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The Use of Naphthalene Fluorescence Probes to Study the Binding Sites on Cyclodextrin Polymers Formed From Reaction of Cyclodextrin Monomers with Epichlorohydrin

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Abstract. Three naphthalene-based fluorescence probes were used as guest molecules to study host/guest binding with cyclodextrin (CD) polymer hosts prepared by treating α-, β-, or γ-cyclodextrin monomers with epichlorohydrin. The fluorescence data indicate that the binding interaction is much stronger for the probes with the CD polymers than with the CD monomers. Moreover, the fluorophore binding site on the CD polymers is also more hydrophobic than that on the CD monomers. Fluorescence lifetime data from one of the bound probes (2-(*N*-methylanilino) naphthalene-6-sulfonic acid) suggest that more than one type of binding site may exist on the CD polymers with this probe. A comparison of fluorescence data using different molecular weight ranges of the CD polymers appear to rule out the possibility of a 1 : 2 host/guest complex, where the two CD units come from the same polymer chain.

Key words: Cyclodextrin polymers, fluorescence probes, fluorescence quenching.

Introduction

Over the past decade considerable effort has been made to understand the nature and effects of the binding of organic molecules to a series of molecules called cyclodextrins (CD). These torroidal-shaped molecules are composed of D(+)-glucopyranose units joined by α-(1,4)-linkages [1]. Three CD molecules are commonly studied, α-CD, β-CD and γ-CD, which consist of six, seven and eight glucopyranose units, respectively. These molecules have a height of 0.78 nm and internal diameters of 0.57 nm for α-CD, 0.78 nm for β-CD and 0.95 nm for γ-CD [2].

Organic molecules form inclusion complexes by binding in the CDs' internal cavities, which are less polar than the surrounding aqueous solution. The great interest in organic molecule : CD complexes arises from the variety of potential applications of these complexes that have been identified by several investigators. These include solubility enhancement of organics in water [3], improvement in

analytical separations through complex formation with CD-bonded phases [4], and CD-modified mobile phases in HPLC [5], as modifiers of photochemical behavior [6], to control dye aggregation equilibria [7], and for models of protein complexes [8].

One restriction on the use of cyclodextrins is their limited solubility, which is lowest for the most versatile of the three CDs, β-CD (0.014–0.016 M). As a consequence, attempts have been made to modify β-CD chemically to improve its solubility. These efforts include the synthesis of water-soluble CD polymers. While several ways have been reported to produce these polymers, the most common procedure involves the use of epichlorohydrin. The resulting CD polymers are a polydisperse mixture containing CD units joined by repeating glyceryl linkers ($-(CH_2-CHOH-CH_2-)_n$). All three of the CD polymers (α-CDP, β-CDP and γ-CDP), which are now available commercially, are highly water soluble.

Several papers have appeared which compare the guest binding ability of β-CDP with that of the parent β-CD [9–14]. However, no systematic comparison of the guest binding ability of all three CDPs with their respective monomer CDs has yet been reported. Our work, reported here, is an attempt to provide this comparison, using three naphthalene-based fluorescence probes: 2-acetylnaphthalene (2-AN), 1-dimethylamino-5-naphthalene sulfonamide (1,5-DNSA) and 2-(N-methylanilino)-naphthalene-6-sulfonic acid, sodium salt (2,6-MANS). The 2-AN probe forms 1 : 1 inclusion complexes with all three of the monomer CDs, including a moderately strong complex with β-CD [15]. The 1,5-DNSA forms a weak 1 : 1 complex with β-CD, while 2,6-MANS forms a very strong 1 : 1 complex with β-CD and a moderately strong 2 : 1 complex (2 β-CDs to one 2,6-MANS) [16]. Thus, the three probes provide a range of binding interactions with the monomer CDs.

The major issue we address in this work is whether the binding sites in the CDPs are similar to those in the CDs. Specifically, we hoped to determine whether the glyceryl linkers affect guest binding and whether there is evidence for cooperative binding interactions between CD units on the same polymer chain.

Experimental Section

The water used in all experiments was deionized, doubly distilled, and passed through a Millipore Milli-Q Water System. The 2-AN and the 1,5-DNSA fluorophores and the three unsubstituted cyclodextrins (α-CD, β-CD and γ-CD) were obtained from Aldrich Chemical Company, Inc. The 2-AN was recrystallized twice from both hexane and ethanol, while β-CD was recrystallized from water. The 2,6-MANS fluorophore was obtained from Molecular Probes, Inc. and used without purification.

The three CD polymers were obtained from Cyclolab R&D Laboratory Ltd. of Budapest, Hungary. The general formula of the polymers is

$$[CD-(CH_2-CHOH-CH_2-O)_nX]_p$$

where CD is α-, β-, or γ-CD; X is H or CD; p is > 1 but < 6–8 and n is > 1 but < 18 (α-CD), < 21 (β-CD), < 24 (γ-CD). The average n value is 12–15, and the reported %CD is 54 (α-CDP), 55 (β-CDP) and 57 (γ-CDP). GPC data from Cyclolab indicate a broad range of molecular weights for these polydisperse CD polymers, up to a maximum of about 11,000. The GPC data show two prominent peaks for β-CDP at about 2000 and 9–10,000; the former is due to polymers containing a single CD unit per polymer chain, while the latter is from polymers containing 4–5 CD units per polymer chain [12]. GPC runs for the α-CDP and γ-CDP polymers show the same two peaks, but the latter is less pronounced than observed with the β-CDP polymer.

Absorption spectra were recorded using a Hewlett-Packard 8454A Diode Array Spectrophotometer, while fluorescence spectra were recorded using a Perkin-Elmer Lambda 5B Spectrofluorometer. This instrument automatically corrects emission spectra for the wavelength dependence of the emission monochromator and detector combination. Slits of width 10 nm were used on both the excitation and emission monochromators. The excitation wavelengths were 340 nm for 2-AN, 324 nm for 1,5-DNSA and 350 nm for 2,6-MANS.

Fluorescence lifetime data were obtained with an LS 100 fluorescence lifetime system from Photon Technology International, Inc. This instrument employs a nitrogen-filled, thyratron-gated flashlamp and an optical boxcar detector. The excitation wavelength employed was 337 nm and the emission wavelengths were chosen to be near the wavelength maxima for the particular 2,6-MANS : CD complex being studied. A dedicated computer provides analysis of the luminescence data by convoluting the lamp scatter peak with a delta-function-generated decay until an appropriate fit is obtained to the observed fluorescence decay curve. The fitting procedure, which can employ from one to four exponentials in the generated decay curve, uses an iterative procedure based on the Marquardt algorithm. A single exponential fit to the lifetime data was deemed appropriate if a double exponential fit gave no improvement in the χ^2 parameter and autocorrelation function. When these conditions existed, the two lifetime values from the double exponential fit were very close to the single exponential fit lifetime.

Stock solutions of fluorophores were prepared by adding a small amount of a given solid fluorophore to water (2-AN) or to 0.1 M phosphate buffer, pH 6–7 (1,5-DNSA, 2,6-MANS) and stirring the solution overnight. The resulting stocks were then passed through 0.2μ disposable syringe filters (Anotec). Solutions for fluorescence measurements were prepared by diluting these stocks with water (2-AN) or 0.1 M phosphate buffer, pH 6–7 (1,5-DNSA, 2,6-MANS) to give fluorophore absorbances of 0.02–0.03 (1 cm cell) at the exciting wavelengths. The required concentration of CD or CDP was obtained by either adding a weighed amount of the solid CD or CDP to an accurately known volume of the fluorophore solution or by dilution from a concentrated stock solution of a given CD or CDP. The concentrations of all CD and CDP solutions are expressed in CD molarity. For the polymers, CD molarities were determined using the %CD values supplied by

Cyclolab (see above). Solutions were allowed to stand for up to several hours to ensure equilibration before measurements were made. Temperature control during the fluorescence experiments was provided by circulating water at a constant temperature (23°C) through a thermostated cell block using a Neslab Endocal Refrigerated Circulating Bath.

To determine how the spectral data would be affected if the heterogeneity of the native CDP mixtures is reduced, we dialyzed α-CDP and β-CDP using dialysis tubing (Spectra/Por CE Molecularporous Dialysis Membrane from Spectrum) having a molecular weight cut-off of 3500. Several grams of either α-CDP or β-CDP were dissolved in 10–20 mL of water, and the solutions were poured into the dialysis tubing and placed in a graduated cylinder containing one liter of water. The contents of the cylinder were magnetically stirred. The water in the cylinder was changed seven to nine times over a five to seven day period. At the end of this period, the dialyzed polymer was recovered by freeze drying the solution remaining in the bag. The mass of the dialyzed polymers recovered was about 50% of the original mass of the native polymers. These polymers are designated α-CDPH and β-CDPH to indicate that only the higher molecular weight components should be present.

Results and Discussion

THE USE OF 2-AN

The fluorescence of 2-AN is observed only when this probe resides in a strong hydrogen-bonding environment, such as water [17, 18]. Consequently, when 2-AN binds in the hydrophobic site of a CD molecule, the 2-AN fluoresence is quenched [15]. The fluorescence lifetime, emission wavelength maximum and spectral shape are unaffected by the presence of CD molecules [15]. This suggests that the quenching must be static, and, if the static complex has a 1 : 1 stoichiometry, the fluorescence intensity data should conform to the modified Stern–Volmer equation:

$$F^0/F = 1 + K[\mathrm{CD}] \tag{1}$$

where F^0 and F are the intensities at the 2-AN fluorescence emission maximum in the absence and presence of a CD, respectively; [CD] is the CD concentration and K is the binding constant for the 1 : 1 complex. Thus, K is equal to the slope of a plot of F^0/F vs. [CD]. Fraiji *et al.* have shown [15] that good linear fits of Equation (1) are obtained for 2-AN with all three monomer CDs and also with the CD derivative, heptakis (2,6-di-*O*-methyl)-β-cyclodextrin. On the other hand, the plot is nonlinear for 2-AN in the presence of 2-hydroxypropyl-β-cyclodextrin, signifying the probable existence of two or more different 2-AN : CD complexes having significantly different K values in this case [15].

Figure 1 shows the modified Stern–Volmer plots (Equation 1) for 2-AN with the three monomer CDs and the three polymer CDs (CDPs). Reasonably good

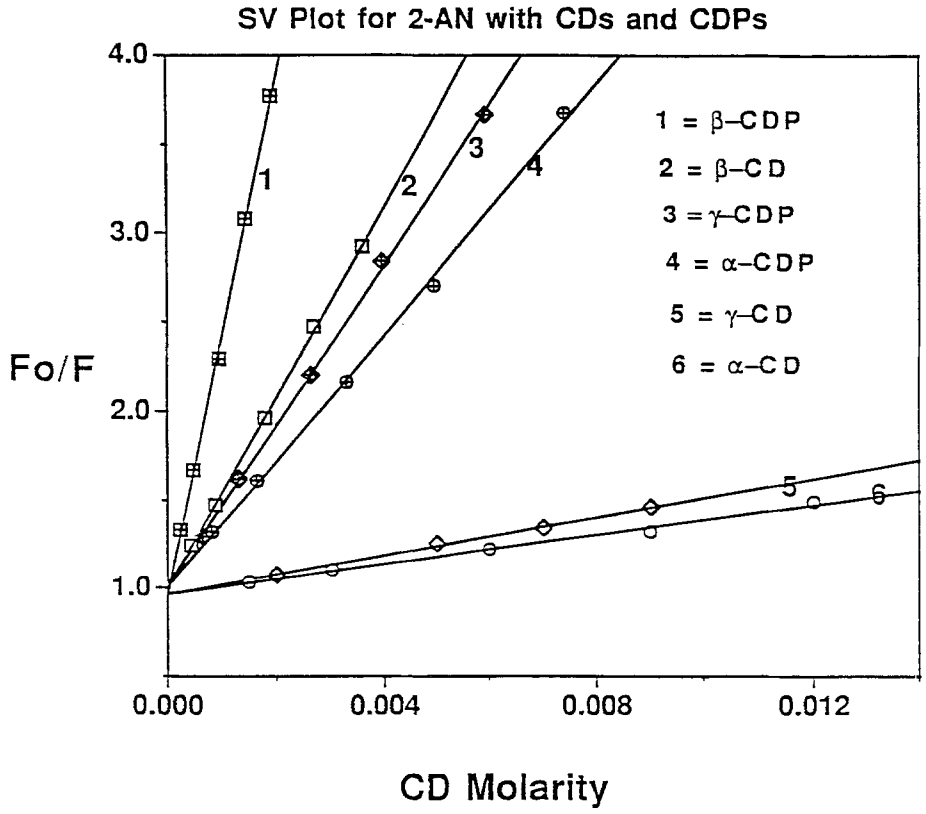

Fig. 1. Stern–Volmer plots for the quenching of 2-AN fluorescence ($\lambda_{em} = 437$ nm) by the three CDs and the three CDPs.

linear fits, with y-intercept values near unity, are obtained for all six of the CD monomers and polymers. This suggests that 2-AN primarily forms 1 : 1 complexes with each of three CDs, regardless of whether the CD is a monomer or a unit of a polymer chain, over the CD concentration ranges employed. It follows that the range of binding constant values for 2-AN and the CD units of a given CDP must be narrow. Otherwise, the linearity would not be observed.

The slopes of the modified Stern–Volmer plots for the quenching of 2-AN fluorescence by each of the CDs or CDPs are listed in Table I. For the CDs, the slopes should be true K values, while, for the CDPs, the slopes may be weighted averages of a narrow range of K values. In any case, the magnitude of the slopes should reflect the relative binding strength for a given 2-AN/CD or 2-AN/CDP interaction. The largest slope among the CD monomers occurs for β-CD : 2-AN because the fit between probe and cavity is best in this case. Similar slope values for the CD : 2-AN complexes were reported by Fraiji *et al.* [15]. The large increase in slope values for the CDPs compared to the slope values for the CDs argues for a prominent role for the glyceryl linkers in the binding interaction. Nonetheless,

TABLE I. Slope values from modified SV plots (294 K)

CD	Slope (±SD)[a]
α-CD	42 (±2)
β-CD	536 (±6)
γ-CD	55 (±2)
α-CDP	354 (±9)
β-CDP	1325 (±28)
γ-CDP	451 (±7)

[a] From standard deviation of slope of SV plot.

TABLE II. Fluorescence behavior of 1,5-DNSA in the presence of CDs and CDPs

CD	CD Molarity	λ_{max} (nm)	F_{rel} (λ_{ex} = 324 nm)
None	0	572	1.00
α-CD	0.00224	572	1.01
β-CD	0.00206	563	1.80
γ-CD	0.00206	568	1.34
α-CDP	0.00207	528, 565	2.44
β-CDP	0.00210	525	5.98
γ-CDP	0.00211	530, 555	4.19

the identity of the CD unit is still a very important factor in determining binding strength for 2-AN among the three CDPs.

THE USE OF 1,5-DNSA

The fluorescence emission spectra of DNSA in the presence of the three CDs and the three CDPs, all normalized to the same maximum intensity to highlight the spectral shifts, are given in Figure 2. The spectrum of DNSA in water is identical to that shown for α-CD. All of the other CDs and CDPs cause significant blue shifts in the fluorescence wavelength maxima, the shift being greater for the CDPs than the CDs.

Table II contains values for the emission wavelength maxima (λ_{max}) and relative intensities (F_{rel}) for 1,5-DNSA in water and in the presence of about 0.002 M of the CDs and CDPs. The F_{rel} values are determined by comparing the areas under the corrected emission spectra. No significant change in either λ_{max} or F_{rel} occurs at higher CD or CDP concentrations.

Blue shifts of λ_{max} and increases in F_{rel} indicate that 1,5-DNSA is entering a more hydrophobic environment [19]. In general, both of these quantities are enhanced with increasing hydrophobicity of the environment [19]. The lack of significant change from the water values for either of these quantities with α-CD

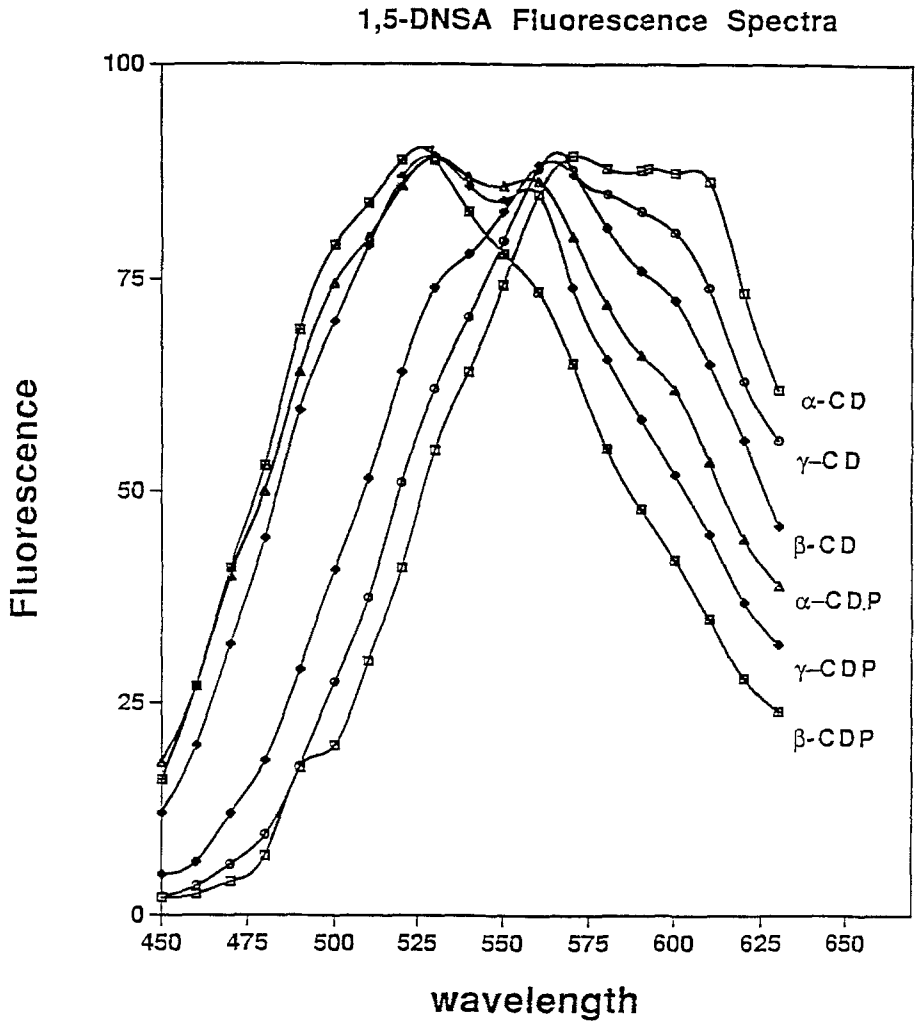

Fig. 2. Fluorescence emission spectra of 1,5-DNSA in solutions containing 0.002 M of one of the three CDs or one of the three CDPs. All spectra are normalized to the same intensity.

indicates virtually no binding occurs between 1,5-DNSA and α-CD, presumably due to the bulkiness of the axially-substituted naphthalene guest. Both β-CD and γ-CD do produce measurable increases in these two quantities, with the former apparently providing the more hydrophobic site.

The data in Table II clearly show that the polymer binding sites are appreciably more hydrophobic than those on the monomer CDs. However, the blue shifts and intensification still depend on the identity of the CD unit in the polymers in the same way they did for the CD monomers. Thus, the polymer binding environment for 1,5-DNSA must include considerable interaction or contribution from both a given CD cavity and the glyceryl linkers.

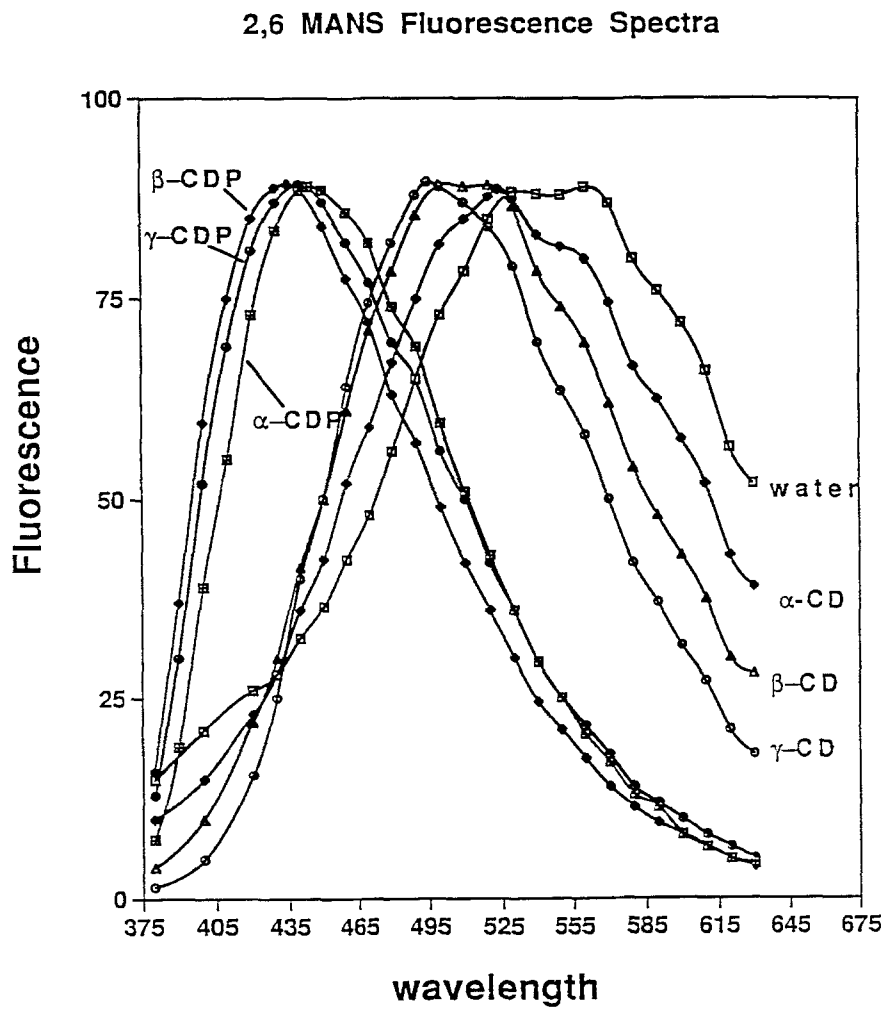

Fig. 3. Fluorescence emission spectra of 2,6-MANS in water and in solutions containing 0.002 M of one of the three CDs or one of the three CDPs. All spectra are normalized to the same intensity.

Harada *et al.* have reported greater blue shifts and enhanced fluorescence intensities for sodium 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) in solution in which epichlorohydrin-linked β-CD polymer is substituted for β-CD [9].

THE USE OF 2,6-MANS

The 2,6-MANS fluorophore also exhibits fluorescence blue shifts and intensification in more hydrophobic environments [20]. Normalized fluorescence spectra of 2,6-MANS in water and in the presence of about 0.002 M of each of the CDs and CDPs are shown in Figure 3. The λ_{max} and F_{rel} for 2,6-MANS in these media

TABLE III. Fluorescence behavior of 2,6-MANS in the presence of CDs and CDPs

CD	CD Molarity	λ_{max} (nm)	F_{rel} ($\lambda_{ex} = 350$ nm)
None	0	560, 530	1.00
α-CD	0.00213	524	1.46
β-CD	0.00202	496	13.3
γ-CD	0.00209	520, 500	5.0
α-CDP	0.00208	444	32.4
β-CDP	0.00208	434	27.4
γ-CDP	0.00213	438	26.2

are given in Table III. The trends in these values among the three monomer CDs are similar to those for 1,5-DNSA, except even α-CD now shows some binding interaction with 2,6-MANS.

The CDPs again provide much more hydrophobic binding environments for the fluorescence probe than those in the CDs. But there is an important difference between the spectral data for 2,6-MANS in the presence of the three CDPs and those of 1,5-DNSA in Table II. The *relative range* of intensification values $((F_{rel,max} - F_{rel,min})/F_{rel,min})$ for 2,6-MANS fluorescence is much smaller than for 1,5-DNSA in Table II. Moreover, in the case of 2,6-MANS, α-CDP actually produces the largest F_{rel} value. Some of the difference between F_{rel} for 2,6-MANS with α-CDP and β-CDP can be accounted for by the combination of a higher background absorbance for the latter at the exciting wavelength and the fact that β-CDP causes a slight decrease in the absorbance of 2,6-MANS at this wavelength. Even so, this combination of effects is not sufficient to change significantly the relative abilities of α-CDP and β-CDP to enhance 2,6-MANS fluorescence. This suggests that the binding environments for 2,6-MANS are much more similar among the three CDPs than is the case for both 1,5-DNSA and 2-AN.

To check this prediction, we measured the fluorescence lifetimes of 2,6-MANS in the presence of β-CD and the three CDPs, all at a concentration of ~ 0.002 M. These data are presented in Table IV. The fluorescence decay in the presence of β-CD appears to be adequately fitted by a single exponential decay by our criteria. By contrast, a double exponential fit gives a much better match to the experimental decay for all three of the CDPs. No significant improvement in fit is observed by including a third exponential component. The bottom line here is that lifetime results suggest the presence of at least two binding environments for the CDPs, all of which are different than that for β-CD. Moreover, the data also indicate that the 2,6-MANS binding environments are quite similar for all three CDPs. From this, we infer that the CDP binding environments for 2,6-MANS are less dependent on the identity of the CD unit than those for 1,5-DNSA and 2-AN. This is consistent with an even greater role for the glyceryl linkers in determining the 2,6-MANS binding sites.

TABLE IV. Fluorescence lifetimes for 2,6-MANS in the presence of CDs and CDPs[a]

CD	τ (nsec)[b]	τ_1 (nsec)[b]	τ_2 (nsec)[b]	F_2[b,c]
none	< 1			
β-CD	6.7(0.1)			
α-CDP		4.4(0.7)	12.2(1.3)	0.61(0.08)
β-CDP		4.6(0.2)	12.1(0.4)	0.45(0.04)
γ-CDP		4.6(0.5)	12.0(0.7)	0.48(0.06)

[a] Concentration for each CD or CDP is ~ 0.0020 M. Solution contains 0.1 M phosphate buffer, pH 6.98.
[b] Uncertainty in parentheses.
[c] F_2 is the fraction of the total fluorescence coming from the longer wavelength component.

TABLE V. A comparison of naphthalene probe data from native and dialyzed CD polymers

2-AN

The slopes of the modified Stern–Volmer plots agree within the limits of experimental error for quenching of 2-AN fluorescence for by both β-CDP and β-CDPH.

1,5-DNSA

CD(0.002 M)	λ_{max} (nm)	F_{rel}
β-CDP	525 nm	1.00
β-CDPH	525 nm	1.03

2,6-MANS

CD(0.002 M)	λ_{max} (nm)	F_{rel}	τ Values (nsec)
β-CDP	434	1.00	4.6, 12.1(0.45)[a]
β-CDPH	432	1.03	4.4, 12.1(0.50)[a]
α-CDP	444	1.00	4.4, 12.2(0.61)[a]
α-CDPH	444	1.07	4.1, 12.1(0.61)[a]

[a] Fraction of total fluorescence from longer lifetime components.

RESULTS WITH DIALYZED CDPS

Table V contains a comparison of spectral data for the three probes in the presence of native and dialyzed polymers. The CD concentration for the dialyzed polymers was determined assuming the same %CD for these polymers as reported for the native polymers [21]. The data in Table V clearly indicate that there is no significant difference in probe fluorescence behavior in the presence of the native and dialyzed polymers. The small increases in F_{rel} for the CDPHs are probably due to a slight reduction in polymer background after dialysis. The dialysis procedure should

effectively remove those polymer chains containing a single CD unit. Since there is no difference in spectral results following their removal, these lighter single-CD containing chains must affect probe spectral behavior in a similar fashion to the higher multiple-CD containing chains. Since single-CD containing chains could not exhibit intrachain cooperative effects, we can infer that cooperative binding interactions involving two CD units on the same polymer chain is not a significant occurrence for these probes.

Conclusions

The fluorescence behavior of these three naphthalene-based probes indicates that binding sites in the CD polymers are considerably more hydrophobic than binding sites on the CD monomers. The slopes of the modified Stern–Volmer plots in Table I also indicate that probe binding is much stronger with the CD polymers than with the CD monomers. These observations leave no doubt as to a major role for the glyceryl linkers in the host/guest binding interaction. One possibility is an expansion of the binding site regions as the linker groups 'wrap around' entrances to the CD cavities to maximize hydrogen-bonding interactions between the glyceryl OH groups and those on the rims of the CD cavities. As the probe moves more into the 'new' regions the binding would become less a function of the particular CD unit. As a consequence, the binding becomes more of a non-inclusional association than true guest/host binding. This could account for the observations with the 2,6-MANS probe. Blyshak *et al.* have suggested the occurrence of noninclusional binding for PAHs bound to α-CD [22].

The linear Stern–Volmer plots for 2-AN and the fact that removal of the polymer chains containing single CD units results in no significant change in probe spectral data argues against cooperative effects between CD units on the same chain. In other words, CD units appear to act independently of one another in binding these probes.

It should be noted that other workers present evidence suggesting the existence of cooperative effects between CD units on epichlorohydrin-linked β-CDs and guest molecules. Harada *et al.* report the existence of a 2 : 1 polymer : TNS complex, in which the two CD units may come from the same chain [9]. Szeman *et al.* show that low molecular weight β-CDP fractions form stronger complexes with small guests than high molecular weight fractions, while the reverse is true for large substrates [11]. Finally, Xu *et al.* suggest that 2 : 1 β-CDP : pyrene 'clam-shell' binding occurs with increasing probability as the length of the linker chains between β-CD units increases [14]. However, based on pyrene I/III ratio data, the pyrene environment in the presence of the β-CD polymer is much more hydrophilic than that observed for clam-shell binding by the β-CD monomer [14, 23].

Thus, there is evidence, from this work and others, that the linker units might serve two functions: as a site for non-inclusional binding and as spacers to hold CD

units in the proper orientation to bind guests. Which of these functions dominates for a given guest may depend on the size, shape and polarity of the guest and the length of the linker chain.

Based on our results, we believe that these CD polymers might be effective mobile phase modifiers in HPLC work at much lower concentrations than required for the CD monomers. We also anticipate significant differences in HPLC selectivity for a given guest analyte between mobile phases containing CDPs and those containing CDs.

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