Investigation of Noncovalent Complexes Between β-Cyclodextrin and Polyamide Acids Containing *N*-Methylpyrrole and *N*-Methylimidazole by Electrospray Ionization Mass Spectrometry

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Electrospray ionization (ESI) mass spectrometry was utilized to investigate noncovalent complexes between β-cyclodextrin (β-CD) and five novel polyamide acids containing *N*-methylpyrrole and *N*-methylimidazole. The 1:1 binding mode was specified by examining the binding stoichiometry from ESI mass spectra. It found that polyamide acids with β-CD have binding affinities in the order: ImImImβCOOH > ImPyImβCOOH > ImPyPyβCOOH > PyPyPyβCOOH > NO_2PyPyPyβCOOH. The method gives, simultaneously, the binding constants between β-CD and polyamide acids based on a novel linear equation. (J Am Soc Mass Spectrom 2006, 17, 9–14) © 2005 American Society for Mass Spectrometry

Recently, polyamides containing *N*-methylpyrrole (Py) and *N*-methylimidazole (Im) have attracted considerable attention from synthetic and biological chemists because of their significant anticancer activity [1, 2]. Five new polyamide acids were synthesized in our laboratory with a convenient method for solution-phase synthesis [3]. Their structures are shown in Figure 1, where Py = *N*-methylpyrrole, Im = *N*-methylimidazole, and β = β-alanine.

β-Cyclodextrin (β-CD or CD) is a very important host molecule. Noncovalent complexes between drug molecules and β-cyclodextrin are capable of improving the stability, water solubility, and bioavailability of some lipophilic drugs [4–10]. Therefore, β-CD and its derivatives have been utilized in agriculture, pharmaceutics, drug formulation, and drug delivery.

A number of different physicochemical methods have been used in analyzing the interactions between small molecules and β-cyclodextrin, such as ^{1}H NMR, conductometric titration, spectrophotometric and fluorometric techniques [11–16]. However, in some instances, there are uncertainties regarding the complex stoichiometries and the lack of structural information using these techniques [17]. Electrospray ionization mass spectrometry (ESI-MS) is a powerful means of studying noncovalent complexes between "host–guest" (for example, small molecules with β-cyclodextrin) with high sensitivity and rapidity, at a very low level of sample consumption [18–34].

In this research, ESI-MS was utilized to study the noncovalent interactions of five novel polyamide acids with β-cyclodextrin, including stoichiometry, stability and binding affinity, and calculation of the binding constants between β-CD and polyamide acids.

Experimental

Chemicals

β-Cyclodextrin (MW = 1134.4) was purchased from Sigma Chemical (St. Louis, MO). Polyamide acids ($\mathbf{P_1}$–$\mathbf{P_5}$) were prepared in our laboratory according to a convenient method for solution-phase synthesis [3]. All other chemicals were of analytical grade.

Mass spectrometry

ESI mass spectra and collision-induced dissociation (CID) spectra were obtained using a Finnigan LCQ Deca XP Plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA); all experiments were performed in negative mode. The mixed solution was directly infused at a flow rate of 2 μl/min into the ion source. ESI-MS conditions were optimized to favor the observation of noncovalent complexes. Spray voltage was 3.0 kV, capillary temperature held at 150 °C, and a doubled drying gas (N_2) was used to ensure efficient

Published online December 9, 2005

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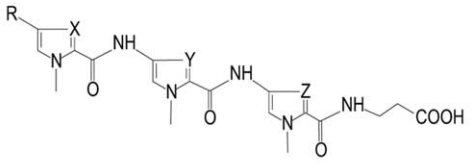
1044-0305/06/$32.00
doi:10.1016/j.jasms.2005.08.011

Received March 16, 2005
Revised August 22, 2005
Accepted August 22, 2005



R=H, X=N, Y=N, Z=N: ImImImβCOOH (P_1)

X=N, Y=C, Z=N: ImPyImβCOOH (P_2)

X=N, Y=C, Z=C: ImPyPyβCOOH (P_3)

X=C, Y=C, Z=C: PyPyPyβCOOH (P_4)

R=NO_2, X=C, Y=C, Z=C: NO_2PyPyPyβCOOH (P_5)

Figure 1. Structures of polyamide acids ($\mathbf{P_n}$).

desolvation. Data were collected and analyzed with the Xcalibur software (ThermoFinnigan), and 10 scans were averaged for each spectrum.

Analysis of Complexation Procedure

Noncovalent interactions of polyamide acids ($\mathbf{P_1}$–$\mathbf{P_5}$) with β-CD were examined by ESI mass spectrometry in the presence of increasing concentrations of β-CD. Aliquots of 0.5–25 μl β-CD (5.0 mM) were mixed with 0.5 μl of polyamide acids (5.0 mM). This solution was diluted with methanol/H_2O (20:80, vol/vol) to 50 μl at room temperature (β-CD–guest ratios were 1:1, 2:1, 5:1, 10:1, 25:1, and 50:1) and then subjected to ESI-MS analyses. Methanol was necessary to obtain good electrospray behavior [35, 36]. All solutions had appropriate concentrations (50–2500 μM) of β-CD and the same initial concentration (50 μM) of polyamide.

Results and Discussion

Complexes Between β-Cyclodextrin and Polyamide Acids

Complexes of β-CD with polyamide acids in the molar ratio 1:1 were detected by negative ion mode; the ESI mass spectra of the three complexes are shown in Figure 2. In the case of ImImImβCOOH ($\mathbf{P_1}$), five ions were observed in the ESI spectrum, i.e., ions at *m/z* 442.3, 566.6, 885.0, 1133.6, and 1576.5, which correspond to $[ImImIm\beta COO]^-$ ($[P_1]^-$), $[\beta\text{-CD}]^{2-}$ ($[CD]^{2-}$), $[2ImImIm\beta COO]^-$ ($[2P_1]^-$), $[\beta\text{-CD}]^-$ ($[CD]^-$), $[complex]^-$ ($[CD + P_1]^-$) of ImImImβCOOH and β-CD, respectively.

ESI mass spectra of complexes of β-CD with polyamide acids show that, in the case of $\mathbf{P_1}$ and $\mathbf{P_2}$, the abundance of $[CD + P_n]^-$ ions is much greater than that of $[P_n]^-$, while the abundance of $[CD + P_5]^-$ is less than that of $[P_5]^-$. This result suggests that the *N*-methylimidazole (Im) ring in polyamide acids is more favorable for binding with β-CD than the *N*-methylpyrrole (Py) ring. The *N*-methylimidazole (Im) moiety has more nitrogen atoms than *N*-methylpyrrol (Py) and, therefore, more interactions, via hydrogen bonding, take place between polyamide acids containing the imidazole group and β-CD. In addition, the existence of nitryl at the end of the polyamides is unfavorable for binding, because it counteracts the superior hydrophobic interactions between β-CD and polyamides.

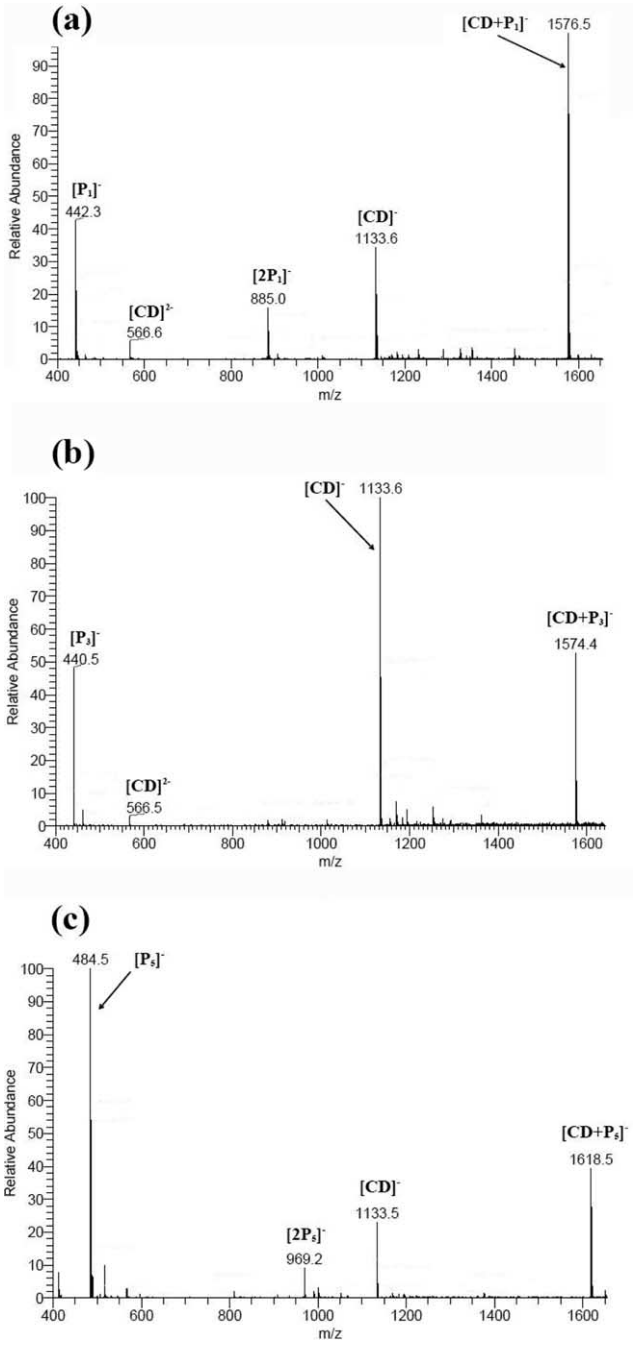


Figure 2. ESI mass spectra of equimolar mixtures of β-CD with (**a**) ImImImβCOOH ($\mathbf{P_1}$), (**b**) ImPyPyβCOOH ($\mathbf{P_3}$), and (**c**) NO_2PyPyPyβCOOH ($\mathbf{P_5}$).

Table 1. Effect of β-CD: P_2 ratio on abundance

Molar ratio	$[P_2]^-$ (%)	$[CD+P_2]^-$ (%)	$\frac{[CD+P_2]^-\%}{[P_2]^-\%+[CD+P_2]^-\%}$
1:1	51	100	0.66
2:1	30	100	0.77
5:1	18	100	0.85
10:1	12	100	0.91
25:1	7	77	0.93
50:1	3	58	0.96

Effects of β-CD Concentration on Relative Intensities of the Respective Complex

Complexes of β-CD with each polyamide acid, at molar ratios ranging from 1:1 to 50:1, were analyzed by ESI-MS in the negative mode. As an example, the effect on binding of β-CD: ImPyIm*β*COOH (**P_2**) molar ratios is listed in Table 1, in which relative abundances listed were normalized to 100% for each spectrum.

Table 1 shows that the abundance of polyamide acid decreased gradually when the molar ratio of β-CD to polyamide acid increased from 1:1 to 50:1. Here, $\frac{[CD+P_n]^-\%}{[P_n]^-\%+[CD+P_n]^-\%}$ (the relative intensity of the complex ion ($[CD + P_n]^-$) is expressed relative to the sum abundance of $[P_n]^-$ and $[CD + P_n]^-$) is introduced as a parameter for analysis of the binding property [29]. Figure 3 shows the relation between $\frac{[CD+P_2]^-\%}{[P_2]^-\%+[CD+P_2]^-\%}$ and $[CD]_t$ (initial concentration of β-CD). At molar ratios ranging from 1:1 to 10:1, the value of $\frac{[CD+P_2]^-\%}{[P_2]^-\%+[CD+P_2]^-\%}$ increased quickly, but the increase is slow in the 25:1 to 50:1 M ratio range. Using this expression, $\frac{[CD+P_n]^-\%}{[P_n]^-\%+[CD+P_n]^-\%}$, the repeatability of the relative intensities of the complex ion is best in all samples (Figure 4), so this parameter (hereafter abbreviated as Ir [29]) was used to determine binding constants.

Considering I_r as a unique parameter, it is better to find a linear relationship between complex ion intensity and $[CD]_t$. Based on the relationship of the function in Figure 3, the reciprocal of I_r was chosen to illustrate the effect of β-CD concentration (the reciprocal of $[CD]_t$), leading to a superior linear progression in Figure 5. Thus, a linear equation, Y = bX + c, could be obtained, which describes the correlation of complex ion intensity and initial β-CD concentrations.

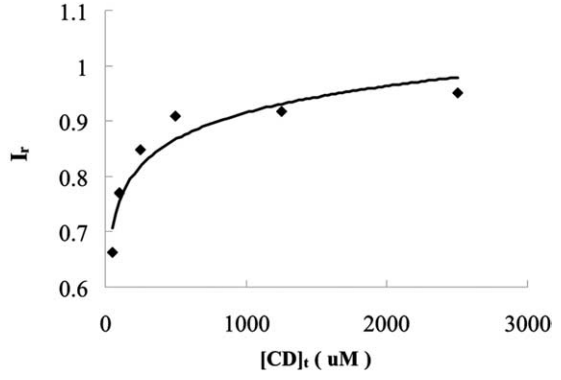


Figure 3. Effect of $[CD]_t$ on relative intensity (I_r) of the complex ion of ImPyIm*β*COOH (**P_2**) with β-CD.

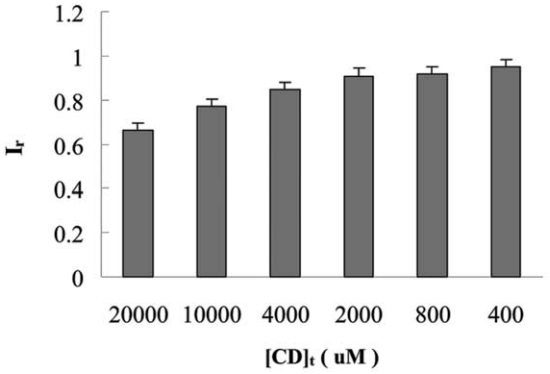


Figure 4. Repeatability of relative intensities (I_r) of the complex ion between ImPyIm*β*COOH (**P_2**) and β-CD ($n = 3$ for each point).

Evaluation of Complex Binding Constants

Based on the linear progression in Figure 5, a double reciprocal linear eq 1 [29] was introduced for the calculation of binding constants, which is another expression of the linear equation, Y = bX + c, and contains a K_{st} factor:

$$\frac{1}{I_r} = \frac{1}{k_c[P_n]_t K_{st}[CD]_t} + \frac{1}{k_c[P_n]_t} \quad (1)$$

where I_r is $\frac{[CD+P_n]^-\%}{[P_n]^-\%+[CD+P_n]^-\%}$ (relative intensity of the complex ion) and $I_r = k_c[CD + P_n]^-$ (at every molar ratio of CD:P); k_c is a proportionality constant, $[CD]_t$ is initial concentration of β-CD, which is the same as that

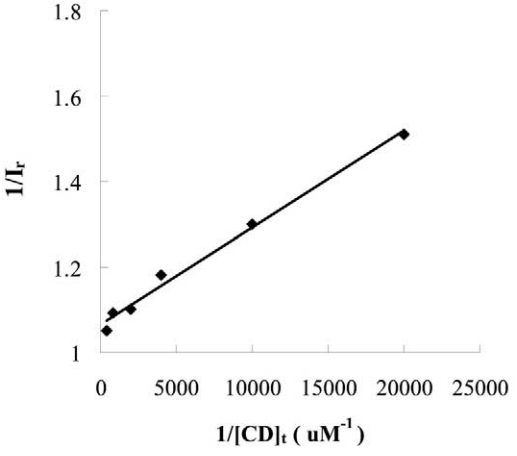


Figure 5. Effect of $1/[CD]_t$ on the reciprocal of the relative intensity ($1/I_r$) of the complex of ImPyIm*β*COOH (**P_2**) with β-CD.

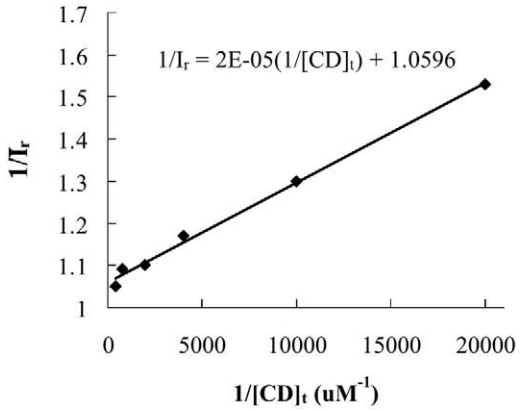


Figure 6. Effect of $1/[CD]_t$ on the relative intensity ($1/I_r$) of the complex ion ($[CD + \mathbf{P_1}]^-$) based on eq 1.

in Figure 5, $[P_n]_t$ is initial total concentration of polyamide acid and K_{st} is the binding constant of a 1:1 complex.

Comparing the two linear equations, $Y = bX + c$ and eq 1, Y, X, b, and c can be defined as follows:

$$Y = \frac{1}{I_r} \tag{2}$$

$$X = \frac{1}{[CD]_t} \tag{3}$$

$$b = \frac{1}{k_c[P_n]_t K_{st}} \tag{4}$$

$$c = \frac{1}{k_c[P_n]_t} \tag{5}$$

where b is the slope and c is the intercept of the equation. The ratio of c to b can be expressed as follow:

$$\frac{c}{b} = \frac{\dfrac{1}{k_c[P_n]_t}}{\dfrac{1}{k_c[P_n]_t K_{st}}} = K_{st} \tag{6}$$

Eq 6 can then be rewritten as:

$$K_{st} = \frac{c}{b} \tag{7}$$

Therefore, the binding constant (K_{st}) can easily be calculated from the ratio of the intercept to the slope of eq 1.

In the evaluation of the complex ($[\mathbf{P_n} + CD]^-$), five linear diagrams could be obtained with excellent linearity. As an example, the case of the $[\mathbf{P_1} + CD]^-$ ion is shown in Figure 6, and a linear equation, $1/I_r = 2E\text{-}05(1/[CD]_t) + 1.0596$, is obtained (where the intercept c is equal to 1.0596, and the slope b is equal to 2×10^{-5}). The binding constant (K_{st}) of $[\mathbf{P_1} + CD]^-$ was calculated, from the intercept and the slope of eq 1, based on eq 7):

$$K_{st} = \frac{c}{b} = \frac{1.0596}{2E-05} = 5.3 \times 10^4 (\mu M^{-1})$$

In the same way, the K_{st} value can be obtained from the intercept and slope of a weighted least-squares regression fit of the data to eq 1 for each complex, as summarized in Table 2.

To validate the results using the double reciprocal linear equation above, a base equation (eq 8) [37] was used to calculate the binding constants. This equation is useful in determining the binding constant of a known system:

$$[CD] + [P_n] \rightleftharpoons [CD + P_n]$$
$$K_{st} = \frac{[CD + P_n]}{[CD][P_n]} = \frac{[CD + P_n]}{[P_n]} \times \frac{1}{([CD]_t - [CD + P_n])} \tag{8}$$

where $[CD + P_n]$, [CD], and $[P_n]$ are the equilibrated concentrations of the complex, β-CD and polyamide acid, respectively. $[CD] = [CD]_t - [CD + P_n]$, $[CD]_t$ is the initial concentration of β-CD. The binding constants (K_{st}) between β-CD and the five polyamide acids were calculated based on eq 8 [37], as listed also in Table 2.

The K_{st} values in Table 2 show good agreement between the two methods. Obviously, the double reciprocal linear eq 1 method is better for precision and simplicity. The r^2 values are >0.93 in all cases, indicating a good correlation between the linear equations and

Table 2. Linear equations and binding constants for polyamide acids with β-CD*

Guest	Linear equation	r^2	K_{st}^a / $\times 10^4$ M^{-1}	K_{st}^b / $\times 10^4$ M^{-1}
P_1	$(1/Ir) = 2E\text{-}05\ (1/[CD]_t) + 1.0596$	0.99	5.3 (±0.2)	5.2 (±1.8)
P_2	$(1/Ir) = 2E\text{-}05\ (1/[CD]_t) + 1.0388$	0.97	5.2 (±0.2)	5.1 (±0.7)
P_3	$(1/Ir) = 4E\text{-}05\ (1/[CD]_t) + 1.2063$	0.99	3.0 (±0.1)	3.3 (±1.2)
P_4	$(1/Ir) = 4E\text{-}05\ (1/[CD]_t) + 1.1756$	0.93	2.9 (±0.1)	2.8 (±0.3)
P_5	$(1/Ir) = 8E\text{-}05\ (1/[CD]_t) + 1.7644$	0.93	2.2 (±0.1)	0.9 (±0.2)

*Value is the average of three measurements.
[a]K_{st} values calculated by eq. (1) and (7).
[b]K_{st} values calculated by eq. (8).

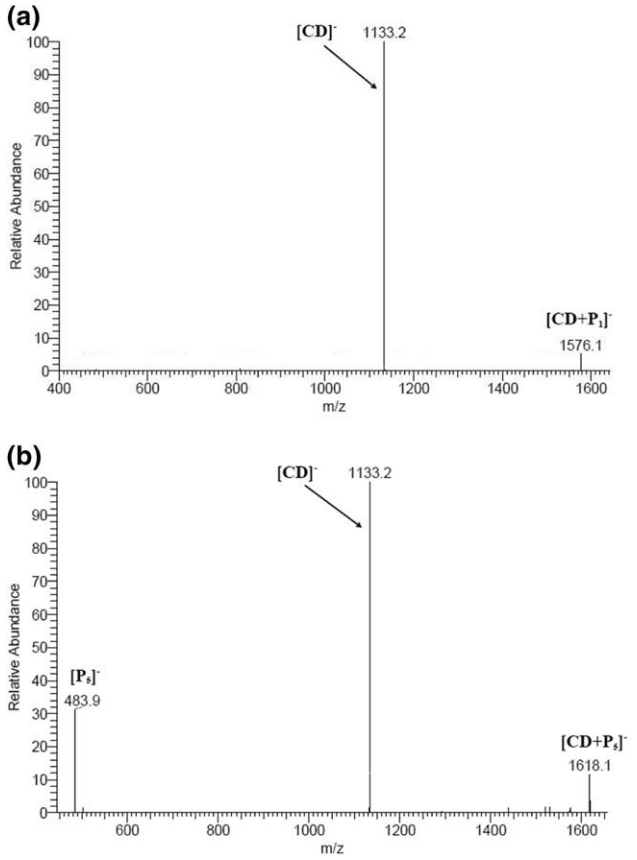


Figure 7. MS/MS spectra of $[CD + P_n]^-$: (**a**) ImImImβCOOH ($\mathbf{P_1}$) and (**b**) NO_2PyPyPyβCOOH ($\mathbf{P_5}$).

experimental values from ESI mass spectra. The K_{st} values of the complexes are in the 10^4 range; the values of $\mathbf{P_1}$ are the maxima, while the minimum is $\mathbf{P_5}$. These K_{st} values also support the fact that *N*-methylimidazole (Im) is more beneficial for binding with β-CD than *N*-methylpyrrole (Py) via hydrogen bonding, and that nitryl is unfavorable for binding of polyamide acid and β-CD.

Property of Complex Ions Between β-Cyclodextrin and Polyamide Acids

Fragmentation and stability of the complex ions were investigated by MS/MS spectra. In MS/MS spectra analysis, the fragmentations of $[CD + P_n]^-$ appeared when the CID energy increased to ~20%, which suggested that the complex ions of β-CD and polyamide acids are stable. Representative MS/MS spectra of the complex ions ($[CD + P_n]^-$) are shown in Figure 7. In the case of ImImImβCOOH ($\mathbf{P_1}$), $[CD + P_1]^-$ dissociated into β-CD and $\mathbf{P_1}$, and only $[CD]^-$ was observed at *m/z* 1133.2. In the case of NO_2PyPyPyβCOOH ($\mathbf{P_5}$), $[CD + P_5^-$ dissociated into $[CD]^-$ and $[P_5]^-$, which were both observed in the MS/MS spectrum. The complex ions of other polyamide acids also generated $[CD]^-$ ion only in the MS/MS spectra of $[CD + P_2]^-$, $[CD + P_3]^-$, and $[CD + P_4]^-$. These results suggested that the nitryl induced polyamide $\mathbf{P_5}$ to yield a negative ion by the loss of a proton.

The use of capillary heating to dissociate the complex could provide additional information regarding the properties of complex ions between β-cyclodextrin and polyamide acids in the gas-phase [38–40]. In this study, capillary temperature was increased from 150 to 400 °C to examine the thermo-stability of $[CD + P_n]^-$. The experimental results show that the complex ions remained dominant in ESI mass spectra, even when the temperature was increased to 400 °C. Consequently, the binding of β-CD and polyamide acids ($[CD + P_n]^-$) is thermodynamically stable.

With respect to the 1:1 complex ion, it was noticeable that the 2:1 or 1:2 complex ions could be observed, but were very weak and were only observed when β-CD or $\mathbf{P_n}$ was in excess. For example, when the ratio β-CD/$\mathbf{P_1}$ was increased to 50:1, the $[2CD + P_1]$ complex ion could be observed (<5%) in ESI spectrum, which indicated that 1:1 complexes were the dominant binding mode compared with the 2:1 complexes, even at the highest β-CD concentration (50:1 M ratio). In addition, the intensities of 2:1 and 1:2 complex ions decreased when capillary temperature increase and, subsequently, disappeared at 300 °C. Therefore, 2:1 and 1:2 complex ions are thermodynamically unstable.

Conclusions

The present work has demonstrated the ability of ESI-MS to provide strong evidence for noncovalent binding between polyamide acids and β-CD. The 1:1 binding mode was indicated initially by examining the binding stoichiometry from ESI mass spectra. The method simultaneously gives binding affinity in the form of binding constants, based on a novel linear equation, and shows that polyamide acids with β-CD have binding affinities in the order: ImImImβCOOH > ImPyImβCOOH > ImPyPyβCOOH > PyPyPyβCOOH > NO_2PyPyPyβCOOH. In addition, sample consumption is less than 1 nmol per analysis, which makes this method useful when only small amounts of sample are available.

Acknowledgments

This project is supported by the National Natural Science Foundation of China (nos. 20272005 and 20472009).

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