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: ImImIm β COOH > ImPyIm β COOH > ImPyPy β COOH > PyPyPy β COOH > NO₂PyPyPy β COOH. 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, pharmaceuticals, 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 ¹H 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 (**P**₁–**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 μ l/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 (N₂) was used to ensure efficient

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R=H, X=N, Y=N, Z=N: ImImIm β COOH (P_1)

X=N, Y=C, Z=N: ImPyIm β COOH (P_2)

X=N, Y=C, Z=C: ImPyPy β COOH (P_3)

X=C, Y=C, Z=C: PyPyPy β COOH (P_4)

R=NO₂, X=C, Y=C, Z=C: NO₂PyPyPy β COOH (P_5)

Figure 1. Structures of polyamide acids (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 (P_1 – 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 μ l β -CD (5.0 mM) were mixed with 0.5 μ l of polyamide acids (5.0 mM). This solution was diluted with methanol/H₂O (20:80, vol/vol) to 50 μ l 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 μ M) of β -CD and the same initial concentration (50 μ M) 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 ImImIm β COOH (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 β COO][−] ($[P_1]^-$), [β -CD]^{2−} ($[CD]^{2-}$), [2ImImIm β COO][−] ($[2P_1]^-$), [β -CD][−] ($[CD]^-$), [complex][−] ($[CD + P_1]^-$) of ImImIm β COOH and β -CD, respectively.

ESI mass spectra of complexes of β -CD with polyamide acids show that, in the case of P_1 and 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*-methylpyrrole (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 nitril 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) ImImIm β COOH (P_1), (b) ImPyPy β COOH (P_3), and (c) NO₂PyPyPy β COOH (P_5).

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

Molar ratio	$[P_2]^-$ (%)	$[CD+P_2]^-$ (%)	$[CD+P_2]^-$ %
			$\frac{[CD+P_2]^-}{[P_2]^- + [CD+P_2]^-}$ %
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 I_r [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

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).

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.

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 $1/[\text{CD}]_t$ on the relative intensity ($1/I_r$) of the complex ion ($[\text{CD} + \text{P}_1]^-$) based on eq 1.

in Figure 5, $[\text{P}_n]_t$ 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:

$$Y = \frac{1}{I_r} \quad (2)$$

$$X = \frac{1}{[\text{CD}]_t} \quad (3)$$

$$b = \frac{1}{k_c[\text{P}_n]_t K_{st}} \quad (4)$$

$$c = \frac{1}{k_c[\text{P}_n]_t} \quad (5)$$

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

$$\frac{c}{b} = \frac{\frac{1}{k_c[\text{P}_n]_t}}{\frac{1}{k_c[\text{P}_n]_t K_{st}}} = K_{st} \quad (6)$$

Eq 6 can then be rewritten as:

$$K_{st} = \frac{c}{b} \quad (7)$$

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 ($[\text{P}_n + \text{CD}]^-$), five linear diagrams could be obtained with excellent linearity. As an example, the case of the $[\text{P}_1 + \text{CD}]^-$ ion is shown in Figure 6, and a linear equation, $1/I_r = 2\text{E-}05(1/[\text{CD}]_t) + 1.0596$, 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 $[\text{P}_1 + \text{CD}]^-$ was calculated, from the intercept and the slope of eq 1, based on eq 7):

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

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:

$$\begin{aligned} [\text{CD}] + [\text{P}_n] &\rightleftharpoons [\text{CD} + \text{P}_n] \\ K_{st} &= \frac{[\text{CD} + \text{P}_n]}{[\text{CD}][\text{P}_n]} = \frac{[\text{CD} + \text{P}_n]}{[\text{P}_n]} \times \frac{1}{([\text{CD}]_t - [\text{CD} + \text{P}_n])} \end{aligned} \quad (8)$$

where $[\text{CD} + \text{P}_n]$, $[\text{CD}]$, and $[\text{P}_n]$ are the equilibrated concentrations of the complex, β -CD and polyamide acid, respectively. $[\text{CD}] = [\text{CD}]_t - [\text{CD} + \text{P}_n]$, $[\text{CD}]_t$ 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	$K_{st}^a / \times 10^4 \text{ M}^{-1}$	$K_{st}^b / \times 10^4 \text{ M}^{-1}$
P ₁	$(1/I_r) = 2\text{E-}05 (1/[\text{CD}]_t) + 1.0596$	0.99	5.3 (± 0.2)	5.2 (± 1.8)
P ₂	$(1/I_r) = 2\text{E-}05 (1/[\text{CD}]_t) + 1.0388$	0.97	5.2 (± 0.2)	5.1 (± 0.7)
P ₃	$(1/I_r) = 4\text{E-}05 (1/[\text{CD}]_t) + 1.2063$	0.99	3.0 (± 0.1)	3.3 (± 1.2)
P ₄	$(1/I_r) = 4\text{E-}05 (1/[\text{CD}]_t) + 1.1756$	0.93	2.9 (± 0.1)	2.8 (± 0.3)
P ₅	$(1/I_r) = 8\text{E-}05 (1/[\text{CD}]_t) + 1.7644$	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 (P_1) and (b) NO₂PyPyPyβCOOH (P_5).

experimental values from ESI mass spectra. The K_{st} values of the complexes are in the 10^4 range; the values of P_1 are the maxima, while the minimum is 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 nitril 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 (P_1), $[CD + P_1]^-$ dissociated into β-CD and P_1 , and only $[CD]^-$ was observed at m/z 1133.2. In the case of NO₂PyPyPyβCOOH (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 nitril induced polyamide 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 P_n was in excess. For example, when the ratio β-CD/ 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 > NO₂PyPyPyβCOOH. In addition, sample consumption is less than 1 nmol per analysis, which makes this method useful when only small amounts of sample are available.

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