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Determination of binding constants of hydrophobically end-capped poly(ethylene glycol)s with β-cyclodextrin by affinity capillary electrophoresis

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Abstract

The formation of inclusion complexes between methoxypoly(ethylene glycol)s (MPEG)s bearing one hydrophobic group (phenyladamantyl) per chain and β-cyclodextrin (β-CD) was studied by capillary electrophoresis (CE). The effect of highly sulphated β-CD (HS-β-CD) on the migration behaviour of the phenyladamantyl-modified MPEG (MPEG-PhAd) analyte was investigated. It was established that the interaction between the modified PEG and β-CD involved a 1:1 stoichiometry. Non-linear regression and three usual linearization methods (*y*-reciprocal, *x*-reciprocal and double reciprocal) were employed to estimate the binding constants. It was demonstrated that the binding constants were similar (around 400 M^{-1}) for two MPEG-PhAd having different chain lengths (2000 and 5000 g/mol).

Keywords: Binding constants; Affinity capillary electrophoresis; Poly(ethylene glycol); Cyclodextrins; Adamantylphenol; Phenols

1. Introduction

β-Cyclodextrins (β-CDs) are cyclic oligosaccharides composed of seven α-1,4 linked D-glucopyranose units. Owing to their hydrophobic cavity surrounded by a hydrophilic outer surface, β-CD are able to form inclusion complexes (host–guest complexes) with a wide range of molecules. Such complexes are formed if the guest molecules possess an hydrophobic group that fits the cavity size of the β-CD [1,2]. Their stability depends on both the hydrophobicity and the size of the guest compound. For example, binding constants higher than $10^5\ M^{-1}$ were reported in the literature for the inclusion of small molecules such as 1-adamantane carboxylic acid into β-CD cavities, demonstrating the stability of adamantyl:β-CD complexes [3].

In a similar way, it was shown in a recent study that the formation of inclusion complexes between β-CD-coated surfaces and polymers bearing adamantyl groups resulted in stable multilayered structures [4]. Such systems were used to bind antibody molecules to gold surfaces via an intermediate adamantyl-modified carboxymethylated dextran layer [5]. The resulting biosensors were stable in aqueous media while the initial β-CD-coated surface could be recovered after exposure to suitable reagents. Since immobilization procedures involving adamantyl:β-CD complexes are carried out under mild conditions, with rapid kinetics, they could be used for the elaboration of chromatographic supports bearing various functionalities.

Recently, the interactions between β-CD and hydrophobically modified polymers were examined using an affinity high performance liquid chromatography (affinity HPLC) method [6]. In this study methoxypoly(ethylene glycol)s (MPEG)s bearing one adamantyl end group per chain (phenyladamantyl-modified methoxypoly(ethylene glycol), MPEG-PhAd) were used as model polymers.

The aim of the present study was therefore to check the previous results obtained by HPLC, using a different separation method such as capillary electrophoresis (CE). Actually, CE can be a useful tool for examining the nature and strength of analyte:ligand interactions. Numerous studies have demonstrated its potential for measuring binding constants [7–10]. One of the major advantages of CE over HPLC, is the small amount of both analyte and ligand which are required for the determination of binding constants.

Affinity capillary electrophoresis (ACE) binding involves the measure of electrophoretic mobility changes caused by

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the addition of a ligand to the running buffer. Indeed, the electrophoretic mobility can be described as:

$$\mu_i = \frac{1}{1+K[L]}\mu_f + \frac{K[L]}{1+K[L]}\mu_c \quad (1)$$

where μ_i is the experimentally determined electrophoretic mobility, μ_f is the electrophoretic mobility of the free analyte and μ_c is the mobility of the analyte:ligand complex. [L] is the equilibrium ligand concentration in the buffer, that can be approximated to the added ligand concentration providing that ligand concentration is much greater than the solute concentration [8]. This assumption may be also valid when the binding constant is small.

In order to estimate binding constants by affinity capillary electrophoresis, several conditions must to be respected. (i) The solute must undergo a change in electrophoretic mobility upon complexation. This means that either the analyte or ligand must be charged. (ii) The equilibrium rate must be faster than the CE separation process. (iii) Fraction of ligand:analyte complex must be between 0.2 and 0.8 (for 1:1 binding ratio) [11].

Accurate binding constant determinations are possible only if the mobility shift is exclusively due to the association between the solute and the additive. One of the important parameters responsible for additional changes in mobility is the increase in buffer viscosity resulting from the increase in ligand concentration. In this case, a correction factor can be introduced in Eq. (1) to normalise the experimental electrophoretic mobilities [12,13]. Another major cause of erronated *K* estimation may be due to secondary equilibria. In particular, possible adsorption of both the analyte and the ligand to the capillary wall must also be accurately checked.

In the present study, as the MPEG is neutral, we chose a highly sulphated cyclodextrin (HS-β-CD) to investigate the affinity between the phenyladamantyl-modified methoxypoly(ethylene glycol) and the β-CD cavities. Before studying this system, we checked that the mobility change was exclusively due to the association between MPEG-PhAd and HS-β-CD. We have therefore investigated the variation of the buffer viscosity with increasing concentration of HS-β-CD and evaluated the possible adsorption of MPEG-PhAd or HS-β-CD to the capillary wall.

2. Materials and methods

2.1. Apparatus

Separations were performed on a Beckman P/ACE MDQ automated CE system (Beckman Coulters, Fullerton, CA, USA), equipped with a capillary cartridge allowing efficient temperature control. Uncoated fused-silica capillaries (50 μm i.d. × 75 μm o.d.) (Beckman Instruments) with effective and total lengths of 21 and 31 cm, respectively, were used. Data were collected using System Gold software (Beckman).

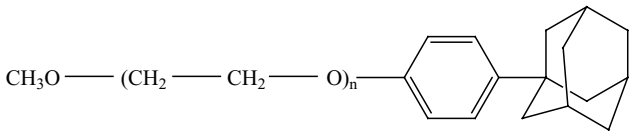

Fig. 1. Structure of phenyladamantyl-modified MPEG (MPEG-PhAd).

2.2. Reagents

All chemicals and solvents used were of analytical grade or of HPLC quality. HS-β-CD (typical substitution 7–11 mol/mol β-CD) and methoxypoly(ethylene glycol) of average molecular masses 2000 and 5000 g/mol (2000- and 5000-MPEG-PhAd), precursor of the phenyladamantyl-modified MPEG, were obtained from Sigma (St. Louis, MO, USA). The structure of phenyladamantyl-modified-MPEG is presented in Fig. 1. As described previously [14], two steps were necessary to synthesise MPEG-PhAd. Briefly, tosylate groups were grafted to MPEG at its OH terminal end to obtain tosyl-modified MPEG (MPEG-Ts). Then, MPEG-PhAd was obtained by nucleophilic substitution of adamantylphenolate on MPEG-Ts.

Aqueous sodium hydroxide solutions (1 and 0.1 M) were purchased from Interchrom (Asnieres, France). Thiourea and all other chemicals used in this study were from Aldrich (Milwaukee, WI, USA).

All buffers and samples were prepared with deionized water obtained from a Milli-Q RG system (Millipore, Bedford, MA, USA). Nylon filters (0.2 μm) used for the filtration of solutes and buffers were purchased from Millipore.

2.3. Methods

2.3.1. Binding constant determination

MPEG-PhAd (average molecular masses 2000 and 5000 g/mol) was dissolved at a concentration of 0.2 mM in water and was analysed threefold using increasing concentrations of HS-β-CD in the running buffer. The separation buffers were 25 mM phosphate solutions at pH 7.0, containing 0 to 17 mM HS-β-CD. Determination of the binding constants (*K*) was achieved by calculation of the electrophoretic mobilities (μ_i) of MPEG-PhAd. Electrophoretic mobilities were calculated subtracting the electroosmotic mobility from the apparent mobilities.

In order to estimate binding constants (*K*) and mobility of the complex (μ_c) non-linear and linear curve fitting methods were used to adjust experimental data. All regressions of the experimental data were performed using Origin 5.0 Software (Microcal Software, Northampton, MA, USA).

2.3.2. ACE conditions

A Thiourea solution at 0.01% (w/w) in water was used as an electroosmotic flow (EOF) marker. New capillaries were conditioned using the following procedure: a 5 min rinse with methanol, followed by deionized water for 5 min, 1 M aqueous sodium hydroxide for 10 min, deionized water for

5 min and finally flushing under pressure for 20 min with the separation buffer. Before each injection, the capillary was rinsed for 5 min with 0.1 M aqueous sodium hydroxide and then equilibrated 5 min with the separation buffer. The samples were introduced into the capillary using a 5 s pressure injection at 0.8 psi (1 psi = 6894.76 Pa). Separations took place at a potential of 4 kV and a temperature of 25 °C. The MPEG-PhAd in solution in phosphate buffer was monitored at 214 nm.

2.3.3. Viscosity measurements

To study the effect of CD addition on the buffer viscosity, all CD-containing buffers were hydrodynamically injected for 5 s with 0.8 psi pressure into the capillary. The times (t) required by the buffer plugs to reach the detector under a constant pressure of 1 psi were determined. Based on Poiseuille's equation, the correction factor υ for variation in viscosity may be estimated by [13]:

$$\upsilon = \frac{\eta}{\eta^0} = \frac{t}{t^0} \quad (2)$$

where η and η^0 are the running buffer viscosities with and without the CD respectively, t and t^0 are the times required for a sample plug to migrate from the injection end of the capillary to the detector with and without the CD, respectively.

2.3.4. Electroosmotic flow (EOF) stability

The possible evolution of EOF resulting from the adsorption of (i) MPEG or (ii) HS-β-CD to the capillary wall was checked using the following procedures. (i) The 5000-MPEG-PhAd was injected several times using a phosphate solution as running buffer. After each injection of polymer, the EOF was measured with Thiourea. (ii) To control the adsorption of HS-β-CD the capillary was rinsed several times with phosphate buffer at 17 mM of HS-β-CD, before measuring EOF. The injection and rinse conditions were the same as reported for the ACE study.

3. Results and discussion

3.1. Preliminary studies

It is well known that poly(ethylene oxide) (PEO) is an efficient coating agent which can be used to control the EOF and surface properties of inner silica capillary walls [15,16]. The possible adsorption of MPEG-PhAd to the capillary wall was, therefore, studied. The variation of the EOF was measured after successive injections of MPEG-PhAd using phosphate buffer at pH 7.0 without HS-β-CD. It was observed that using a simple between-run rinse procedure, the EOF remained constant over 25 consecutive injections of MPEG-PhAd. So, it was assumed that the adsorption of MPEG-PhAd to the capillary wall was negligible. It should be noticed that efficient PEO coating procedures required generally a full protonation of the silanol groups. This may explain why under our experimental conditions (phosphate buffer at pH 7 with very small amounts of MPEG-PhAd injected) no significant adsorption was observed.

It was expected that the ligand adsorption on the silica wall was also negligible because both the buffer additive (HS-β-CD) and the silanol groups are negatively charged at pH 7. By a similar procedure than for MPEG-PhAd, it was confirmed that HS-β-CD did not interact with the capillary wall.

The concentration of HS-β-CD added to the running buffer ranged between 0 and 17 mM. Thus, the impact of the HS-β-CD concentration on the buffer viscosity, and therefore, on the electrophoretic mobilities was examined. In this study, the correction factor υ (Eq. (2)) was evaluated using the procedure described in materials and methods. Its value fluctuated from 1 to 1.02 over the concentration range investigated in this study. So it can be reasonably assumed that the electrophoretic mobility of solutes was not affected by the viscosity when the ligand concentration increased. No correction of the electrophoretic mobilities by υ was applied in this study.

3.2. Binding isotherms

First, the mobility of MPEG-PhAd in the free form (μ_f) was measured to determine both the binding constant and the complex mobility using non-linear regressions of ($\mu_i - \mu_f$) versus [HS-β-CD] (Eq. (1)). The MPEG-PhAd polymer being a neutral analyte, its free mobility should be theoretically zero. This zero mobility was confirmed by EC since the apparent electrophoretic mobility of MPEG-PhAd was equal to the electroosmotic flow determined using Thiourea.

As PEG molecules studied in this work (MPEG-PhAd) were modified by a PhAd group at one extremity and a methoxy group at the other one, we have investigated two models of interaction between PEG and CD. A model assuming a 1:1 interaction was first tested for the MPEG-PhAd:HS-β-CD system in the capillary electrophoretic study. The non-linear regressions of ($\mu_i - \mu_f$) versus [HS-β-CD] are presented in Fig. 2 for 2000- and 5000-MPEG-PhAd. The data points are in good agreement with the theoretical binding isotherm ($R^2 = 0.993$), but as reported by Bowser et al., high correlation coefficients alone are not sufficient to prove that 1:1 model is the best model to describe the data [17]. Corresponding values of association constants and complex mobilities are reported in Table 1.

As Harada et al. [18] showed that no interaction occurred between the OCH_3-modified PEG and β-CD, we concluded that, in our study, the phenyladamantyl group was the only group responsible for the interaction with the HS-β-CD.

A careful examination of the non-linear regression obtained for 2000-MPEG-PhAd, at the highest concentrations of HS-β-CD (Fig. 2), revealed a slight deviation from the 1:1 model. A model assuming both 1:1 and 1:2 stoichiometries, described by Eq. (3) [17], was therefore,

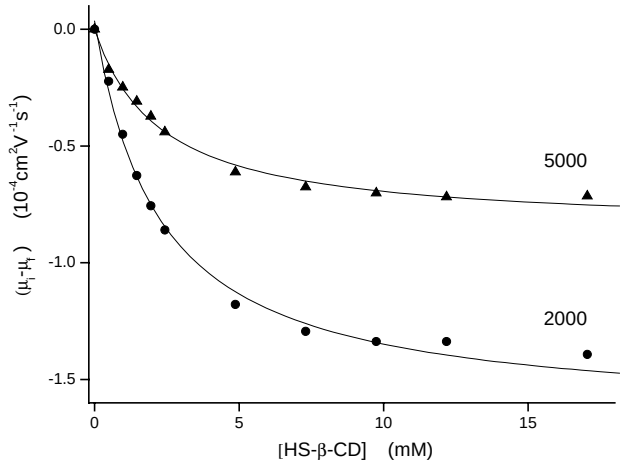

Fig. 2. The non-linear fits (Eq. (1)) for the binding isotherms for 2000-MPEG-PhAd (●) and 5000-MPEG-PhAd (▲) using all the experimental data. Experimental conditions: samples, 0.2 mM 2000- or 5000-MPEG-PhAd in water; electrolyte, 25 mM phosphate solutions at pH 7.0 containing 0–17 mM of HS-β-CD; pressure injection, 0.8 psi for 5 s; applied voltage, 4 kV; temperature, 25 °C; capillary, 31 cm (21 cm effective length); detection, 214 nm.

tested to fit the data points obtained for 2000- and 5000-MPEG-PhAd.

$$\mu_i - \mu_f = \frac{(\mu_c - \mu_f)K_1[L] + (\mu_{c_2} - \mu_f)K_1K_2[L]^2}{1 + K_1[L] + K_1K_2[L]^2} \quad (3)$$

where K_1, K_2 and μ_c, μ_{c_2} are the macroscopic equilibrium constants and the electrophoretic mobilities, respectively, corresponding to the analyte singly or doubly complexed to the ligand.

In this model, both the PhAd and the OCH_3 end groups were supposed to form inclusion complexes with HS-β-CD cavities. Although non-linear regression curves were in good agreement with the experimental data over the range of concentrations investigated (0 mM < HS-β-CD < 17 mM), some macroscopic equilibrium constants and complex mobilities determined from the non-linear regressions were inconsistent. For instance, negative K_2 values were obtained and in some cases, μ_{c_2} was found to be positive (data not reported). For these reasons, the hypothesis of both 1:1 and 1:2 stoichiometries was definitively rejected. These results are in good agreement with a previous work on the affinity between MPEG-PhAd and β-CD investigated by HPLC [6], demonstrating that only the hydrophobic PhAd extremity was able to form inclusion complexes with the immobilised β-CD cavities. Similar results were obtained on β-CD-coated gold surfaces with a surface plasmon resonance technique [4].

Table 1
Binding constant estimation of MPEG-PhAd to HS-β-CD (K) and electrophoretic mobilities of the free (μ_f) and complexed (μ_c) MPEG-PhAd using all the experimental data

	Molecular mass of MPEG-PhAd (g/mol)	
	2000	5000
K (M^{-1})	438 ± 38	437 ± 39
μ_f (10^{-4} $cm^2/(V\,s)$)	0 ± 0.05	0 ± 0.02
μ_c (10^{-4} $cm^2/(V\,s)$)	−1.63 ± 0.25	−0.85 ± 0.13
R^2	0.993	0.993

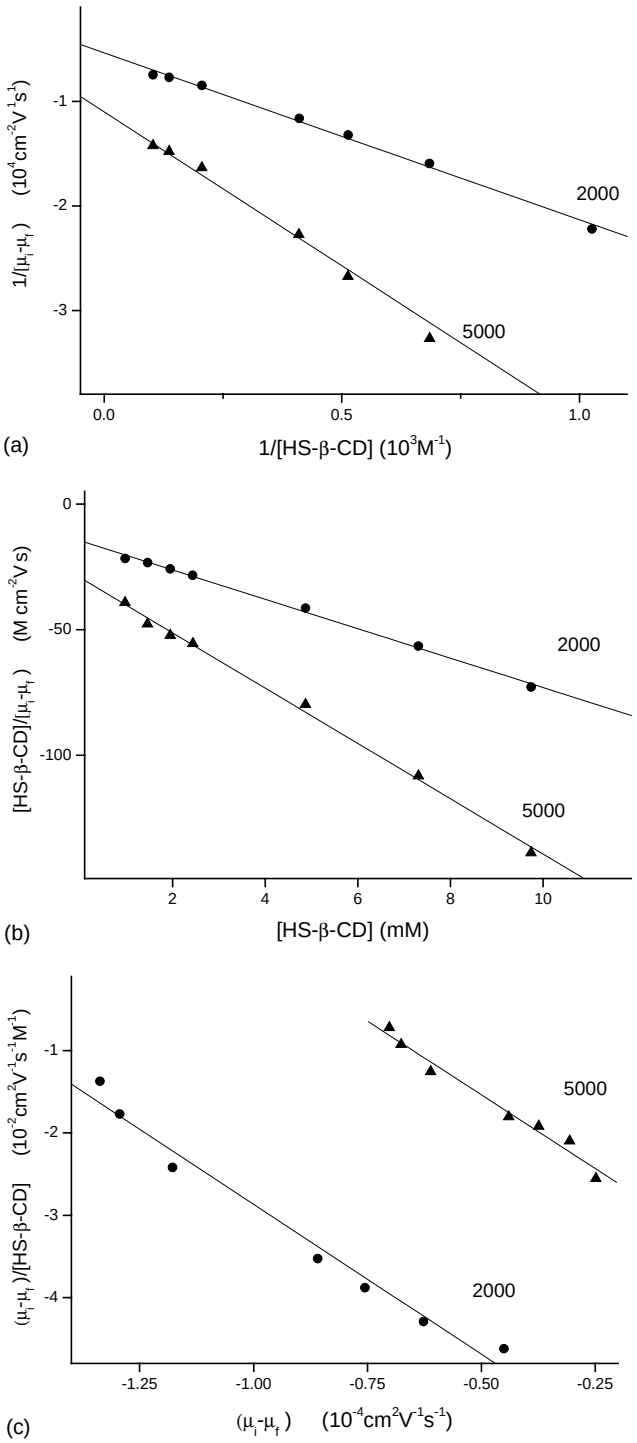

Fig. 3. The double reciprocal (a), *y*-reciprocal (b) and *x*-reciprocal (c) plots for 2000-MPEG-PhAd (●) and 5000-MPEG-PhAd (▲) using data corresponding to *f* ranging from 0.2 to 0.8. The experimental conditions are the same as in Fig. 2.

3.3. Determination of K and μ_c values

Assuming a 1:1 interaction between MPEG-PhAd and HS-β-CD and in order to minimise the error in the calculated constants, the range of HS-β-CD concentration investigated has been checked. Indeed, in the case of a 1:1 model, Deranleau [11] suggested that data should be collected at ligand concentrations where the fraction of analyte in the complexed form (*f*) is between 0.2 and 0.8. When *f* is lower than 0.2 or higher than 0.8, changes in mobility resulting from complexation are small compared to the random error. At high ligand concentrations, the analyte is, indeed, almost completely in the complexed form, while at low concentration values, the fraction of analyte in the complexed form is not high enough to give sufficient informations about *K*.

In the present study, when the concentration of HS-β-CD ranged from 0.97 to 9.7 mM, the fraction of analyte in the complexed form *f* ranged from 0.28 to 0.80 and from 0.26 to 0.78 for 2000-MPEG-PhAd and 5000-MPEG-PhAd, respectively. Then, in order to minimise the error on the calculated constants and mobilities, non-linear and linear regressions were established in the range 0.97–9.7 mM of HS-β-CD concentration. The double reciprocal, *y*-reciprocal and *x*-reciprocal equations [19] are plotted for the selected range of concentration in Fig. 3. The values of the binding constants and complex mobilities determined from both the linear and non-linear regressions are reported in Table 2.

It appears from Fig. 3 that the double reciprocal, *y*-reciprocal and *x*-reciprocal plots obtained from experimental data were linear with $R^2 > 0.988$. This result confirms that the interaction between MPEG-PhAd and β-CD cavities can be described as in HPLC, by a 1:1 model within the range of HS-β-CD concentrations investigated in this study.

Table 2 compares the binding constants and mobilities determined for 2000- and 5000-MPEG-PhAd from the various graphical techniques. It should be noted that linear regression methods were applied without using weighted least squares analysis reported in previous studies [13,19]. In spite of this, linear plotting methods yielded parameters similar to those obtained from the non-linear curve fitting method (Table 2). For 5000-MPEG-PhAd, the association constant was found equal to $368 \pm 27\ M^{-1}$. When compared to values determined from linear plots, non-linear data did not differ over 5%. For 2000-MPEG-PhAd, the observed difference was generally lower than 10%. Thus, it could be assumed that no weighting procedure on electrophoretic mobility was needed in the linear regressions [19].

As shown in Table 2, the binding constants obtained for MPEG-PhAd of 2000 and 5000 g/mol molecular weight were similar. In a previous study, the affinity of hydrophobically end-capped MPEG for hydroxypropyl-β-CD (HP-β-CD) was studied by HPLC [6]. It was demonstrated that affinity constants in solution remained similar when

Table 2
Binding constants of MPEG-PhAd (2000 and 5000 g/mol) to HS-β-CD (*K*) and electrophoretic mobilities of the complexed MPEG-PhAd (μ_c) determined by non-linear and linear regressions using data restricted to *f* from 0.2 to 0.8

	Non-linear regression		Double reciprocal		*y*-reciprocal		*x*-reciprocal	
	2000	5000	2000	5000	2000	5000	2000	5000
K (M^{-1})	398 ± 28	368 ± 27	337 ± 27	375 ± 36	402 ± 32	376 ± 28	364 ± 24	358 ± 25
μ_c (10^{-4} $cm^2/(V\,s)$)	−1.72 ± 0.20	−0.92 ± 0.11	−1.86 ± 0.09	−0.91 ± 0.05	−1.71 ± 0.04	−0.91 ± 0.02	−1.79 ± 0.19	−0.93 ± 0.10
R^2	0.994	0.994	0.996	0.992	0.996	0.996	0.978	0.976

increasing the molecular weight of MPEG-PhAd from 2000 to 5000 g/mol. Thus, the present ACE study confirms the HPLC results.

The association between MPEG-PhAd and HP-β-CD determined using the HPLC method was around 1000 M^{-1}. Lower values are reported in the present study for the formation of inclusion complexes between MPEG-PhAd and HS-β-CD. This can be easily explained since the interaction between MPEG-PhAd and the neutral HP-β-CD is probably stronger than that with the negatively charged HS-β-CD. In a recent study, a stability constant around 200 M^{-1} was determined by Wang et al. for the complex formed between neutral β-CD and a charged solute, adamantadine hydrochloride. This result demonstrates again the influence of the charge on the formation of inclusion complexes [20].

When comparing the electrophoretic mobilities of the 2000- and 5000-MPEG-PhAd:HS-β-CD complexes, more negative values were obtained for the polymer with the lowest molecular weight (Table 2). This result is in good agreement with the charge/mass ratios of both complexes since the 2000-MPEG-PhAd:HS-β-CD inclusion complex migrates more rapidly towards the anode than the 5000 inclusion complex.

4. Conclusion

ACE appeared to be a satisfactory method to study the stoichiometry and the strength of the interaction between MPEG-PhAd and HS-β-CD. It was demonstrated that the complexation between MPEG-PhAd and HS-β-CD involved a 1:1 stoichiometry. The binding constants determined for 2000- and 5000-MPEG-PhAd from linear and non-linear regressions were similar (around 400 M^{-1}). The results of the ACE study are in good agreement with the previous HPLC study of the complexation of MPEG-PhAd to HP-β-CD.

It would be interesting to compare more precisely HPLC results to binding constants determined by an indirect capillary electrophoresis method described by Wang et al. [20]. This indirect method, based on two competitive chemical equilibria, is indeed valid for both charged and neutral solutes and ligands.

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