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Binary and Ternary Complexes Between Lauryl Hexaoxyethylene, Benzoate and Cyclodextrin. Part II. β-CD

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Abstract

The characterization of binary and ternary complexes of benzoate, lauryl hexaoxyethylene ($C_{12}E_6$) and β-CD is presented. The complexation equilibrium was characterized by UV-Vis spectrophotometry, titration microcalorimetry, capillary electrophoresis, and 2D ROESY ^{1}H-NMR. Results suggested that β-CD forms one complex with $C_{12}E_6$ in the stoichiometric ratio of β-CD : $C_{12}E_6$ 1.5 : 1, with a stability constant 1.3×10^5 $M^{-1.5}$. The 2-D ROESY ^{1}H-NMR spectrum indicated that $C_{12}E_6$ is included inside the β-CD cavity. The primary binding site of $C_{12}E_6$ is on the lauryl subunit of this molecule. Analogous to a previously reported study of α-CD, the combination of β-CD and $C_{12}E_6$ precipitated from the solution. Addition of benzoate seemed to dissolve the precipitate and nearly doubled the apparent stability constant of the complex. Results from the various techniques supported formation of ternary complexes between β-CD, $C_{12}E_6$, and benzoate.

Abbreviations: β-CD – beta cyclodextrin; $C_{12}E_6$ – lauryl hexaoxyethylene; CE – capillary electrophoresis; cmc – critical micellar concentration; ^{1}H-NMR – proton nuclear magnetic resonance; 2D – two dimensional rotating frame Overhauser; ROESY – enhancement spectroscopy

Introduction

Lauryl hexaoxyethylene ($C_{12}E_6$) belongs to the family of nonionic surfactants containing oligooxyethylene units. It is a less complex analog of Triton X-100, in which the bulky *p*-tertiary butyl phenoxy group is replaced with a much smaller lauryl group (C_{12}). Several studies of interactions of mixtures of alkylphenyl surfactants [1, 2] and pure triton surfactants [3–7] with β-CD have been published. Reported stability constants were large, (10^5) and the reported stoichiometries of these complexes were 2 : 1 or higher (cyclodextrin : surfactant). It has been suggested that the β-CD preferentially complexed with the *p*-tertiary octyl phenoxy group [5–7] of the Triton surfactants. Most of the oligooxyethylene tail was thought to be outside of the cavity and coiled on the cyclodextrin external surface.

Previously, binary and ternary complexes of α-CD, $C_{12}E_6$, and benzoate were described and characterized [8]. It was determined that α-CD formed two complexes with $C_{12}E_6$. β-CD has a larger and wider cavity size than α-CD. Therefore, it was anticipated that formation of ternary complexes between β-CD, $C_{12}E_6$, and benzoate should be more favorable than for α-CD, $C_{12}E_6$, and benzoate.

This paper reports on a study of the binary complexation of β-CD and $C_{12}E_6$, and ternary complex formation of β-CD, $C_{12}E_6$, and benzoate. Titration microcalorimetry and capillary electrophoresis proved to be reliable techniques in characterization of such complexation [8]. These techniques were also employed for this study. Because NMR is a direct structural technique and has been used for studying inclusion of optically inactive surfactants [9, 10], 2-D ROESY ^{1}H-NMR experiments were added to provide a better understanding of the structure of the binary and ternary complexes of β-CD, $C_{12}E_6$, and benzoate.

Experimental

Materials

All chemicals were of analytical grade and used as is. For all experiments, distilled and deionized water with resistance higher than 17 MΩ was used. Benzoate, 6M hydrochloric acid (AA grade), and sodium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA), β-CD was purchased from Acros Organics (Pittsburgh, PA, USA), bis-tris propane (1,3-bis[trishydroxymethyl]aminopropane - BTP) and lauryl hexaoxyethylene ($C_{12}E_6$) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The D_2O

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for the NMR experiments was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). The water content in β-CD was measured via Karl-Fisher titration and accounted for in the calculation of exact concentration.

Instrumentation

Titration Microcalorimetry and Capillary Electrophoresis

For calorimetric measurements, a MicroCal Omega-ITC microcalorimeter was used following standard instrumental procedures as described previously [8]. A Bio-Rad 3000 capillary electrophoresis equipped with a fast-scanning UV-Vis detector (Bio-Rad Laboratories, Inc., Hercules, CA, USA) interfaced to a Gateway 2000 PC was used for all the CE measurements. In both techniques, the experimental set-up and extraction of stoichiometries and stability constants were similar to previously reported studies [8, 11, 14].

UV-Vis

The UV-Vis experiments were performed using a Hewlett-Packard 8452A diode array spectrophotometer and a 1 cm quartz cell. Samples were prepared in 0.1M BTP buffer adjusted to pH = 7 with HCl. The background signal of the buffer was subtracted from all samples.

2D-ROESY ^{1}H-NMR

The 2D-ROESY ^{1}H-NMR experiments were performed on a Bruker DRX 500 spectrometer operating at 500.13 MHz for protons. Samples were dissolved in 99.96% D_2O. An external standard was not added to the samples because common standards have been found to experience shifts in the presence of cyclodextrin either due to a complexation or different H-bond network within a solution [12]. In all cases, the residual signal of HDO at 4.83 ppm was taken as the secondary reference [13].

Results and discussion

Titration microcalorimetry

Experimental considerations and calculations have been described in detail previously [8, 11, 14]. The binding isotherm of β-CD and $C_{12}E_6$ can be found in Figure 1. The fit of the binding isotherm to the experimental data indicated that only one binary complex between β-CD and $C_{12}E_6$ was formed. The calculated stoichiometric ratio of β-CD : $C_{12}E_6$ in this binary complex was found to be 1.5 : 1. The stability constant of this complex was $1.3 \pm 0.1 \times 10^5$ $M^{-1.5}$. To insure validity of the stoichiometry and stability constant, the titration microcalorimetry experiment was repeated with the cyclodextrin titrated with $C_{12}E_6$. The stability constant of the binary complex obtained in this experiment fell within a standard deviation of the value reported above and the stoichiometry of $C_{12}E_6$: β-CD was 1 : 1.5. Turco Liveri *et al.* [3]

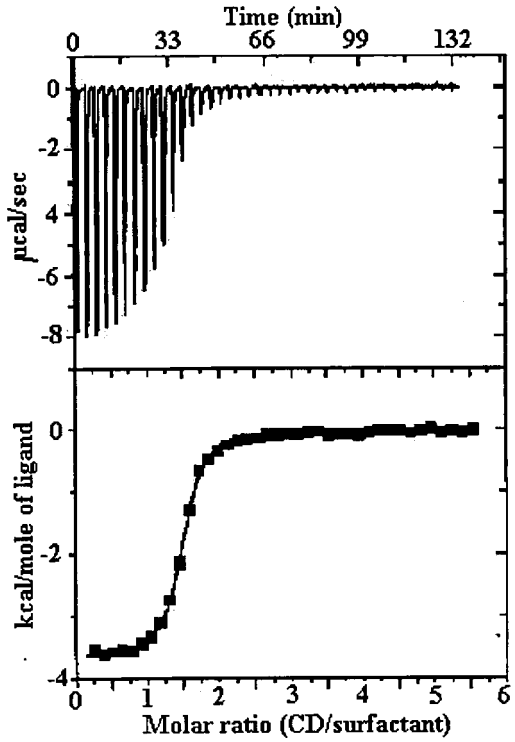

Figure 1. Binding isotherm of β-CD and $C_{12}E_6$ in BTP buffer.

studied complex formation between Triton surfactants and cyclodextrins via calorimetric measurements. The β-CD was found to form a complex with Triton X-100 in a 1 : 2 ratio (surfactant : cyclodextrin) and a stability constant 1.8 x 10^5. Other authors [4–6] also determined that Triton X-45 and Triton X-100 forms stable complexes with stoichiometric ratios equal to 1 : 2 (surfactant : cyclodextrin). Accompanying NMR experiments suggested that the *p*-tertiary butyl phenoxy group of Triton X-100 includes tightly inside the β-CD cavity [5].

Analogous to a previous report describing the interaction of α-CD with $C_{12}E_6$, the value of the stability constant K, for the β-CD : $C_{12}E_6$ complex also depended on the presence and concentration of benzoate in the ligand solution [8]. Figure 2 shows the dependence of the apparent K calculated for the binary complex β-CD : $C_{12}E_6$ on the molar ratio of benzoate : β-CD in the ligand solution. As can be seen from this figure, as the molar ratio of benzoate : β-CD increased, the stability constant of the complex also increased. At a molar ratio of benzoate: β-CD of 1:1, the overall stability constant reached a maximum, suggesting stoichiometric interaction of $C_{12}E_6$, β-CD and benzoate. The data is summarized in Table 1.

UV-Vis spectroscopy

Figure 3 shows the UV-Vis spectra of benzoate, binary mixtures of benzoate with β-CD and benzoate with $C_{12}E_6$, and the ternary mixture of benzoate with β-CD and $C_{12}E_6$. The concentration of benzoate was the same in all solutions. Thus, the broadening of the absorption spectrum and the increased absorptivity of the mixture of benzoate and β-CD vs. that of pure benzoate indicated a change in the microen-

Table 1. Stability constants of binary and ternary complexes of β-CD, $C_{12}E_6$, and benzoate determined from titration microcalorimetric data

Ligand β-CD	K ($M^{-1.5}$)	Ratio $C_{12}E_6/\beta$-CD	Ligand $C_{12}E_6$	K ($M^{-1.5}$)	Ratio $C_{12}E_6/\beta$-CD
Binary Complex [(CD-$C_{12}E_6$]	$1.3 \pm 0.1 \times 10^5$	1 : 1.5	Binary Complex [β-CD-$C_{12}E_6$]	$1.5 \pm 0.3 \times 10^5$	1 : 1.5
Ternary Complex [(CD-$C_{12}E_6$-benzoate]	$2.5 \pm 0.2 \times 10^5$	1 : 1.5	Ternary Complex [β-CD-$C_{12}E_6$-benzoate]	$3.1 \pm 0.5 \times 10^5$	1 : 1.5

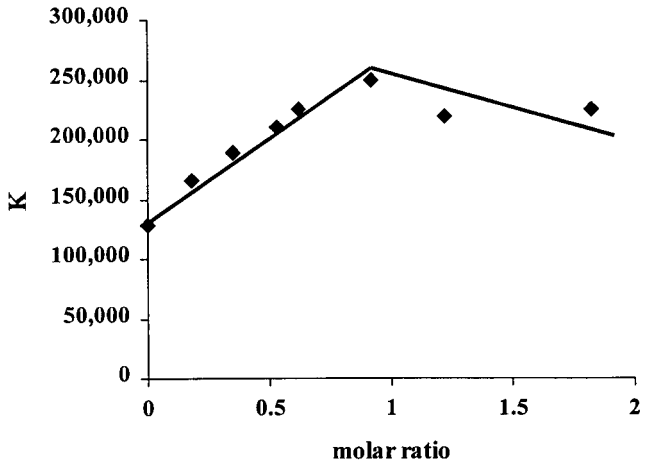

Figure 2. Plot of stablility constant K calculated for the binary complex β-CD-$C_{12}E_6$ vs the molar ratio of benzoate : β -CD in the ligand solution.

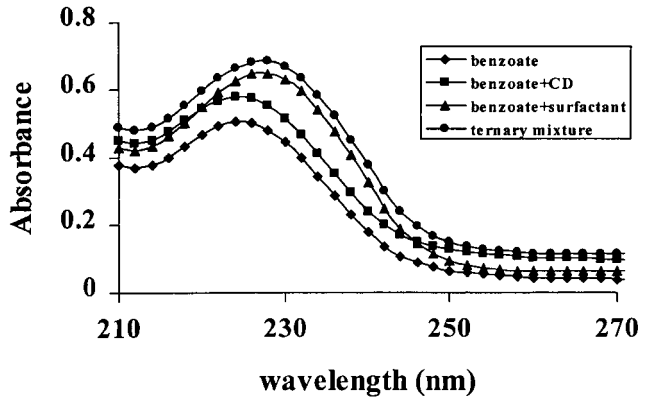

Figure 3. UV-Vis spectra of aqueous solutions of benzoate in binary and ternary mixtures of benzoate (3.64×10^{-5} M), β-CD (5.75×10^{-5} M), and $C_{12}E_6$ (1.01×10^{-2} M).

vironment of benzoate, presumably through inclusion of the phenyl ring inside the β-CD cavity. A further increase in the absorption maximum of benzoate and a red-shift of the peak maxima was observed for the binary mixture of benzoate and $C_{12}E_6$ when compared to the binary mixture of benzoate and β-CD. However, the largest shift change in intensity and broadening was obtained with the ternary mixture. Thus, the microenvironment of benzoate in a ternary mixture was influenced synergistically by both β-CD and $C_{12}E_6$, suggesting ternary complex formation. This finding is consistent with findings of other authors in which benzoate and its derivatives were found to be included inside the β-CD cavity together with polyethylene glycol [15].

Capillary electrophoresis

Capillary electrophoresis was used to quantify the free cyclodextrin in the samples containing both surfactant and cyclodextrin. It requires that the total (initial) concentrations of the individual interacting species be known. It can be shown that for complex stoichiometry 1 : 1, the stability constant for the binary complex can be determined from the slope of the following relationship,

$$K^*(c_S - c_{CD} + [CD]) = \frac{c_{CD} - [CD]}{[CD]} \qquad (1)$$

in which c_S is total concentration of a surfactant, c_{CD} is the total concentration of cyclodextrin, K is the stability constant and [CD] is the concentration of free cyclodextrin. Similarly, relationships for more complex stoichiometries could be derived. For example, the stoichiometry of the cyclodextrin : surfactant complex equal to 3 : 2 can be written as:

$$K^*\left(c_S - \frac{2c_{CD}}{3} + \frac{2[CD]^2}{3}\right) = \frac{c_{CD} - [CD]}{3[CD]^3}. \qquad (2)$$

If the assumed stoichiometry is correct, then a plot of ($c_S - 2/3c_{CD} + 2/3[CD]^2$) vs. ($c_{CD} - [CD]$)/(3*$CD]^3$) should yield a straight line with an intercept of zero and a slope equal to the stability constant. Systematic or nonsystematic deviation of the data from the calculated line or a non-zero intercept could indicate the formation of more than one complex, or incorrect stoichiometry.

For the quantitation of free β-CD in the mixtures of β-CD and $C_{12}E_6$ a modified method for separation of α-, β-, and γ-CD was implemented [8, 16]. A crucial component of the separation buffer was benzoate because its presence allowed indirect detection of the UV-transparent cyclodextrin. Benzoate also complexed with β-CD thereby conferring a partial negative charge thus allowing β-CD to be displaced from the zone containing other neutral components of the sample.

Figure 4 shows electropherograms of mixtures of βCD and $C_{12}E_6$ in a buffer containing 20 mM benzoate. Figure 4a is the electropherogram obtained with only β-CD in water as a sample. Two peaks were observed. The first peak (labeled as A) corresponds to water; the second peak (labeled as C) corresponds to β-CD. Figure 4b is the electropherogram obtained from the injection of $C_{12}E_6$ in water. Under the experimental conditions $C_{12}E_6$ was neutral; thus if no interaction occurred with benzoate, $C_{12}E_6$ should have eluted in

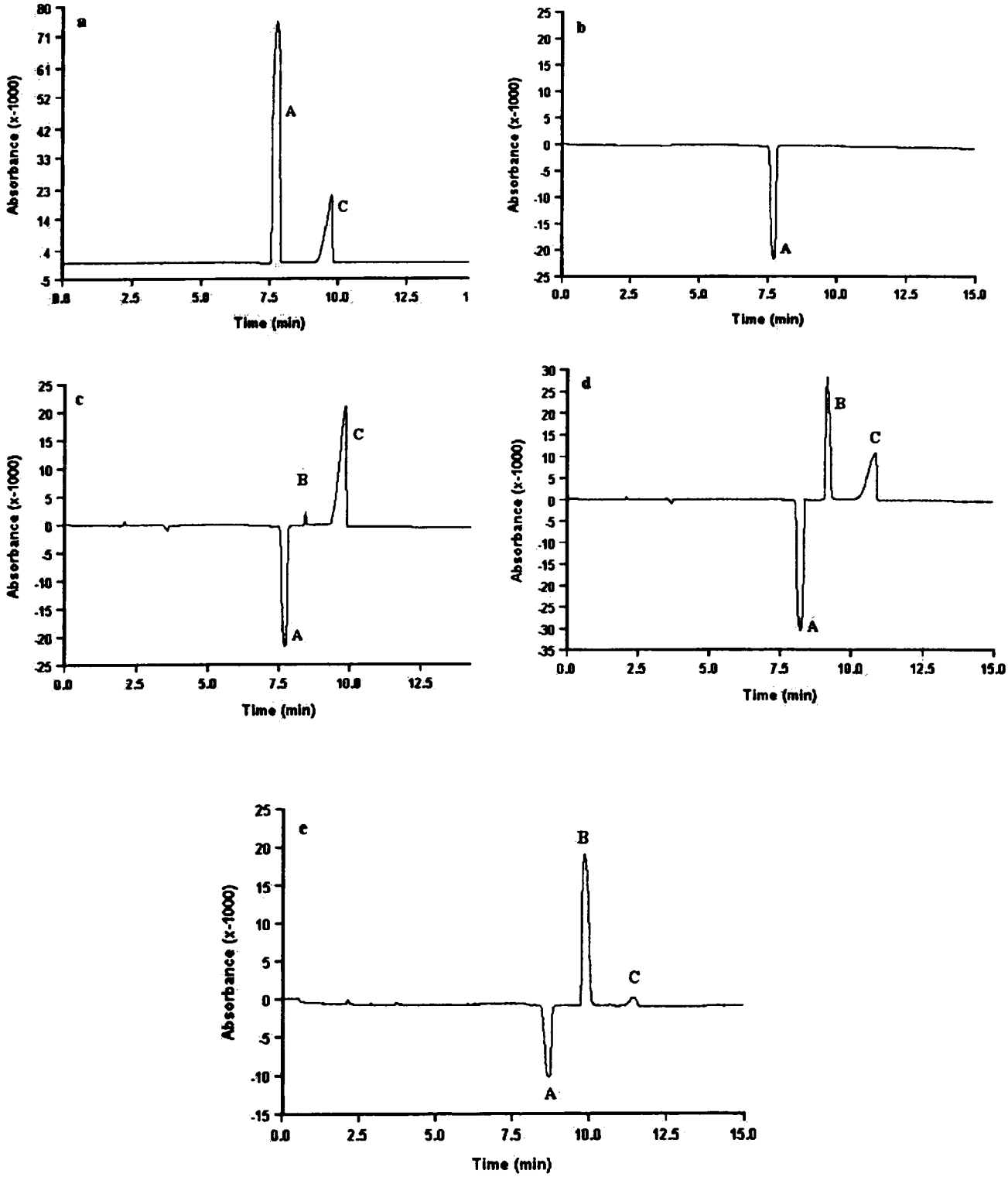

Figure 4. Electropherograms of (a) 2.8 mM β-CD; (b) $C_{12}E_6$; (c) mixture of β-CD : $C_{12}E_6$ = 7 : 1; (d) mixture of β-CD : $C_{12}E_6$ = 2 : 1; (e) mixture of β-CD : $C_{12}E_6$ = 1.1 : 1.0. Buffer 20 mM benzoate adjusted to pH = 7 with BTP, U = 17 kV.

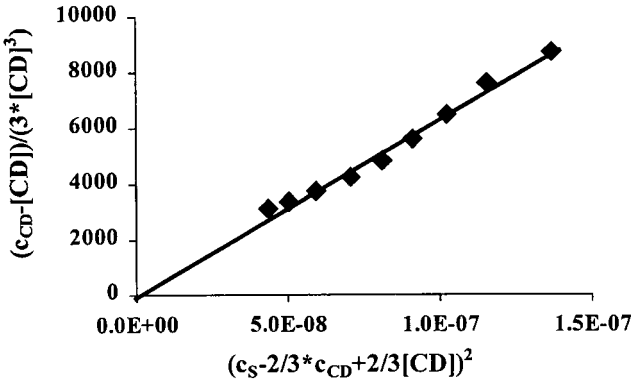

Figure 5. Determination of the stability constant and stoichiometry for complex formation of β-CD and $C_{12}E_6$ with assumed stoichiometry of β-CD : $C_{12}E_6$ = 3 : 2.

Table 2. Log of the apparent stability constants (pK) for β-CD-$C_{12}E_6$ in the complex with stoichiometric ratio equal to 1.5 : 1. Shown values of pK were measured in the separation buffers with various concentration of benzoate

c of benzoate in the CE buffer (mM)	pK calculated for [β-CD : $C_{12}E_6$] =1.5 : 1
0 (extrapolated)	4.3
10	4.6
20	4.8
30	5.0
40	5.1
50	5.4
60	5.8

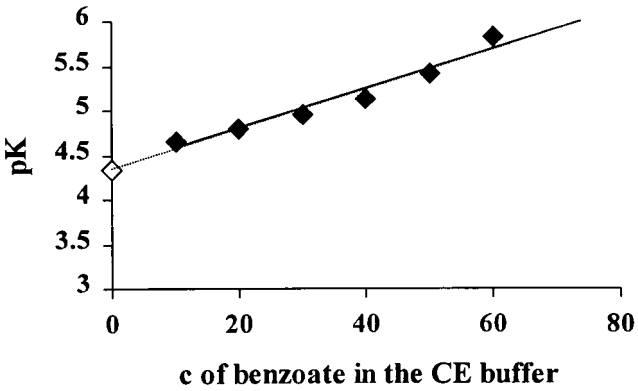

Figure 6. Dependence of the stability constant K calculated for binary complex β-CD and $C_{12}E_6$ on concentration of benzoate in the separation buffer. Because calculated stability constants are average values of the stability constants of the binary and ternary complex, extrapolation to zero concentration of benzoate gives the apparent value of the binary complex.

the neutral zone. As Figure 4b shows, elution of only one peak was observed. Figure 4c shows the electropherogram obtained for a sample containing β-CD : $C_{12}E_6$ in the molar ratio of 7 : 1 in water. The new peak (labeled as B) appearing on the electropherogram corresponds to the complex formed between β-CD and $C_{12}E_6$. It should be noted that this complex must also include benzoate. Figure 4d shows an electropherogram for the mixture of β-CD : $C_{12}E_6$ in a molar ratio of 2 : 1. As the concentration of $C_{12}E_6$ in the sample increased, peak B also increased, while the peak area of peak C (β-CD) decreased. Assuming complex stoichiometry of β-CD : $C_{12}E_6$ 1.5 : 1, peak C should diminish when the molar ratio is smaller than 1.5 : 1. Figure 4e shows the electropherogram of a mixture of β-CD : $C_{12}E_6$ equal to 1.1 : 1. Indeed, it can be seen in this figure that the β-CD peak (peak C) has almost disappeared. When $C_{12}E_6$ was present in a large excess of β-CD, the β-CD peak (peak C) disappeared entirely and peak B remained constant regardless of further increases in the concentration of $C_{12}E_6$. It is important to note that baseline resolution of the binary complex between β-CD with $C_{12}E_6$ from the zone of neutral components strongly supports the interaction of the binary complex with benzoate. Additionally, the appearance of an additional peak was not observed upon the injection of $C_{12}E_6$ alone. The fact that the peak area of the binary complex (peak B) did not change when a large excess of $C_{12}E_6$ was added to the sample also suggests stochiometric binding of benzoate to the binary complex.

Figure 5 shows a plot of $(c_S - 2/3{*}c_{CD} + 2/3[CD])^2$ vs. $(c_{CD} - [CD])/(3{*}[CD]^3)$. The slope of this dependence provides a measure for the stability constant with complex stoichiometry of 3 : 2 (β-CD : $C_{12}E_6$) at the given concentration of benzoate.

A plot of stability constants vs. benzoate concentration (as determined from CE measurements in the buffers with various benzoate concentration) is linear and can be extrapolated to zero concentration of benzoate. Results are shown in Table 2 and Figure 6.

Table 3 shows comparison of stoichiometries and stability constants of binary and ternary complexes of β-CD, $C_{12}E_6$ and benzoate acquired in titration microcalorimetry and capillary electrophoresis experiments. The complex stoichiometry was identical in both techniques. Stability constants determined from CE experiments were of the same order of magnitude as those measured by titration microcalorimetry even though a bias might be observed in calculated stability constants in titration microcalorimetry experiments. The discrepancy between the two techniques may arise from a variety of sources. It might be argued that CE may be a more direct technique than titration microcalorimetry because it measures concentration of "free" CD, while titration microcalorimetry is an indirect technique and records only average effects. In addition, it should be noted that the experimentally determined stability constant from titration microcalorimetry might include other effects such as secondary micelle formation or heat of demicellization induced by the presence of cyclodextrin [4–6].

2D-ROESY

Inter- and intra-molecular spatial proximity of groups of atoms are often determined using 2D-T-ROESY [17] NMR spectroscopy. The diagonal, in the two-dimensional plot, represents the normal 1D spectrum of the sample. Clusters of peaks off the diagonal represent atoms that are spatially close regardless of the presence or the absence of scalar couplings.

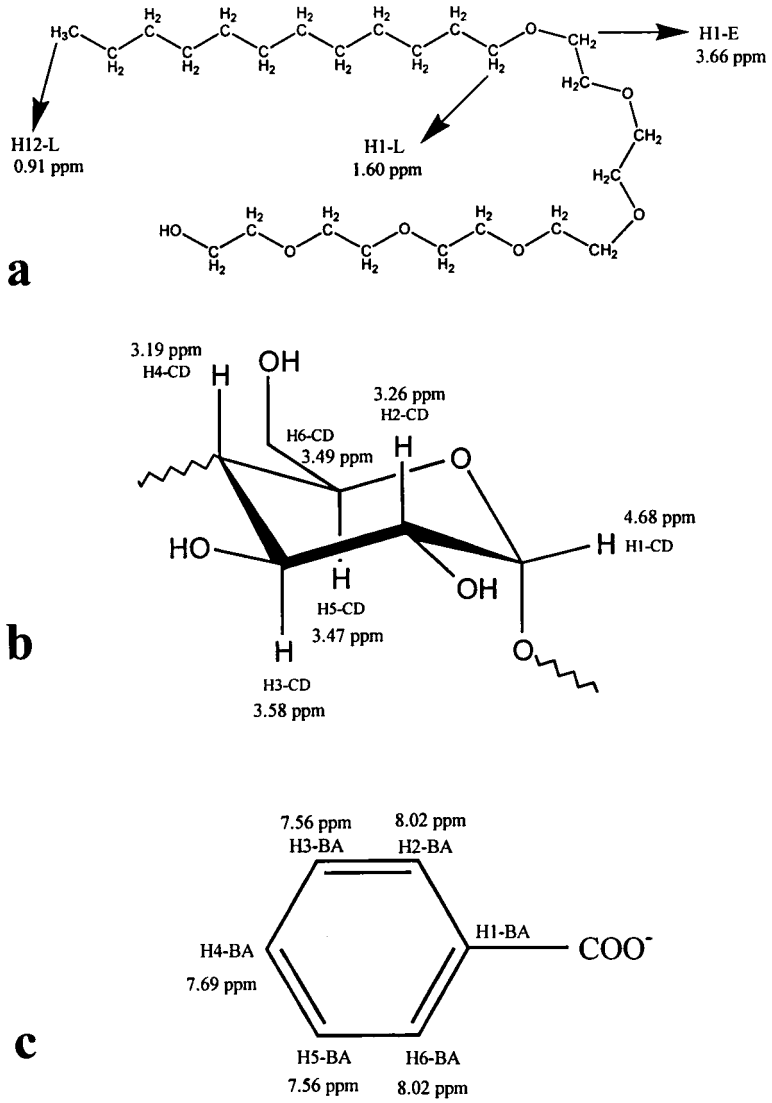

Figure 7. Proton assignments used for ^{1}H-NMR. $C_{12}E_6$ protons (a), β-CD protons (b), and benzoate protons (c).

Table 3. Comparison of titration microcalorimetry experiments with capillary electrophoresis experiments for K_{stab} and stoichiometries of the system β-CD-$C_{12}E_6$

	Titration Microcalorimetry	CE*
Stoichiometric ratio	1.5 : 1	1.5:1
pK_{stab} of binary β-CD-$C_{12}E_6$	5.11	4.34 (extrapolated)
pK_{stab} of ternary β-CD-$C_{12}E_6$-benzoate	6.38	6.0

*Actual stoichiometric ratio determined directly from CE experiments was 3 : 2; which is numerically the same as 1.5 : 1. For the purposes of comparison and consistency, all apparent stability constants are calculated for a stoichiometric ratio 1.5 : 1.

Figure 7 shows assignment and chemical shifts of the protons in β-CD [18], $C_{12}E_6$ [19], and benzoate. The H3-CD and H5-CD protons of β-CD are directed towards the inside of the β-CD cavity, while the H1-CD, H2-CD, H4-CD and H6-CD protons are directed toward the outside of the cyclodextrin cavity. Figure 8 shows the spectrum generated for the mixture of $C_{12}E_6$ and β-CD in the 2D-T-ROESY experiment. Two sets of off-diagonal clusters are observed, labeled as A and B. Cluster A represents the spatial proximity of the H3 and H5 protons of the CD molecule with the lauryl protons of the $C_{12}E_6$ molecule. It indicates that the lauryl moiety of $C_{12}E_6$ is included inside the β-CD cavity. Cluster B, which also appears in the spectrum of uncomplexed β-CD represents the spatial proximity of H1-CD of one glucose unit of β-CD with H2-CD of the same unit and with H4-CD proton of the next one as shown in Figure 9.

Analysis of the spectrum also revealed that the group of protons at 3.66 ppm corresponding to C1-E in $C_{12}E_6$ (see Figure 7) experiences a significant downfield shift when β-CD is present (data not shown). The significant downfield shift of the C1-E protons suggests that a strong modification of the micellar structure of the $C_{12}E_6$ occurs as a result of the interaction with β-CD. Since the molecule is in the micellar form in the absence of β-CD, inclusion of the lauryl moiety would "liberate" the oligooxyethylene moiety and affect chemical shifts.

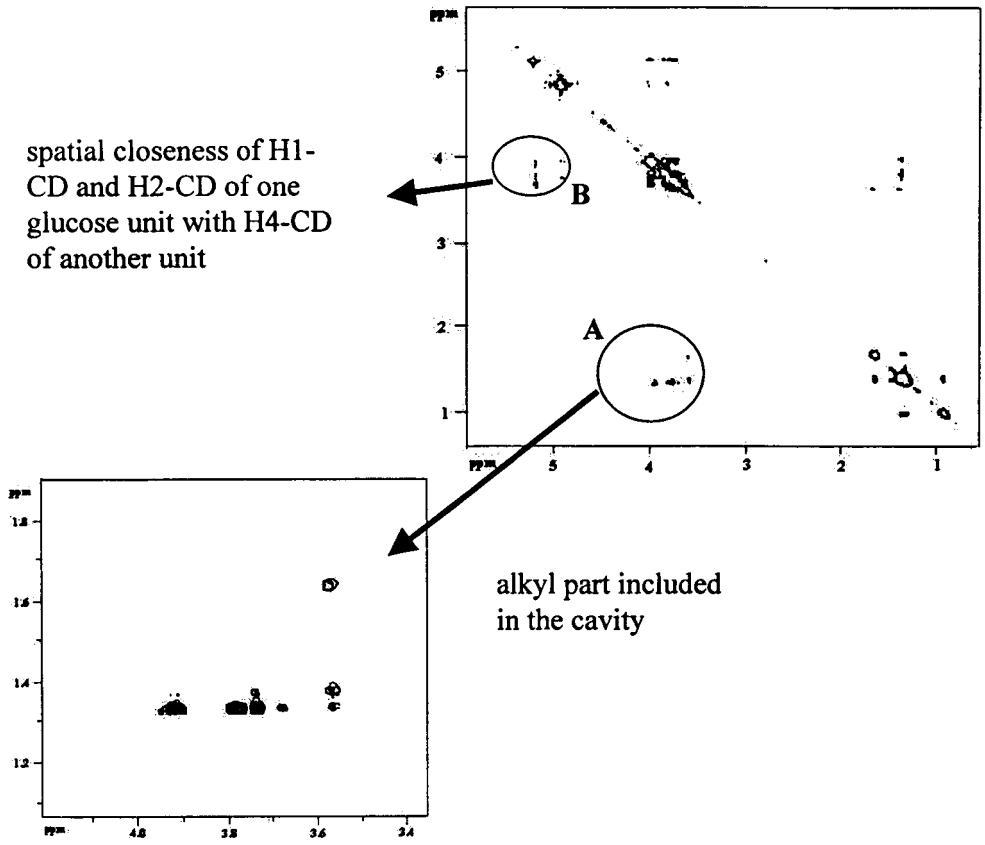

Figure 8. 2D-T-ROESY experiment of the mixture of β-CD and $C_{12}E_6$ in D_2O. See details in the text.

Closer inspection of the T-ROESY map at very low levels shows weak cross-peaks between benzoate and oligooxyethylene protons suggesting the formation of ternary complex. This also implies that the benzoate does not expel the lauryl chain from the cavity. However, dipolar interactions between the protons of benzoate and the β-CD are in the signal-to-noise range indicating that although benzoate may be in close proximity to the cavity of β-CD, it remains mainly in the oligooxyethylene domain. It also indicates that the benzoate is not strongly competing with the lauryl chain for inclusion inside of the β-CD cavity.

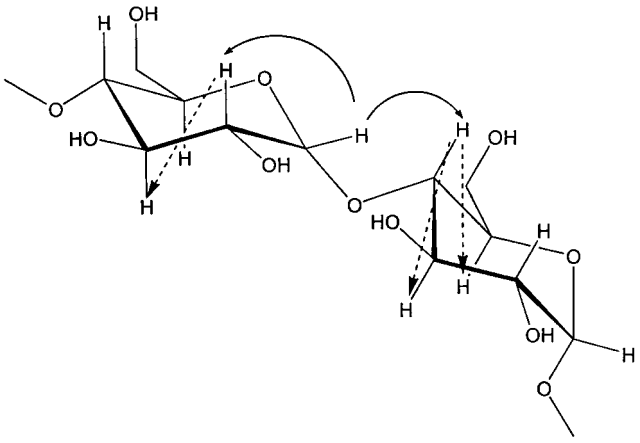

Figure 9. Origin of cluster B of the 2D-ROESY experiment of the mixture of β-CD and $C_{12}E_6$ in D_2O. Details for the origin of cluster B in Figure 8. In T-ROESY experiments, H1 protons experience dipolar coupling (only due to spatial proximity) with H2 of the same unit and H4 of the next one as indicated by full lines. Dotted lines indicate possible artifacts due to further transfer through scalar couplings. They appear as very weak cross peaks compared to the pure dipolar ones.

Conclusions

The stoichiometry, stability constants, and structure of the binary and ternary complexes between β-CD, benzoate, and $C_{12}E_6$ were investigated. UV-Vis provided only qualitative spectroscopic information supporting formation of binary and ternary complexes.

Titration microcalorimetry suggested that the stoichiometric ratio of β-CD : $C_{12}E_6$ is 1.5 : 1, with pK = 5.11. The ternary complex of β-CD : $C_{12}E_6$: benzoate appears to have the stoichiometric ratio 1.5 : 1 : 1.5, and pK = 6.38. Capillary electrophoresis confirmed the stoichiometry of the binary complex (1 : 1.5); however, stability constants determined by CE for the binary (pK = 4.34) and ternary complex (pK = 6.0) were somewhat lower than those measured in titration microcalorimetry experiments. This inconsistency may arise from differences in the measurements of CE and titration microcalorimetry. While CE is a more direct technique measuring 'free' and complexed β-CD, measurement of the stability constant by titration microcalorimetry might be perturbed by other solution effects, such as demicellization and/or aggregate formation.

Structural information of the binary and ternary complexes was gathered by using 2D-ROESY ^{1}H-NMR. The

data indicated that the lauryl moiety of $C_{12}E_6$ is included inside the β-CD cavity in both the binary and the ternary complexes. In addition, it was determined that in the ternary complex, although benzoate resides in close proximity to the β-CD cavity, it remains mainly in the oligooxyethylene domain.

Acknowledgments

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