Table 3: Legend of Abbreviations for β-CDs.

Abbreviation	Type of β-CD	Source
β-CD	β-CD	Cerestar
6-O-G	6-O-α-D-Glucosyl	TCI
6-O-M	6-O-Maltosyl	Pitha
HP	Hydroxypropyl	Amaizo
RMP	Randomly-Methylated	Pitha
RMC	Methylated	Cerestar
HDM	Heptakis-(2,6-di-o-methyl)	Aldrich
P125	P125 Product	Pitha
β-CDP	β-CD Polymer	Cyclolab

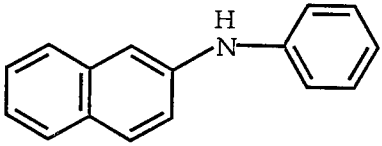
The binding of 2-acetylnaphthalene (2-AN) to methylated β-CD derivatives was also studied in the course of this work (Figure 4). CD complexes with naphthalene derivatives have been studied due to the enhancement of fluorescence, which accompanies the complex formation.[12] Naphthalene / β-CD complexes are generally 1:1 in stoichiometry. Complexes that are formed with naphthalene derivatives are also 1:1 with various β-CD derivatives. The 2-AN shows only static quenching when it binds to CDs; thus the Stern-Volmer plot can be utilized for determining K.[12] The goal for this work was to determine how the K values for 2-AN bound to substituted β-CDs compare with the K values for 2-AN bound to β-CD. The K value reported for the 2-AN / β-CD complex is 581 ± 6 by Fraiji et al.[12]

The final study conducted was with N-phenyl-2-naphthlamine (NP-2-NA) bound to unsubstituted β-CD (Figure 4). The goal for this work was to determine how the K value for NP-2-NA bound to β-CD compares with that for 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) bound to β-CD. In the case of 2,6-ANS, the closest molecule to our NP-2-NA sample, studied by Bright et al., the K value recorded was 2080 ± 20, (Figure 4).[13] We hoped to reach some conclusions about whether or not the SO_3H group in 2,6-ANS affects binding by comparing Bright's results with 2,6-ANS with our result with NP-2-NA, which does not have the SO_3H group.

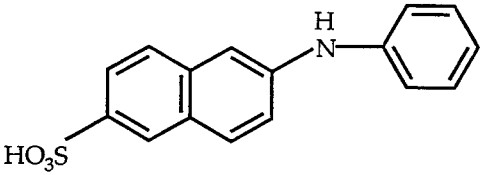
Figure 3: The Structures of Other Fluorophores Studied.



2-AN



NP-2-NA



2,6-ANS

EXPERIMENTAL

Reagents. The water used in all experiments was deionized, doubly distilled, and passed through a Millipore Milli-Q Water System. Pyrene (99+%), 2-acetylnaphthalene (99+%), N-phenyl-2-naphthalamine (97+%), KI (99+%) and 2,2,3,3,3-pentafluoro-1-propanol (PFP) were all obtained from the Aldrich Chemical Company, Inc. The β-cyclodextrin, was obtained from Cerestar U.S.A., Inc.

β-CD was recrystallized three times from water; 2-AN was recrystallized three times in ethanol and water; NP-2-NA was recrystallized three times in ethanol; pyrene was recrystallized twice from ethanol.

The β-CD Polymer was obtained from Cyclolab R&D Laboratory Ltd. of Budapest, Hungary. The following substituted β-CD's were all gifts from Josef Pitha: Randomly-Methylated-β-CD (RMP), 6-O-Maltosyl-β-CD, and the P125 Product (P125). These three derivatives were described in a letter sent to us by Dr. Pitha.[10] Table 4 gives sample composition information for RMP and P125. Other substituted β-CDs studied were Methylated-β-CD from Cerestar U.S.A., Inc., Heptakis-(2,6-di-o-methyl)-β-CD from Aldrich Chemical Company, Inc., Hydroxypropyl-β-CD from American Maize-Products Company (Amaizo), and 6-O-α-D-Glucosyl-β-CD from the TCI Company.

Table 4: Molar Percentages of Glucopyranosal Residues from Alditol Acetate Analyses.[10]

Substitution	RMP	P125
Unsubstituted	6.7	60.0
Cyclic Substitution 2^{o} and $3^{o\dagger}$		19.9
Substitution on 2^{o*}	14.7	4.6
Substitution on 3^{o*}	4.7	10.9
Substitution on 6^{o*}	14.5	1.9
Disubstitution on 2^{o}, 3^{o*}	7.0	
Disubstitution on 3^{o}, 6^{o*}	9.5	
Disubstitution on 2^{o}, 6^{o*}	27.8	
Trisubstitution on 2^{o}, 3^{o}, 6^{o*}	15.4	

† Substitution crosslinks adjacent hydroxyl groups

* The 2- and 3-positions are on the wide rim of the β-CD; the 6-position is on the narrow rim

(Based on results of analyses performed at Arrhenius Laboratory of Stockholm University)

Instrumentation. UV absorption spectra were recorded with a Hewlett-Packard 8453 Diode Array Spectrophotometer. The spectra were collected over a range of 300 - 500 nm for pyrene, 200 - 400 nm for 2-AN and 200 - 500 nm for NP-2-NA.

Fluorescence spectra were taken with a Photon Technology International QuantaMaster Spectrofluorometer using Felix, version 1.1 operating software.

Solutions containing pyrene were excited at 320 nm, and the emission spectra were collected from 360 – 500 nm with excitation slits at 2 nm and emission slits set at 1 nm. For the solutions containing 2-AN, the excitation wavelength was 340 nm, and the emission spectra were acquired from 370 – 600 nm with excitation and emission slits set at 2 nm. Finally, solutions made with NP-2-NA were excited at 300 nm, and emission spectra were taken from 350 – 580 nm with excitation and emission slits set at 2 nm. For all of the experiments conducted, the integration time used was 1 sec. Corrected emission spectra were obtained for all of these data.

Fluorescence lifetime data were collected using the Photon Technology International (PTI) LS-100 Lifetime Instrument. This instrument employs a nitrogen-filled, thyratron-gated flashlamp and an optical boxcar detector, which is a special photomultiplier that can be turned on at varying delay times after the lamp is flashed.[7] The excitation wavelength used was 337 nm, and the emission wavelength was 384 nm for the pyrene solutions. The fitting procedure for the lifetime decay curve allows for 1 – 4 exponentials, meaning that there can be between one and four emitting species in solution. In our work only single and double exponential fits were acquired. The decay curve is generated by an iterative procedure based on the Marquardt algorithm. In order to generate the curve fit, one is required to enter "an approximation" of two values for

each possible emitting species. These two values are A_i, the weighing factor for a given component, and τ_i, the fluorescence lifetime (ns) of the species, i. The true fluorescence decay (D(t)) for a single exponential fit can be summarized in equation (1). If the fluorescence lifetime decay that is collected requires other than a single exponential fit, the total fluorescence of each component in solution (F_i) can be defined mathematically in equation (2):

(1) $$D(t) = \Sigma A_i e^{-t/\tau_i}$$

(2) $$F_i = A_i\tau_i / \Sigma A_i\tau_i$$

The parameters used to determine whether or not the curve fit was acceptable were the appearance of the residuals, the χ^2 value, and Durbin-Watson (DW) parameter. A satisfactory fit is indicated by the residuals showing a random pattern, a χ^2 value as close to 1.000 as possible, and a D'W of > 1.7 for a single exponential, and > 1.75 for a double.[6]

Solutions. The pyrene, 2-AN, and NP-2-NA stock solutions were all prepared by placing a small amount of the fluorophore in a small brown bottle full of water and stirring overnight. The brown bottle was chosen as the container to protect each of the samples from light to minimize photodecomposition.[4] After stirring, the stock solutions were filtered using a 25 mm, 0.20 µm inorganic membrane syringe filter, Whatman Anotop 25. The first 20 mL of filtered solution was discarded to "saturate" the filter with the fluorophore to prevent all of the fluorophore in solution from being adsorbed on the filter. Absorbance measurements were taken of both the unfiltered and filtered stock solutions to ensure adequate absorbance values were seen. Each of these stock solutions were only used on the day of the preparation to guarantee the freshness of the solution and to avoid any complications from spoiled stocks.

The solutions of β-CD derivatives / pyrene were prepared by weighing out a certain amount of a solid β-CD and adding it to a known volume of the pyrene stock solution.[7] The mixture was then stirred for at least an hour to ensure the CD was completely dissolved. For quenching studies, increasing amounts of [I⁻] were added to the β-CDs / pyrene solutions. To observe any effect due to the added alcohol (PFP), pyrene solutions were made 1% in PFP (by volume). The fluorescence properties of these PFP-containing solutions were compared with those of solutions not containing PFP.

The 2-AN solutions contained methylated β-CD concentrations ranging from 0 – 0.00225 M, increasing by 0.00050 M. The lowest concentration of β-CD was 0.00025 M. In order to make these solutions, 1.00 mL of 2-AN stock was placed in six different 10.00 mL volumetric flasks; then the appropriate volume of 0.0025 M methylated β-CD solution was added and diluted with water to 10.00 mL. Each of these six solutions was prepared in duplicate for each of the three methylated β-CD derivatives. The mixtures were shaken and allowed to stand for at least one hour before measurements were taken.

For the experimentation with NP-2-NA, nine different sets of data were acquired, (Table 5). The first set was prepared by adding 1.00 mL of NP-2-NA stock to six different 10.00 mL volumetric flasks, then the appropriate volume of 0.010 M β-CD was added and diluted with water to 10.00 mL. In the second and third sets, again 1.00 mL of NP-2-NA stock was placed into six different 10.00 mL volumetric flasks, the appropriate volume of 0.0050 M β-CD was added and diluted to 10.00 mL. For the fourth and fifth sets, 5.00 mL of NP-2-NA stock were placed in ten different 10.00 mL volumetric flasks,

the appropriate volume of 0.010 M β-CD was added and diluted to 10.00 mL. In the sixth, seventh, eighth, and ninth sets, 5.00 mL of NP-2-NA stock were placed in thirteen different 10.00 mL volumetric flasks, the appropriate volume of 0.0050 M β-CD was added and diluted to 10.00 mL. All of the flasks, in every set of solutions, were shaken and allowed to stand for at least one hour before measurements were taken.

Table 5: NP-2-NA Solution Concentrations.

Data Set	NP-2-NA Stock Date	[β-CD] Range (M)
1	9-15	0.001 – 0.009
2	9-29	0.001 – 0.003
3	9-30	0.001 – 0.003
4	10-6	0.0002 – 0.0005
		0.00075 – 0.003
5	10-7	0.0002 – 0.0005
		0.00075 – 0.003
6	10-29	0.0002 – 0.0005
		0.0006 – 0.0015
7	11-2	0.0002 – 0.0005
		0.0006 – 0.0015
8	11-9 A	0.0002 – 0.0005
		0.0006 – 0.0015
9	11-9 B	0.0002 – 0.0005
		0.0006 – 0.0015

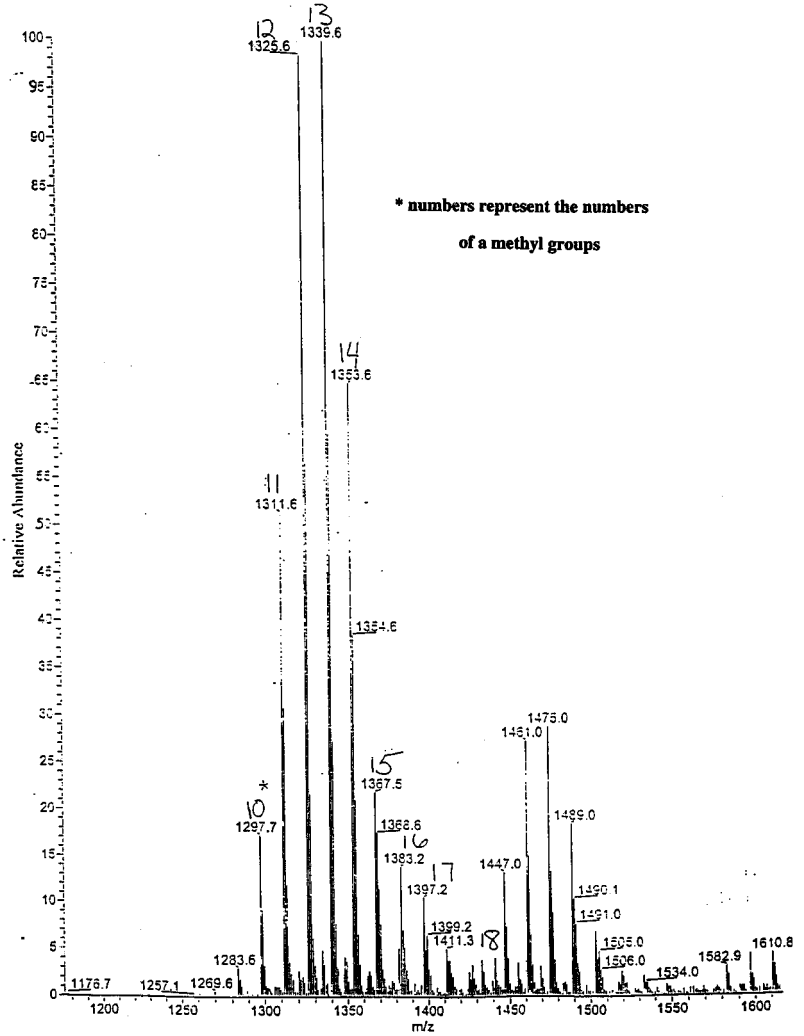
RESULTS

ESI-MS Data. Samples of all three of the methylated β-CD derivatives were collected and sent to Professor Laszlo Prokai, at the University of Florida, to obtain the Electrospray Ionization-Mass Spectra (ESI-MS) of the methylated β-CDs. The data collected showed that all three derivatives are significantly different from each other, in the range of methylation of the CD. The HDM sample showed a greater range of methylation from 14-17 methyl groups. The RMP sample showed a range of methylation from 10-18 methyl groups, both above and below the value reported by Dr. Josef Pitha. Finally, the RMC sample was shown to have a range of methylation from 10-17 methyl groups. There is an average of 12-14 methyl groups per CD, seen in all three derivatives (Figure 6). This accounts for about two-thirds of the -OH groups being replaced by methyl groups per CD. These data also reveal that the methylated β-CD derivatives are heterogeneous mixtures in nature and differ in the extent of methylation depending on the source. All of the data received can be seen in Figures 5 and 6 and Tables 6 and 7.[14]

Pyrene. The binding of pyrene with the nine different β-CDs was studied using the shape and lifetime of pyrene fluorescence and the effect on pyrene fluorescence of added iodide quencher and alcohol. Tables 8, 9, and 10 show the limiting I / III ratios for all the β-CDs as well as the other data collected throughout these pyrene experiments. The limiting I / III ratio for pyrene in the presence of β-CD and H_2O in our research is 0.71, while the value previously published by Muñoz et al. is 0.62.[5] The aggregation of β-CD on standing makes this a problematic value to confirm.

Previous work done by Warner et al. shows the presence of alcohols to have a profound affect on the I / III ratio for pyrene bound to β-CD.[15] In this work, PFP was added so that its volume was 1% of the total volume of the solution. It has been suggested that this alcohol "caps" the narrower end of the CD which protects the pyrene further from the water solvent and makes its binding environment more hydrophobic.[6,15] Alcohols can also participate in the complex, as a third component, which significantly enhances the formation constant and slows down the "in / out" rate of the complex.[15] The PFP essentially burrows into the binding site creating a more favorable environment for the pyrene. The PFP may in fact be positioned in and extend into the open end of the cavity, which in turn eliminates some of the water and reduces the interaction between the fluorophore and water in solution. Higher concentrations of PFP would have produced a slight increase in the fluorescence intensity, but not enough to drastically affect the I / III ratio.[8] The I / III ratios found for pyrene in the presence of the β-CDs and PFP can be seen in Table 8.

Figure 4: ESI-MS Data Example for RMP.



*** numbers represent the numbers of a methyl groups**

Table 6: ESI-MS Data for RMP.

Mass (g / mol)	# Methyl Groups	Intensity	Relative Abundance
1275*	10	1.93E+05	17.09
1289	11	5.89E+05	52.21
1303	12	1.12E+06	98.97
1317	13	1.13E+06	100.00
1331	14	7.42E+05	65.73
1345	15	2.49E+05	22.09
1360	16	1.56E+05	13.81
1374	17	1.18E+05	10.44
1376	18	5.58E+04	4.95
	Total Relative Abundance =		**385.29**

* The ESI-MS data show a peak at 1298 for this molecular ion. The ion is $(M + Na)^+$. So, subtracting the mass of Na from the indicated mass gives the mass of M.

Table 7: Determining Molecular Weight from ESI-MS Data for RMP.

Mass (g / mol)	Fraction of Rel. Abund.*	Frac. x Mass (g / mol)
1275	0.0444	56.61
1289	0.1355	174.7
1303	0.2569	334.7
1317	0.2595	341.8
1331	0.1706	227.1
1345	0.0573	77.07
1360	0.0358	48.69
1374	0.0271	37.24
1376	0.0128	17.61
	Sum (Frac. x Mass) =	**1316****

* Frac. = rel. abund. at each mass / total rel. abund.

** Sum of all Frac. x Mass = molecular weight

Figure 5: ESI-MS Data.

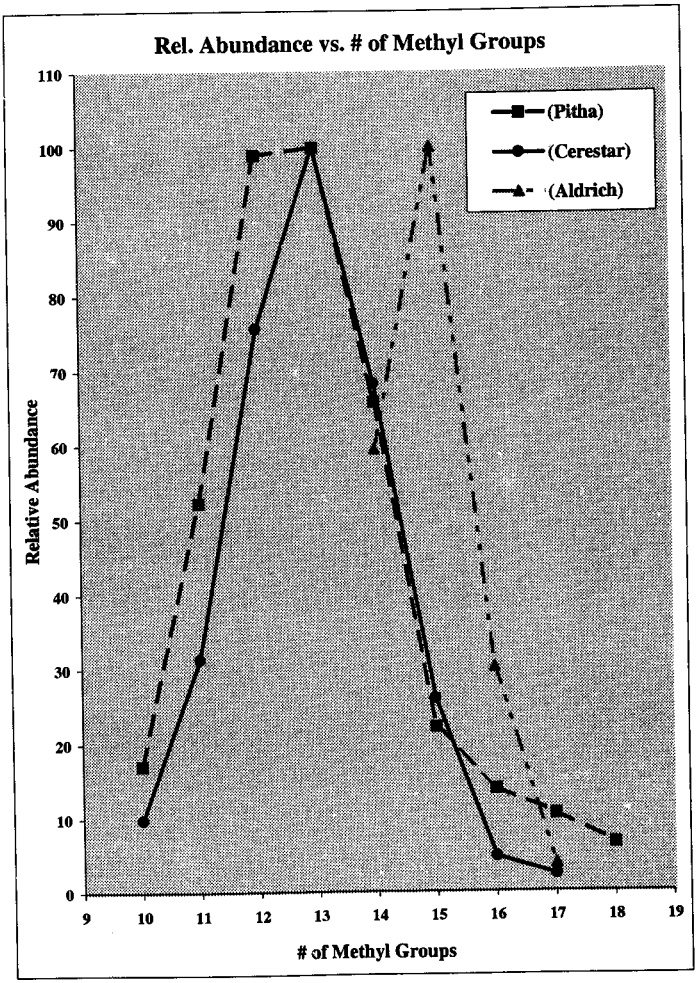


Table 8: β-CDs / Pyrene Complexes I / III Ratio Data.

Type of β-CD	Conc. mg/mL (M)	I / III* (H_2O)	I / III* (1% PFP)	I / III* Quenched (I^-)
β-CD†	17 (0.015)	0.72	0.52	0.64
	20 (0.018)	0.69		
6-O-G	52 (0.040)	0.87		0.79
	58 (0.045)	0.95	0.44	
6-O-M	27 (0.015)	0.92		
	40 (0.022)	0.89	0.46	0.83
	50 (0.028)		0.46	
	60 (0.033)	0.89		
HP	4 (0.0028)	1.58	1.63	1.56
	17 (0.012)	1.57		
RMP	20 (0.015)	1.58		1.56
	50 (0.038)	1.54	1.17	
RMC	50 (0.038)	1.49	1.16	1.43
HDM	50 (0.038)	1.55	0.77	1.51
P125	50	1.61	0.95	
	77	1.76	0.77	1.43
β-CDP	5	1.76	1.72	1.75
	10	1.72		

† Due to the insolubility problem, it is difficult to get repeatable results

* Data show results from multiple experiments, average value reported

The next parameter that was investigated with these β-CDs / pyrene solutions was the effect of iodide quenching. Increasing amounts of iodide ion were added to these solutions to determine the limiting I / III for pyrene in the presence of β-CDs and iodide, (Table 8). Using the resulting data, the area under each curve could be collected. This area data was then used to prepare F_o / F vs. [Iodide] plots, with F_o, the area under the curve with no iodide, and F, the areas under all of the other curves with increasing amounts of iodide, (Figure 7).

(3) $$F_o / F = 1 + K_q[\Gamma]$$

The slope of the trendline fit from the resulting data points determines the K_q value. An example of a resulting K_q value can be seen in Figure 7. A summary of the resulting K_q values can be seen in Table 9. Other ways of viewing the data collected throughout the course of the quenching part of this experiment is to look at the limiting I / III as a function of total [Γ] added, (Table 10).

Table 9: Stern-Volmer Plot Data (F_o / F vs. [I⁻]) for All β-CD Derivatives.

Type of β-CD	Sample	Slope	Intercept	R^2	Ave. K_q* (Ave. Dev.)
β-CD	1	22	1.25	0.828	22 (± 0)
	2				
6-O-G	1	112	1.16	0.991	112 (± 0)
	2				
6-O-M	1	22	1.14	0.970	18 (± 4)
	2	14	1.04	0.971	
HP	1	156	1.17	0.990	135 (± 21)
	2	114	1.03	0.998	
RMP	1	331	1.10	0.998	320 (± 11)
	2	309	0.84	0.983	
RMC	1	192	0.91	0.994	210 (± 18)
	2	228	0.94	0.998	
HDM	1	67	1.12	0.985	67 (± 1)
	2	66	1.12	0.984	
P125	1	206	1.46	0.991	206 (± 0)
	2				
β-CDP	1	114	1.13	0.990	116 (± 2)
	2	117	1.09	0.996	

* K_q is found directly from the slope of the trendline fit for each data set using area under the curve for F_o and F

Figure 6: Stern-Volmer Quenching Plot Example.

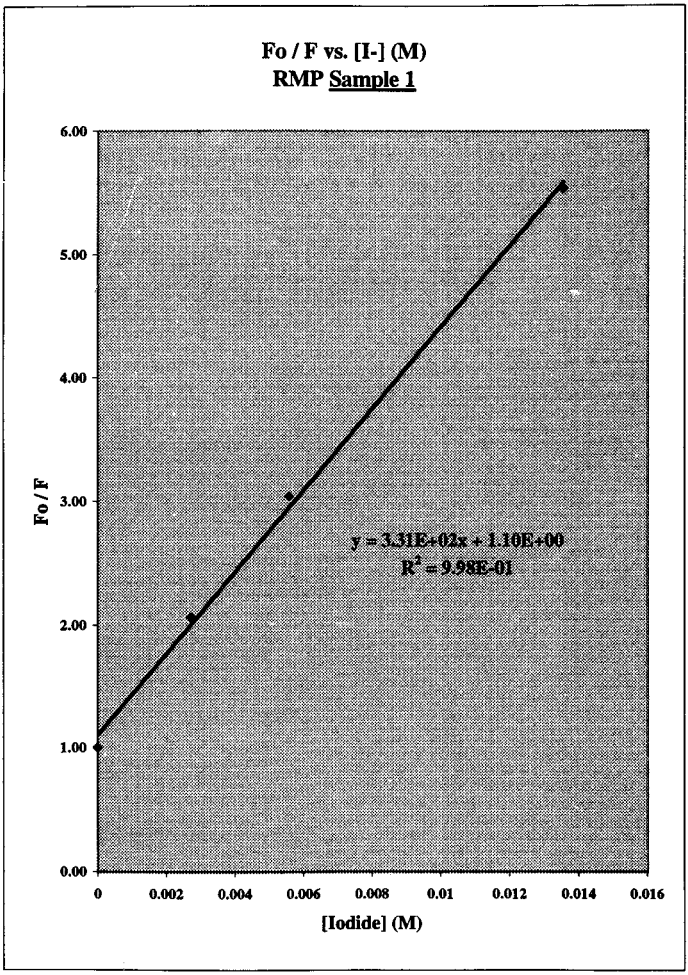


Table 10: Limiting Pyrene I / III Ratio for
All β-CDs as a Function of Total [I⁻] Added.

Type of β-CD	Ave. I / III (H_2O)	Limiting [I⁻] (M)	Ave. I / III† (at Limiting [I⁻])
β-CD**	0.71	0.0235	0.64
6-O-G**	0.91	0.0283	0.83
6-O-M*	0.90	0.0269	0.83
HP*	1.58	0.0236	1.56
RMP*	1.56	0.0131	1.56
RMC*	1.49	0.0151	1.43
HDM*	1.55	0.0267	1.51
P125**	1.69	0.0313	1.43
β-CDP*	1.74	0.0223	1.75

† Since there was minimal I / III change between 0 – 0.0268 M [I⁻] and virtually none after the limiting [I⁻], the I / III ratios are confirmed

* Result of one run of data

** Result of two runs of data averaged

Fluorescence lifetime (τ) measurements were made for the pyrene in the presence of all of the β-CDs, using the solution condition that produced the limiting I / III. A sample lifetime plot can be seen in Figure 8, while Table 11 contains the lifetime data that were collected in the course of the experiments. The value in parenthesis for τ_2 is the fraction of the fluorescence (F_2) that is due to this particular emitting species. For all of our β-CD data a single exponential fit gave a τ similar to the τ_2 value found for the double exponential fit. Moreover, the fraction (F_2) is near unity in the case of the double exponential fit.

Figure 7: Fluorescence Lifetime Sample Plot for Pyrene in the Presence of β-CD.

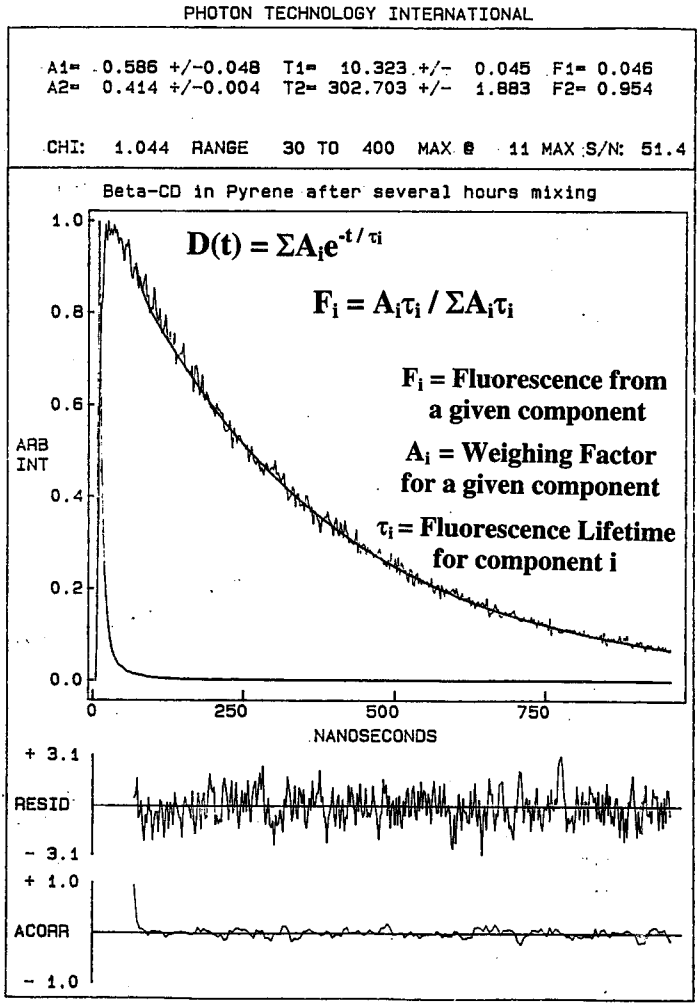


Table 11: β-CDs / Pyrene Complexes Lifetime Data.

Type of β-CD	Conc. mg/mL (M)	τ^{*}	τ_2^{**}
β-CD	17 (0.015)	303	303 (0.954)
6-O-G	52 (0.040)	280	291 (0.988)
	58 (0.045)	279	298 (0.975)
6-O-M	40 (0.022)	303	315 (0.97)
	60 (0.033)	306	Short
HP	4 (0.0028)	209	212 (0.978)
	17 (0.012)	221	222 (0.996)
RMP	50 (0.038)	207	
RMC	50 (0.038)	209	216 (0.944)
HDM[†]	50 (0.038)	247	249 (0.986)
P125	50	214	213 (0.97)
	77	209	213 (0.957)
β-CDP	5		251 (0.97)

[†] Average of two measurements

[*] Result from single exponential fit

[**] Result from double exponential fit (longer component)

2-Acetylnaphthalene (2-AN). Fluorescence emission spectra were also used to study the binding of 2-AN with three of the β-CD derivatives: HDM, RMC, and RMP. 2-AN is known to form a 1:1 inclusion complex with β-CD.[15] When it binds inside the hydrophobic cavity of a CD, its fluorescence is quenched.[6] Since we know that the resulting quenching is static, and a 1:1 complex is seen, the intensity information should conform to the Stern-Volmer equation.[16,17]

(4) $$F_o / F = 1 + K[CD]$$

Fraiji et al. showed that the slope value (K) form the Stern-Volmer equation for 2-AN quenched by β-CD to be 581 ± 6.[6,12] The procedure described in this article was followed for each of the three β-CD derivatives and the data collected were tabulated in Figure 9 and Table 12.

Figure 8: Stern-Volmer 2-AN Plot Example.

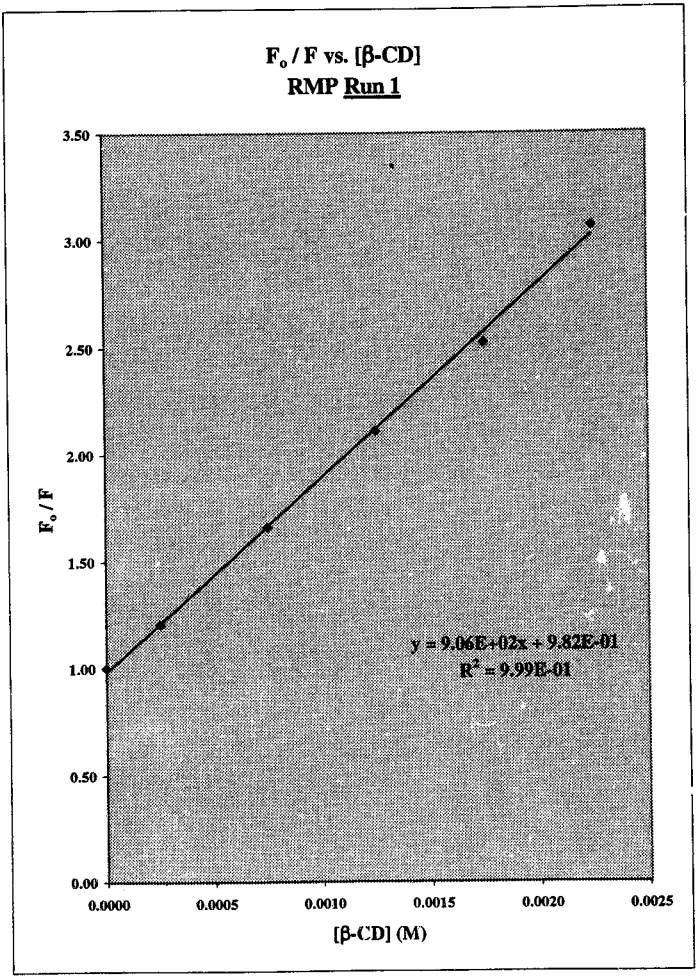


Table 12: Stern-Volmer Plot Data (F_o / F vs. [β-CD]) for 2-AN in the Presence of Methylated β-CD Derivatives.

Type of β-CD	Run	K* (Rate Constant)	R^2
HDM	1	1190	0.974
	2	1190	0.997
		Ave. K = 1190	**Ave. R^2 = 0.986**
		Ave. Dev. = ± 0	**Ave. Dev. = ± 0.012**
RMC	1	1600	0.962
	2	1030	0.993
		Ave. K = 1345	**Ave. R^2 = 0.978**
		Ave. Dev. = ± 285	**Ave. Dev. = ± 0.016**
RMP	1	906	0.999
	2	1000	0.990
		Ave. K = 953	**Ave. R^2 = 0.995**
		Ave. Dev. = ± 47	**Ave. Dev. = ± 0.005**

* Slope using area under curve for F_o and F

N-Phenyl-2-naphthlamine (NP-2-NA). Fluorescence spectroscopy was once again utilized in the analysis of NP-2-NA binding to β-CD. Several sets of solutions were prepared to test the effect of varying concentrations of β-CD on the fluorescence of NP-2-NA. Data collected were plotted as 1 / Area under the curve vs. 1 / [β-CD]. Plots of this type are designated as double reciprocal plots. The equation showing these plots is as follows, where S is NP-2-NA, C_S is the analytical concentration of S, B is β-CD, C_B is the analytical concentration of B and SB is the β-CD / NP-2-NA complex:

(5) $$K = [SB] / [S][B]$$

(6) $$K = [SB] / (C_S - [SB])(C_B - [SB])$$

$$\text{Since } C_B >> [SB]$$

(7) $$K = [SB] / [C_S - [SB])\, C_B$$

$$\text{Let } [SB] = kF$$

where k is a constant and F is the fluorescence intensity. Then:

(8) $$K = kF / (C_S - kF)C_B$$

(9) $$K(C_S - kF)(C_B) = kF$$

(10) $$(KC_S - KkF)C_B = kF$$

(11) $$KC_SC_B - KkFC_B = kF$$

(12) $$KC_SC_B = kF + KkFC_B$$

(13) $$KC_SC_B = kF(1 + KC_B)$$

(14) $$KC_SC_B / (1 + KC_B) = kF$$

(15) $$1 / kF = (1 + KC_B) / KC_SC_B$$

(16) $$1 / kF = 1 / KC_SC_B + 1 / C_S$$

(17) $$1 / F = k / KC_SC_B + k / C_S$$

(18) $$\text{Intercept / Slope} = (k / C_S) / (k / KC_S) = K$$

The linear region for this plot is characteristic of 1:1 complex formation. If the plot is not well defined as a single straight line, and can be best described by two linear parts, then two different K values can be found. The initial linear portion at low concentrations of the β-CD defines K_2 for the 2:1 complex, while the second linear portion at high concentrations accounts for K_1 for the 1:1 complex.[13] All of the data collected can be seen in Figures 10 and 11 and Tables 5 and 13.

Figure 9: Double Reciprocal NP-2-NA Plot for Low Concentrations of CD. (Finding K_2)

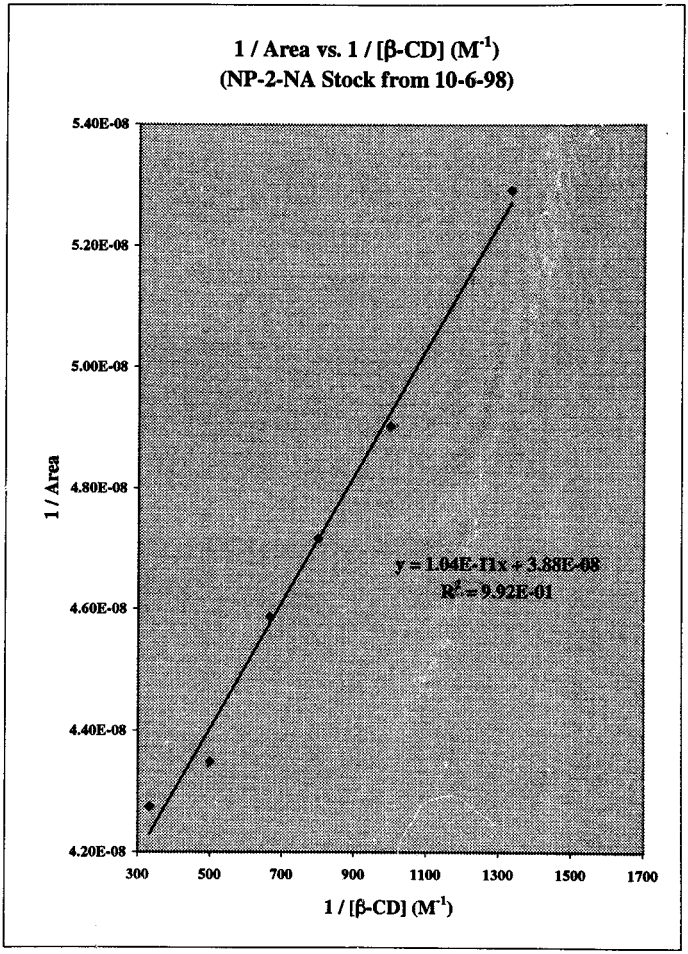


Figure 10: Double Reciprocal NP-2-NA Plot for High Concentrations of CD. (Finding K_1)

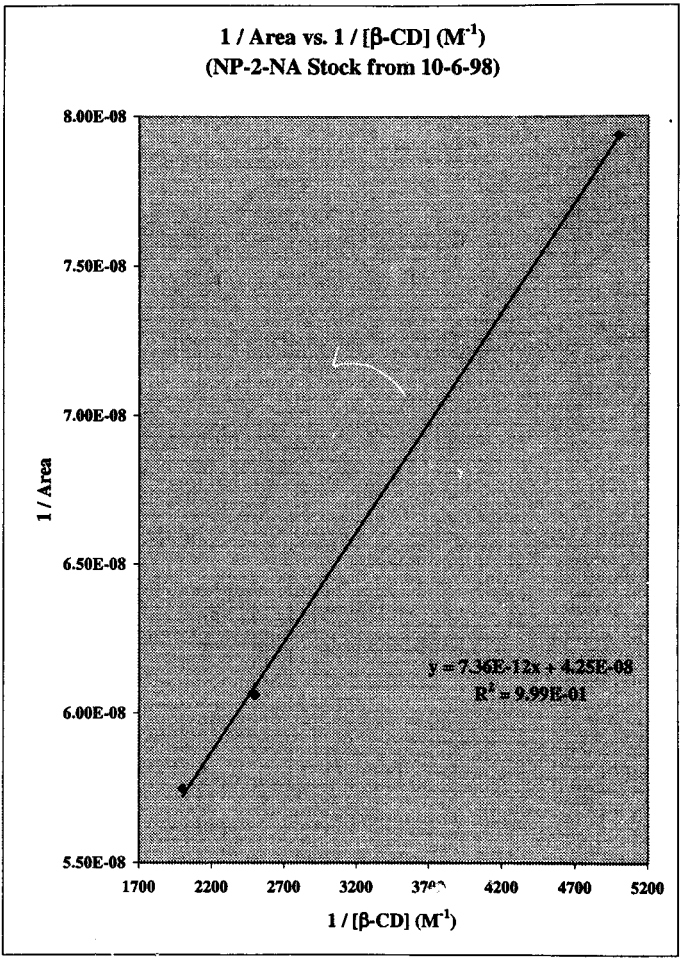


Table 13: Double Reciprocal Plot Data (1 / Area vs. 1 / [β-CD]) for NP-2-NA with Varying Concentrations of β-CD.

Data Set	CD Conc. End	Slope	Intercept	R^2	K_1*	K_2*
1	Low†	2.76E-11	7.15E-08	**0.995**		**2590**
2	Low	2.45E-11	1.12E-07	0.905		4570
3	Low	2.50E-11	8.94E-08	0.908		3370
4	High††	7.36E-12	4.25E-08	**0.999**	**5770**	
	Low	1.04E-11	3.88E-08	**0.992**		**3730**
5	High	4.22E-12	3.02E-08	**0.999**	**7160**	
	Low	7.65E-12	2.53E-08	**0.966**		**3310**
6	High	1.80E-12	2.49E-08	0.908	13800	
	Low	5.58E-12	1.65E-08	**0.996**		**2960**
7	High	1.39E-12	2.79E-08	0.799	20100	
	Low	7.88E-12	1.66E-08	0.923		2110
8	High	2.26E-12	2.25E-08	**0.977**	**9960**	
	Low	4.11E-12	1.83E-08	0.882		4450
9	High	2.78E-12	2.59E-08	0.921	9320	
	Low	5.90E-12	1.95E-08	**0.982**		**3310**
		Ave. K value with $R^2 > 0.950$ =			**7630**	**3180**
				Ave. Dev. =	**± 1550**	**± 320**

* K = intercept / slope

† Concentration range of 0 - 1700 M^{-1} = K_2

†† Concentration range of 1700 - 5200 M^{-1} = K_1

DISCUSSION

ESI-MS Data. The extent of methylation could be determined from the ESI-MS data obtained for samples of all three of the methylated β-CD derivatives. The data show that all three derivatives are significantly different from each other, in the extent of methylation of the CD. All of the data received can be seen in Figures 5 and 6 and Tables 6 and 7.[14]

Pyrene. The 6-O-M and the 6-O-G derivatives were the only two β-CD derivatives studied having substitution only on the narrow rim of the CD cavity. These derivatives are also the only two that exhibit spectral behavior that resembles that seen when pyrene binds to unsubstituted β-CD. For example, the limiting I / III ratios in H_2O (Table 8) of pyrene in the presence of β-CD, 6-O-M, and 6-O-G are found to be 0.71, 0.89, and 0.87, respectively. The I / III ratio for free pyrene in water is 1.96, which is considerably higher than the I / III ratios seen for these two derivatives and the β-CD monomer. These lower I / III values indicate that pyrene is experiencing a rather hydrophobic environment in each case.

Table 8 also contains pyrene I / III results with the other β-CDs, all of which have some wide rim substitution. These derivatives have larger I / III ratios in H_2O, ranging from 1.49 – 1.69, in comparison to those for β-CD, 6-O-M, and 6-O-G, which can be seen above. As a consequence, it can be assumed that the nature of β-CD complexation with pyrene is dependent on whether or not the wide rim is substituted.

Warner et al. reported a I / III value of 0.38 for pyrene in β-CD and 1% PFP and suggest that the PFP "caps" the narrower end of the CD cavity protecting the pyrene

further from the water solvent and making the binding environment more hydrophobic.[6,15] The I / III ratios found for pyrene in the presence of 1% PFP and β-CD, 6-O-M, and 6-O-G were 0.49, 0.46, and 0.44, respectively, (Table 8). In contrast, when 1% PFP was added to pyrene in the presence of HP, RMP, RMC, HDM, P125, and β-CDP, the I / III ratios found were 1.63, 1.17, 1.16, 0.77, 0.86, and 1.72, respectively, (Table 8). From these data it is clear that there is different behavior between the pyrene and the β-CD derivatives without wide rim substitution in comparison to those with wide rim substitution.

Another parameter that was investigated with these β-CDs / pyrene solutions was the effect of I^- quenching (Tables 8, 9, and 10). The iodide quenching experiment was conducted in order to take a closer look at the range of binding sites for the β-CD derivatives, especially the methylated β-CDs. A more exposed environment leads to a higher I / III ratio. If there is a range of binding or methylation, the I / III ratio should be reduced as quenching occurs because the less exposed pyrene should be quenched last. Since there was not a noticeable lowering of the I / III values found for the β-CDs, including the three methylated derivatives, the conclusion that a similar environment for the β-CDs regardless of the extent of methylation or substitution could be made. The quenching study was used strictly to examine the environment that pyrene experiences when binding to the several β-CD derivatives studied, not to distinguish between a 2:1 or 1:1 complex formation.

The I / III ratio results seen for all of the β-CDs in the presence of 1% PFP are consistent with the trends described earlier, with one exception: P125. The average I / III ratios found for P125 in the presence of H_2O and 1% PFP are 1.69 and 0.86, respectively.

Table 4 indicates that very little 6-substitution or narrow rim substitution is present for the P125 sample. Using this fact, a speculation about a possible reason for the behavior with P125 can be made. It is possible that the PFP is able to penetrate or bind with the narrow rim of the P125 more readily than for the other β-CDs due to the lower degree of substitution on the narrow rim. As a result, the environment that pyrene experiences becomes more hydrophobic, less exposed, which in turn decreases the I / III ratio in the presence of 1% PFP.

The I / III ratio results for I^- quenching also show P125 to be an exception. All of the other β-CDs exhibit very little change between the I / III ratio in H_2O and in I^-, but the P125 sample again deviates from the trend. The average I / III ratios found for P125 in the presence of H_2O and I^- are 1.69 and 1.43, respectively. There is the possibility that a range of binding environments for pyrene exists for P125. Twenty percent of the P125 molecules have bridged 2,3-OH groups. It is possible that these P125 derivatives bind pyrene differently than the eighty percent without such bridging. Since there was no significant change in the I / III ratios for the methylated β-CDs, it would lead one to believe that the pyrene binding sites are very similar among the various methylated components of these heterogeneous samples.

Fluorescence lifetime measurements were made for the pyrene in the presence of all of the β-CDs, using solutions that produced the limiting I / III. Table 11 shows the lifetime data that were collected in the course of the experiment. The lifetime, τ, for free pyrene in water is 130 ns, which can be used for comparison to the other lifetime data collected throughout this experiment.[4] The first thing that is clear from all of the collected fluorescence lifetime data is that τ is lengthened when the pyrene is bound to all

the β-CD derivatives. This increased lifetime is a result of a more buried, less exposed environment; the pyrene is being hidden from the oxygen quencher present in the water surroundings. Since there is a longer lifetime with respect to the β-CD derivatives with no substitution on the wide rim, it can be concluded that the pyrene is in a more hidden environment than in the complexes formed with the β-CD derivatives having substitution on the wide rim. Reported lifetime data for β-CD is $\tau = 141$ ns and $\tau_2 = 363$ ns, which is comparable to those found for 6-O-M and 6-O-G.[7] These similar lifetime values support the I / III data, indicating that β-CD / pyrene, 6-O-M / pyrene, and 6-O-G / pyrene form similar complexes.

Another noticeable difference between the 6-O-M, 6-O-G, and the other derivatives is the lifetime value. The found lifetimes for 6-O-M are $\tau = 303$ ns and $\tau_2 = 315$ ns, while for 6-O-G they are $\tau = 280$ ns and $\tau_2 = 291$ ns, (Table 11), which are considerably larger than the lifetime of free pyrene. All of the other derivatives show an increase in lifetime also, but not as drastic as the 6-O-M and 6-O-G. Fluorescence lifetime values for the β-CD derivatives with substitution on the wide rim range from 207 – 249 ns, (Table 11). The τ values for the derivatives with substitution on the wide rim are shorter than those for the derivatives without substitution, which is consistent with a more exposed environment in comparison with β-CD, 6-O-M, and 6-O-G.

The proposal of the binding structure for β-CD and pyrene is a 2:1 complex, where two β-CD molecules engulf one pyrene molecule in a "clam shell" or "sandwich" structure. Since all of our data indicate that β-CD / pyrene is forming the same type of complex for the 6-O-M / pyrene and 6-O-G / pyrene complexes, we suggest that these complexes are also 2:1. The maltosyl groups in 6-O-M and the glucosyl group in 6-O-G

are only on the narrow rim of the CD cavity, so they do not interfere with binding and do not prevent the 2:1 complex from emerging. Our evidence, therefore indicates that a 2:1 complex is formed when there is no substitution on the wider rim of the β-CD molecule. If some substituents are present on both the narrow and wide rims or only the wide rim of the β-CD molecule, then a 1:1 complex is likely to result.

Thus, in conclusion, the β-CD derivatives with no substitution on the wide rim: β-CD, 6-O-G, and 6-O-M, have a lower I / III in H_2O, a lower I / III in the presence of 1% PFP, and longer lifetime. The spectral behavior of pyrene with these CDs is consistent with a more hydrophobic, less exposed environment, which is as expected for a 2:1 complex. The two structures seen in Figure 3 clearly show that substituents on the wide rim would sterically hinder the binding. There is no way to determine which of the two complexes is formed, "clamshell" or "sandwich," but our data are consistent with the idea that the 2:1 complex does indeed exist for those CDs with no substitution on the wide rim.

The β-CD derivatives with substitution on the wide rim: HP, RMP, RMC, HDM, P125, and β-CDP have a higher I / III in H_2O, a higher I / III in the presence of 1% PFP, and shorter lifetime. The spectral behavior of pyrene with these CDs is consistent with a less hydrophobic, more exposed environment, which is the expected trends if a 1:1 complex is formed in these cases. Thus, pyrene can only form a 2:1 complex with a β-CD derivative that contains no substitution on the wide rim. It is likely that a 1:1 complex will form in any other situation.

2-Acetylnaphthalene (2-AN). Fluorescence emission spectra were also used to study the binding of 2-AN with three of the β-CD derivatives. Fraiji et al. showed that the slope value (K) from the Stern-Volmer equation for 2-AN quenched by β-CD to be 581 ± 6.[6,12] The values for K that were determined in our study were 1190 ± 0 for HDM, 1345 ± 285 for RMC, and 953 ± 47 for RMP (Table 12). This increase in K value is consistent with the fact that the presence of the methyl groups allows for an expanded hydrophobic cavity. Therefore, there is a noticeable difference in the K values for 2-AN bound to substituted β-CDs compared to the K values previously reported for 2-AN bound to β-CD.

N-Phenyl-2-naphthlamine (NP-2-NA). Fluorescence spectroscopy was once again utilized in the analysis of NP-2-NA. Bright et al. has reported that the K value for 2,6-ANS of about 2080 ± 20, where we are seeing an average K_1 value of 7630 ± 1550 and an average K_2 value of 3180 ± 320 for NP-2-NA (Table 13). The only difference between the NP-2-NA molecule that we studied and the 2,6-ANS molecule studied by Bright et al., was the addition of a SO_3H group in the 2,6-ANS.

The question that was investigated entailed whether or not the SO_3H group in the 2,6-ANS affects the binding. After careful consideration of the data, it can be concluded that having an attached SO_3H group causes different complexes to form than those seen without it. It seems that the SO_3H results in 1:1 β-CD binding. Bright et al. only report a 1:1 complexing pattern between the β-CD and 2-ANS, with no mention of a possible 2:1 complex. Our data show that NP-2-NA forms a 2:1 β-CD / NP-2-NA complex, as well as a 1:1 complex. It seems NP-2-NA likes a more buried, less exposed environment, which is consistent with the 2:1 complex. The greater water solubility with the added SO_3H

group allows the 1:1 complexation to be sufficient to make the 2,6-ANS "happy," while the NP-2-NA requires less exposure to be "happy." Both of our K values are greater than those reported by Bright for 2-ANS. Thus, with greater binding constants for the NP-2-NA, it can be shown that the SO_3H does not enhance binding in either case, since 2-ANS does not even form a 2:1 complex as NP-2-NA does.

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