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Study on the inclusion complexes of cyclodextrin and sulphonated azo dyes by electrospray ionization mass spectrometry

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Abstract

The inclusion complexes of α-, β-cyclodextrin (α-, β-CD) and sulphonated azo dyes ligands (Orange II, Ponceau SX, Allura red AC and Tartrazine) were studied by electrospray ionization mass spectrometry (ESI-MS) and the dissociation constants (K_D) of the inclusion complexes were determined. A new method to obtain the dissociation constants of CD–ligand inclusion complexes without curve fitting was developed. Once the total concentrations of CD and ligand have been known, K_D can be calculated from the sum peak intensities of free CD and inclusion complex and the number of binding site can be obtained from the mass spectrum. Ponceau SX, Allura red AC and Tartrazine binding to α-CD form 1:1 inclusion complexes with K_D values of 1.33×10^{-5} mol L^{-1}, 4.85×10^{-6} mol L^{-1} and 7.47×10^{-5} mol L^{-1}, respectively. The obtained K_D values of the inclusion complexes of above-mentioned three sulphonated azo dyes ligands binding to β-CD in turn are 3.93×10^{-6} mol L^{-1}, 6.50×10^{-6} mol L^{-1} and 1.12×10^{-4} mol L^{-1}, respectively. The 1:1 and 1:2 inclusion complexes are found in the systems of CD and Orange II. $K_{D,1}$ and $K_{D,2}$ of α-CD and Orange II inclusion complexes are 4.05×10^{-4} mol L^{-1} and 4.60×10^{-7} (mol L^{-1})^2, respectively. 3.94×10^{-5} mol L^{-1} and 1.72×10^{-7} (mol L^{-1})^2 are the $K_{D,1}$ and $K_{D,2}$ of β-CD and Orange II inclusion complexes, respectively. The competition experiments were performed to validate the results obtained by one ligand. According to the proposed method, the K_D values of inclusion complexes regardless of any stoichiometric relation of host and guest can be obtained.

Keywords: Inclusion complex; Cyclodextrin; Sulphonated azo dye; Electrospray ionization mass spectrometry; Dissociation constant

1. Introduction

Sulphonated azo dyes have been widely used as coloring agents in foodstuffs, paper and cosmetic and so on. However, some of these dyes pose a potential risk to human health and are even carcinogenic [1,2]. α- and β-cyclodextrins (α- and β-CDs) are cyclic (α-1,4) linked oligosaccharides consisting of 6 and 7 α-D-glucopyranose molecules, respectively. The exterior sides of them are hydrophilic for the outward hydroxyl groups, while the interior cavities are hydrophobic, so they can behave as hosts. Organic and inorganic molecules of appropriate dimensions and properties can be included into the cavities of CDs to form inclusion complexes selectively. The ability of CDs has been validated for various purposes. CDs are known to affect the spectral and chemical properties of guest dramatically such as increasing the solubility, stability or bioavailability [3], weakening the bitter taste [4] and so on. Incorporated into the CD's cavity, guest molecule could be released lingeringly. So it is worthy to study CD–ligand complex that can provide valuable information. The dissociation constant can reflect the binding equilibrium, so it is of great worth to determine the dissociation constants of inclusion complexes. As far as know, there is no report about determination of the dissociation constants of CD-sulphonated azo dye inclusion complexes by ESI-MS.

There are many studies on the interaction of CD and ligands with various methods such as UV–vis spectrometry [5–7], HPLC [8], fluorescence spectrometry [9–12], NMR [13] and so forth [14,15]. As a soft ionization technique, electrospray ionization mass spectrometry (ESI-MS) has been acting as an important tool in research of non-covalent complexes in recent years due to

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many advantages over other techniques. High sensitivity, specificity and speediness are the proverbial virtues. Furthermore, ESI-MS can provide the stoichiometric information of the complex directly and detect multiple components simultaneously. However, many researchers encountered the following problems in determining dissociation constants by ESI-MS [16]. It may be ambiguous whether non-covalent complexes will dissociate when they are transferred from the solution phase to the gas phase. The question if the behavior in gas phase represents that in solution is under dispute. Consequently many studies of determining the dissociation constant of complex by ESI-MS made some assumptions. Despite all that, a lot of examples of determining dissociation constant of complex of protein or peptide and guest were reported in the literature [17–26]. For the system of CD–ligand, the studies on qualitative determination were widely carried out by ESI-MS and the quantitative determination of the dissociation constant of CD–ligand is insufficient. The determination of the binding constant of β-cyclodextrin and three-model "guest" molecules by ESI-MS was reported [27].

In this study, we made the same assumptions as were reported in the literature [28]: first, no change happens when the mixture is transferred from the solution phase to the gas phase and the dissociation of bound ligand hardly occurs. Secondly, the measured peak intensities of both free CD and its complexes are directly proportional to their equilibrium concentrations in the solution. Thirdly, free CD and CD–ligand inclusion complex share similar ionization efficiencies, namely the ionization percent of molecule, so we assumed that CD and inclusion complex with equal concentrations in solution would occupy the same intensities in mass spectrum. According to the three assumptions, we can say that the ratio of the equilibrium concentrations of free CD and CD–ligand inclusion complex is equal with that of the intensities of them. And according to the proposed method, the dissociation constants of the inclusion complexes of four ligands (L), Orange II (L_O), Ponceau SX (L_P), Allura red AC (L_A) and Tartrazine (L_T) (Fig. 1) binding to CD (α- and β-CD), respectively, have been obtained. According to the proposed method, in spite of the inclusion complex combination in any stoichiometric relation, the dissociation constants can be obtained without curve fitting.

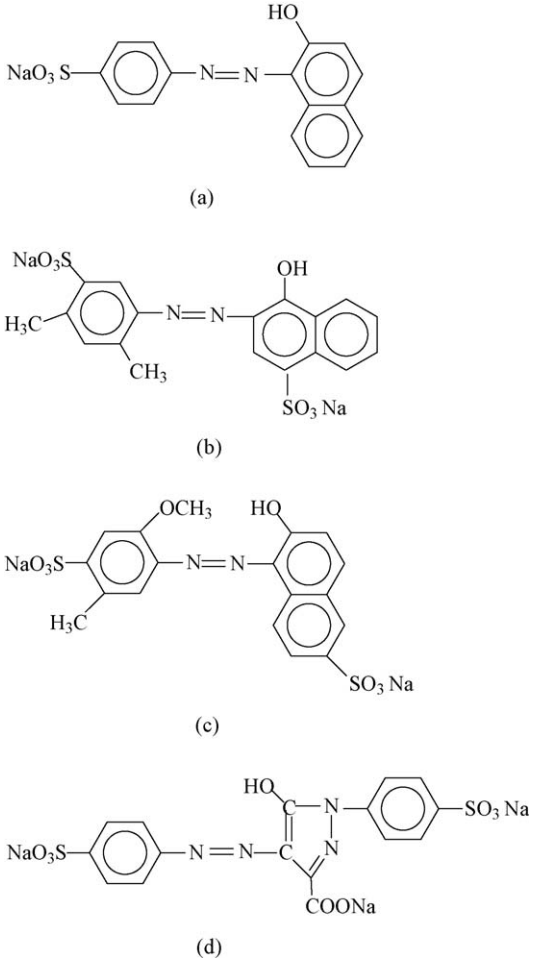

Fig. 1. Structure of (a) Orange II, (b) Ponceau SX, (c) Allura red AC and (d) Tartrazine.

2. Experimental

2.1. Chemicals

α-Cyclodextrin (α-CD, MW = 972) and β-cyclodextrin (β-CD, MW = 1135) were provided by Shanxi Liquan Chemicals Enterprise Co. Ltd. (Liquan, China) and Shanghai Sanpu Limited Company (Shanghai, China), respectively. Orange II (MW = 350.33, CI 15510), Ponceau SX (MW = 480.4, CI 14700) and Allura red AC (MW = 496.43, CI 16035) were obtained from TCI (Tokyo, Japan). Tartrazine (MW = 534.37, CI 19140) was purchased from Fluka. The deionized water was purified using a Milli-Q system (Millipore, Belford, MA, USA). All chemicals were dissolved in water and prepared into 1.0×10^{-3} mol L^{-1} stock solutions.

2.2. Instruments

An ABI Q-Trap Mass Spectrometer (Applied Biosystems Sciex, Foster City, USA) and an ABI Q-Star Hybrid Quadrupole TOF mass spectrometer (Applied Biosystems Sciex, Foster City, USA) were used. The instruments are equipped with electrospray ionization (ESI) source and interfaced to a computer running Applied Biosystems Analyst version 1.4 software. The former is capable of recording ions up to *m/z* 1700 and the latter can determine higher mass complexes. Nitrogen is used for nebulizer gas and curtain gas.

2.3. Procedures

For each system, a series of samples with the constant concentration of CD and the different concentrations of ligand were prepared. After mixed evenly, the mixture was then incubated at room temperature of 23 ± 1 °C for an appropriate period of time to bring into the state of equilibrium. The solution was filtered through a 0.45 μm membrane prior to transferring into ESI-MS. All samples were operated in negative polarity mode.

In the competition experiment of one CD and two ligands, the concentrations of CD and one ligand were kept constant and

Table 1
The experimental parameters for ESI-MS

CD	Ligand (s)	Ionspray voltage (V)	Nebulizer gas (psi)	Declustering potential (V)
α-CD	Orange II	−3000	30	−80
α-CD	Ponceau SX	−4000	25	−50
α-CD	Allura red AC	−3700	30	−50
α-CD	Tartrazine	−3000	30	−30
β-CD	Orange II	−3000	30	−80
β-CD	Ponceau SX	−4000	25	−50
β-CD	Allura red AC	−3700	25	−70
β-CD	Tartrazine	−3000	30	−50
α-CD	Ponceau SX and Allura red AC	−4000	30	−40
β-CD	Ponceau SX and Allura red AC	−4000	30	−40
α-CD	Orange II and Tartrazine	−3600	25	−60

the concentration of another ligand changed. The other operation processes were the same processes as procedure mentioned above.

It is important to optimize the ESI-MS parameters to avoid destroying the non-covalent binding in the gas phase. For different systems, the optimal instrument conditions are not uniform completely. For all systems, curtain gas was maintained at 20 psi, and collision gas was chosen "high". Entrance potential and collision energy were −10 V and −10 eV, respectively. Other parameters, such as ionspray voltage, nebulizer gas and declustering potential are different for various systems of CD and ligands as shown in Table 1. The scan rate is 1000 amu s^{-1}. All samples were injected via a syringe pump of 4.61 mm diameter at a rate of 5 μL min^{-1}. The data of each sample were acquired for 3 min in some appropriate mass range.

3. Theories

3.1. One ligand

In this paper, the calculation method of K_D is modified based on the above-mentioned assumptions. For a system containing one kind of ligand (L) binding and CD with one or many binding sites,

$$CDL \overset{K_{D,1}}{\rightleftharpoons} CD + L \quad CDL_2 \overset{K_{D,2}}{\rightleftharpoons} CD + 2L \cdots$$
$$CDL_n \overset{K_{D,n}}{\rightleftharpoons} CD + nL$$

the dissociation constants are defined as:

$$K_{D,1} = \frac{[CD][L]}{[CDL]} \tag{A.1}$$

$$K_{D,2} = \frac{[CD][L]^2}{[CDL_2]} \tag{A.2}$$

$$\vdots$$

$$K_{D,n} = \frac{[CD][L]^n}{[CDL_n]} \tag{A.3}$$

where [CD], [L] and [CDL] are the equilibrium concentration of CD, L and CDL, respectively, and n is the binding site number which can be observed from the mass spectrum. $[CD]_0$ is the total concentration of CD in the solution and $[CD]_0 = [CD] + [CDL] + [CDL_2] + \cdots + [CDL_n]$. $[L]_0$ is the total concentration of ligand in the solution and $[L]_0 = [L] + [CDL] + 2[CDL_2] + \cdots + n[CDL_n]$. According to the assumptions above-mentioned, the relative abundances obtained from the mass spectral data are a reflection of the concentrations in the solution phase: $[CD]_0 \propto$ total intensities of CD and CDL ($\sum$CD); $[CDL] \propto$ total intensity of CDL ($\sum$CDL) and $[CDL_n] \propto$ total intensity of CDL_n ($\sum CDL_n$). These formulas can be obtained:

$$a_1 = \frac{[CDL]}{[CD]_0} = \frac{\sum CDL}{\sum CD + \sum CDL + \sum CDL_2 + \cdots + \sum CDL_n} \tag{A.4}$$

$$a_2 = \frac{[CDL_2]}{[CD]_0} = \frac{\sum CDL_2}{\sum CD + \sum CDL + \sum CDL_2 + \cdots + \sum CDL_n} \tag{A.5}$$

$$\vdots$$

$$a_n = \frac{[CDL_n]}{[CD]_0} = \frac{\sum CDL_n}{\sum CD + \sum CDL + \sum CDL_2 + \cdots + \sum CDL_n} \tag{A.6}$$

the total intensities of CD ($\sum$CD), CDL ($\sum$CDL), ⋯, CDL_n ($\sum CDL_n$) are easy to be determined by ESI-MS and then the $a_i(a_1, a_2, \cdots, a_n)$ values can be calculated. By rearranging [CDL], $[CDL_2]$ and $[CDL_n]$ in Eqs. (A.4)–(A.6), we can have

$$[CDL] = a_1[CD]_0 \tag{A.7}$$

$$[CDL_2] = a_2[CD]_0 \tag{A.8}$$

$$\vdots$$

$$[CDL_n] = a_n[CD]_0 \tag{A.9}$$

$$[CD] = [CD]_0 - a_1[CD]_0 - a_2[CD]_0 - \cdots - a_n[CD]_0 = (1 - a_1 - a_2 - \cdots - a_n)[CD]_0 \tag{A.10}$$

$$[L] = [L]_0 - [CDL] - 2[CDL_2] - \cdots - n[CDL_n] = [L]_0 - a_1[CD]_0 - 2a_2[CD]_0 - \cdots - na_n[CD]_0 \quad (A.11)$$

Then substituting Eqs. (A.7)–(A.11) into Eqs. (A.1)–(A.3), respectively, we get

$$K_{D,1} = \frac{(1 - a_1 - a_2 - \cdots - a_n)([L]_0 - a_1[CD]_0 - 2a_2[CD]_0 - \cdots - na_n[CD]_0)}{a_1} \quad (A.12)$$

$$K_{D,2} = \frac{(1 - a_1 - a_2 - \cdots - a_n)([L]_0 - a_1[CD]_0 - 2a_2[CD]_0 - \cdots - na_n[CD]_0)^2}{a_2} \quad (A.13)$$

$$\vdots$$

$$K_{D,n} = \frac{(1 - a_1 - a_2 - \cdots - a_n)([L]_0 - a_1[CD]_0 - 2a_2[CD]_0 - \cdots - na_n[CD]_0)^n}{a_n} \quad (A.14)$$

More simply, the Eqs. (A.12)–(A.14) can be represented as a general equation:

$$K_{D,m} = \frac{(1 - a_1 - a_2 - \cdots - a_n)([L]_0 - a_1[CD]_0 - 2a_2[CD]_0 - \cdots - na_n[CD]_0)^m}{a_m} = \frac{(1 - \sum a_n)([L]_0 - [CD]_0 \sum na_n)^m}{a_m} \quad (A.15)$$

According to Eq. (A.15), K_D of CDL_m ($1 \le m \le n$) can be obtained for a system containing one kind of ligand.

3.2. Multiple ligands

For a system containing multiple ligands (L_A, L_B, $\cdots$, and L_X) and CD with one or many binding sites, such as x kinds of ligands and n biding sites,

$$CDL_A \overset{K_{D,A1}}{\rightleftharpoons} CD + L_A \quad CD(L_A)_2 \overset{K_{D,A2}}{\rightleftharpoons} CD + 2L_A \cdots \quad CD(L_A)_n \overset{K_{D,An}}{\rightleftharpoons} CD + nL_A$$

$$CDL_B \overset{K_{D,B1}}{\rightleftharpoons} CD + L_B \quad CD(L_B)_2 \overset{K_{D,B2}}{\rightleftharpoons} CD + 2L_B \cdots \quad CD(L_B)_n \overset{K_{D,Bn}}{\rightleftharpoons} CD + nL_B$$

$$\vdots$$

$$CDL_x \overset{K_{D,X1}}{\rightleftharpoons} CD + L_X \quad CD(L_X)_2 \overset{K_{D,X2}}{\rightleftharpoons} CD + 2L_X \cdots \quad CD(L_X)_n \overset{K_{D,Xn}}{\rightleftharpoons} CD + nL_X$$

In the same way, we consider the followings:

$$a_n = \frac{[CD(L_A)_n]}{[CD]_0} = \frac{\sum CD(L_A)_n}{\sum CD + \sum CDL_A + \sum CD(L_A)_2 \cdots + \sum CD(L_A)_n} \quad (B.1)$$

$$b_n = \frac{[CD(L_B)_n]}{[CD]_0} = \frac{\sum CD(L_B)_n}{\sum CD + \sum CDL_B + \sum CD(L_B)_2 \cdots + \sum CD(L_B)_n} \quad (B.2)$$

$$\vdots$$

$$x_n = \frac{[CD(L_X)_n]}{[CD]_0} = \frac{\sum CD(L_X)_n}{\sum CD + \sum CDL_X + \sum CD(L_X)_2 \cdots + \sum CD(L_X)_n} \quad (B.3)$$

$$[CD] = [1 - (a_1 + a_2 + \cdots + a_n) - (b_1 + b_2 + \cdots + b_n) - \cdots - (x_1 + x_2 + \cdots + x_n)][CD]_0 \quad (B.4)$$

$$[L_A] = [L_A]_0 - (a_1 + 2a_2 + \cdots + na_n)[CD]_0 \quad (B.5)$$

$$[L_B] = [L_B]_0 - (b_1 + 2b_2 + \cdots + nb_n)[CD]_0 \quad (B.6)$$

$$\vdots$$

$$[L_X] = [L_X]_0 - (x_1 + 2x_2 + \cdots + nx_n)[CD]_0 \quad (B.7)$$

For ligand A, the $K_{D,Am}$ of CDL_{Am} ($1 \le m \le n$) can be calculated with following equation:

$$K_{D,Am} = \frac{(1 - \sum a_n - \sum b_n - \cdots - \sum x_n)([L_A]_0 - [CD]_0 \sum na_n)^m}{a_m} \quad (B.8)$$

The Eq. (B.8) is also suitable for the other ligands. In the fact, the Eq. (A.15) is a special form of Eq. (B.8) at special conditions and the Eq. (B.8) can be widely applied.

4. Results and discussion

4.1. The mass spectra for CD and sulphonated azo dyes

4.1.1. The mass spectra for CD

In the negative mode, ions of $[CD - H]^-$ and $[CD + Cl]^-$ are in great abundance. Peaks at *m/z* 971.5 and 1007.5 represent that of $[\alpha\text{-CD} - H]^-$ and $[\alpha\text{-CD} + Cl]^-$, respectively. The *m/z* 1133.8 and 1169.8 are $[\beta\text{-CD} - H]^-$ and $[\beta\text{-CD} + Cl]^-$, respectively. The doubly charged state of ions, at *m/z* 485.3 and 566.4, represented α-CD and β-CD, respectively, can also be observed in the mass spectra.

4.1.2. The mass spectra for sulphonated azo dyes

Of the above-mentioned four sulphonated azo dyes, Orange II has one Na, and Ponceau SX and Allura red AC has two and Tartrazine has three. For Orange II, the ions at *m/z* 327.2 is $[L_O - Na]^-$. When the concentration of Orange II increases, the aggregation occurs and can be confirmed by the mass spectrum. For Tartrazine, there are ions at *m/z* 533.3 and 511.3 ($[L_T - H]^-$ and $[L_T - Na]^-$). The peaks of Ponceau SX are at *m/z* 457.2 and 435.1 ($[L_P - Na]^-$ and $[L_P - 2Na + H]^-$). The base peak of Allura red AC is at *m/z* 451.1 ($[L_A - 2Na + H]^-$). The mass spectra of sulphonated azo dyes are not shown independently and the peaks are higher than the peaks of CD and inclusion complexes in the mass spectra of mixture.

4.2. The mass spectra for CDL

4.2.1. One CD and one ligand

For α-CD and Orange II, the peaks of $[\alpha\text{-CD} + L_O - Na]^-$ and $[\alpha\text{-CD} + 2L_O - Na]^-$ are found in Fig. 2(a). $[\beta\text{-CD} + L_O - Na]^-$ and $[\beta\text{-CD} + 2L_O - Na]^-$ are observed from the mass spectra for the system of β-CD and Orange II as shown in Fig. 2(b). There are very weak peaks of $[\alpha\text{-CD} + L_O - H]^-$ and $[\beta\text{-CD} + L_O - H]^-$ in the mass spectra. For Ponceau SX and Allura red AC, only 1:1 inclusion complexes form in the mixed system, which are all doubly charged state of ions of $[CD + L - 2Na]^{2-}$ as shown in Fig. 2(c–f). Ions at *m/z* 704.2 and 785.4 represent the 1:1 inclusion complexes of Ponceau SX binding to α-CD and β-CD, respectively. Ions at *m/z* 712.2 and 792.9 represent the 1:1 inclusion complexes of Allura red AC binding to α-CD and β-CD, respectively. In the systems of Tartrazine and CD, besides $[CD + L_T - 2Na]^{2-}$, there are also $[CD + L_T - 3Na + H]^{2-}$ as shown in Fig. 2(g and h). It is obvious from the experimental results above-mentioned that Na is easier disintegrated from sulphonated azo dye than H. All the doubly charged state of ions can be conformed by the zoom-scan negative ESI mass spectra of the ions of inclusion complexes and the spectra are inserted in Fig. 2(c–h).

4.2.2. One CD and two ligands

In the competition experiment, two kinds of ligands were added into the system containing one kind of CD. From the mass spectra, two kinds of inclusion complexes are observed. The relative abundances of peaks of α-CD and Orange II decrease and that of Tartrazine increases with the increasing of Tartrazine concentration when the concentrations of α-CD and Orange II are constant. For the systems of α-CD, Ponceau SX and Allura red AC, two kinds of inclusion complexes are observed from Fig. 3(a). And the same result is observed for the system of β-CD, Ponceau SX and Allura red AC as shown in Fig. 3(b). For the system of α-CD, Orange II and Tartrazine, besides the peaks of free α-CD and ligands, two inclusion complex peaks of α-CD and Orange II, α-CD and Tartrazine, are found in the mass spectra as shown in Fig. 3(c).

4.2.3. Negative ESI tandem mass spectra

The tandem mass spectra of all ions of inclusion complexes were performed and the product ions are CD and sulphonated azo dyes. Fig. 4 showed the tandem mass spectrum of $[\alpha\text{-CD} + L_O - Na]^-$ ion at *m/z* 1299.4 and the tandem mass spectra of other ions were also obtained in the experiment.

4.3. Calculation of K_D of inclusion complexes

4.3.1. One ligand and one binding site

The relative peak abundance of inclusion complex increases with the increasing ligand concentration and then increases slightly or leaves off. The all peak relative abundances are obtained from the mass spectra data. According to the Eq. (A.15), K_D value can be calculated after calculating the a_i value. Table 2 shows the K_D of CD and Ponceau SX. For other reaction systems, the results obtained by the same calculation method as mentioned above are shown in the Table 3. It is seen from Tables 2 and 3 that the R.S.D. values were satisfying at the constant concentrations of CD and ligand and a conclusion can be drawn that the K_D value increases with the concentration ratio of ligand to CD when the concentration of CD is constant and the wider the concentrations range of the ligand is, the higher the R.S.D. of K_D values obtained is. So the concentrations of CD and ligands should be chosen appropriately.

4.3.2. One ligand and two binding sites

In the mass spectra of the mixture of CD and Orange II, 1:1 and 1:2 stoichiometric inclusion complexes of CD and ligand can be found obviously. The relative peak intensities are comparatively reproduceable. For the inclusion complexes of α-CD and β-CD, the resulting $K_{D,1}$ and $K_{D,2}$ values obtained with the Eq. (A.15) are shown in Table 4.

4.3.3. Two ligands and one binding site

In the competitive experiment, two ligands compete with each other for one binding site. On obtaining the relative abundances of peaks by ESI-MS, the K_D values of two inclusion complexes of CD and two kinds of ligands can be calculated according to

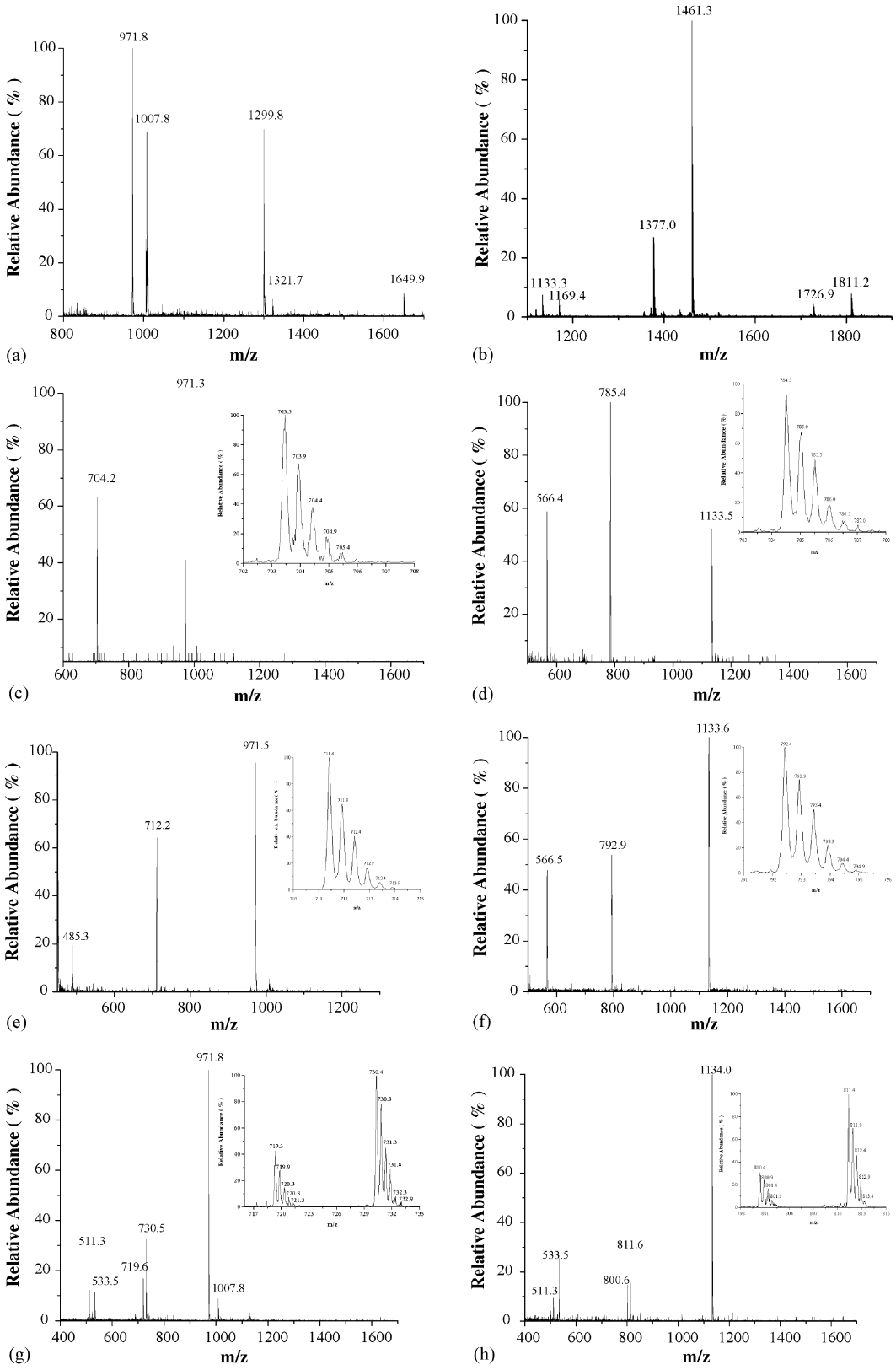

Fig. 2. Negative ESI mass spectra obtained for mixtures of (a) α-CD and Orange II, (b) β-CD and Orange II, (c) α-CD and Ponceau SX, inset shows the zoom-scan negative ESI mass spectrum of $[\alpha\text{-CD} + L_p - 2Na]^{2-}$ ion at *m/z* 703.5, (d) β-CD and Ponceau SX, inset shows the zoom-scan negative ESI mass spectrum of $[\beta\text{-CD} + L_p - 2Na]^{2-}$ ion at *m/z* 784.5, (e) α-CD and Allura red AC, inset shows the zoom-scan negative ESI mass spectrum of $[\alpha\text{-CD} + L_A - 2Na]^{2-}$ ion at *m/z* 711.4, (f) β-CD and Allura red AC, inset shows the zoom-scan negative ESI mass spectrum of $[\beta\text{-CD} + L_A - 2Na]^{2-}$ ion at *m/z* 792.4, (g) α-CD and Tartrazine, inset shows the zoom-scan negative ESI mass spectrum of $[\alpha\text{-CD} + L_T - 2Na]^{2-}$ and $[a\text{-CD} + L_T - 3Na + H]^{2-}$ ions at *m/z* 719.3 and 730.4 and (h) β-CD and Tartrazine, inset shows the zoom-scan negative ESI mass spectrum of $[\beta\text{-CD} + L_T - 2Na]^{2-}$ and $[\beta\text{-CD} + L_T - 3Na + H]^{2-}$ ions at *m/z* 800.4 and 811.4.

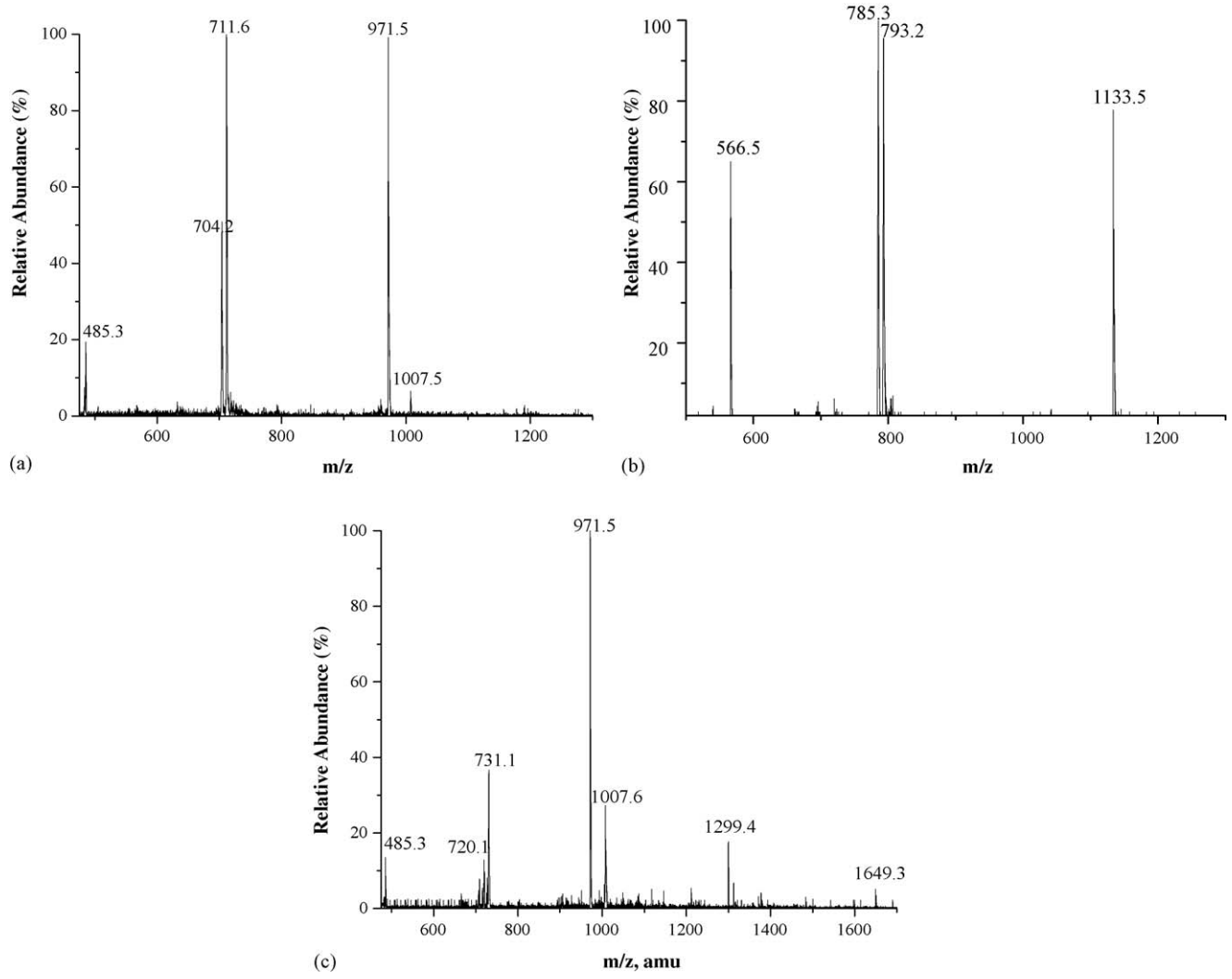

Fig. 3. Negative ESI mass spectra for (a) α-CD, Allura red AC and Ponceau SX, (b) β-CD, Allura red AC and Ponceau SX and (c) α-CD, Tartrazine and Orange II.

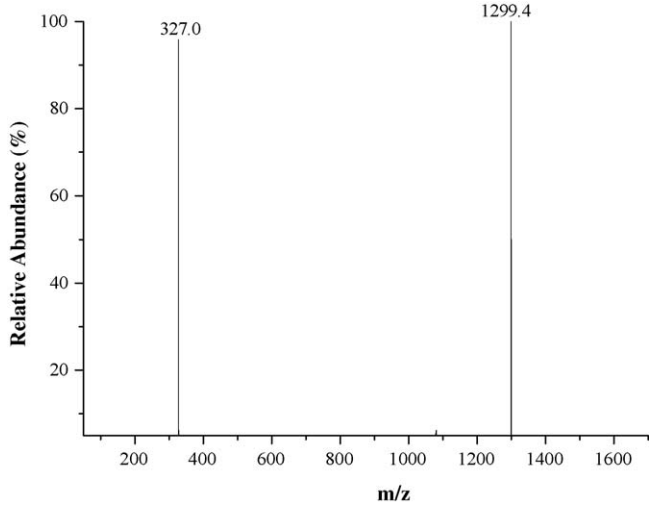

Fig. 4. Negative ESI tandem mass spectra of $[\alpha\text{-CD} + L_O - Na]^-$ ion at *m/z* 1299.4.

the Eq. (B.8). The results for the systems of α-CD, Ponceau SX and Allura red AC are shown in Table 5. For other competitive systems obtained by the same method as mentioned above, the results are shown in Table 3. According to the theory proposed, K_D can be calculated in spite of several ligands. For the ligand with constant concentration, the K_D values of inclusion complexes are in good precision, while for the ligands whose concentrations change, R.S.D. values of K_D values obtained are high.

4.3.4. Two ligands and two binding sites

It is applicable to the case for the system of two ligands of Orange II and Tartrazine binding to α-CD. According to the Eq. (B.8), the dissociation constant can be obtained as shown in Table 6.

The K_D values obtained from competition experiment are different from those obtained from the system of one CD and one kind of ligand (Table 3). However, compared with the difference of the K_D values obtained from one CD containing different concentration ligand, the difference is not significant. The exper-

Table 2
K_D of the inclusion complex of α-, β-CD and Ponceau SX

	$[CD]_0$ (10^{-6} mol L^{-1})	$[L_P]_0$ (10^{-5} mol L^{-1})	$\sum$CD	$\sum$CD − L_P	a	K_D (10^{-5} mol L^{-1})
α-CD	8.00	0.80	100.00	47.53	0.322	1.14
	8.00	1.20	100.00	62.34	0.384	1.43
	8.00	1.60	100.00	81.00	0.448	1.53
	8.00	2.00	66.40	100.00	0.601	1.01
	8.00	2.40	60.77	100.00	0.622	1.16
	8.00	2.80	65.81	100.00	0.603	1.53
	8.00	3.20	49.59	100.00	0.669	1.32
	8.00	3.60	50.69	100.00	0.664	1.55
Average						1.33
S.D.						0.209
R.S.D. (%)						15.71
β-CD	6.00	0.40	100.00	78.25	0.439	0.175
	6.00	0.80	65.32	100.00	0.605	0.285
	6.00	1.20	48.63	100.00	0.673	0.387
	6.00	1.60	33.95	100.00	0.747	0.390
	6.00	2.00	29.67	100.00	0.771	0.457
	6.00	2.40	22.25	100.00	0.818	0.425
	6.00	2.80	18.38	100.00	0.845	0.421
	6.00	3.20	20.16	100.00	0.832	0.545
	6.00	3.60	14.59	100.00	0.873	0.448
Average						0.393
S.D.						0.107
R.S.D. (%)						27.23

imental results indicate that the competitive experiment can be applied to obtain the K_D values of CD and different ligands at the same time.

In the systems of one ligand and one binding site, it is shown that the K_D values increase with the concentration of ligand when CD concentrations are constant. The result obtained in the work is consistent with that mentioned in the literature [29]. In the systems of one ligand and two binding sites, the $K_{D,1}$ values are in good precision and the $K_{D,2}$ values show a tendency to change with the concentration change of one kind of ligand when CD and other ligand concentrations are kept constant. And the same trend exits in the systems of two ligands and one binding site. The reason maybe is that the ionization is not sufficient when the polymerization of ligand occurs at higher ligand concentration and that there is maybe unspecific binding of the ligand to the CD.

Studying on the molecular structure of four ligands shown in Fig. 1, the main difference between Orange II and other three is the substituent. The substituent number in Orange II is the least and the steric hindrance is the smallest. Subsequently it is easy to enter the cave of cyclodextrin and the cave of cyclodextrin can contain two Orange II molecules, so Orange II results in

Table 3
the K_D values obtained from different system and method

Ligand	CD	K_D (mol L^{-1})[a]	R.S.D. (%)	K_D (mol L^{-1})[b]	R.S.D. (%)	K_D (mol L^{-1})[c]
Orange II	α-CD	4.05×10^{-4}	27.90	1.44×10^{-3}	15.63	–
		4.60×10^{-7}[d]	36.74	1.66×10^{-7}[d]	10.48	–
Tartrazine	α-CD	7.47×10^{-5}	29.45	2.23×10^{-5}	30.67	7.68×10^{-5}
Ponceau SX	α-CD	1.33×10^{-5}	15.71	5.48×10^{-6}	30.20	1.32×10^{-5}
Allura red AC	α-CD	4.85×10^{-6}	16.80	2.49×10^{-5}	10.06	4.86×10^{-6}
Orange II	β-CD	3.94×10^{-5}	25.63	–	–	–
		1.72×10^{-7}[d]	40.29	–	–	–
Tartrazine	β-CD	1.12×10^{-4}	27.23	–	–	1.11×10^{-4}
Ponceau SX	β-CD	3.93×10^{-6}	27.23	5.51×10^{-6}	66.45	3.64×10^{-6}
Allura red AC	β-CD	6.50×10^{-6}	20.15	1.81×10^{-5}	36.97	6.58×10^{-6}

[a] The K_D values obtained from the system of one kind of ligand binding to CD.
[b] The K_D values obtained from the system of two kind of ligand binding to CD.
[c] The K_D values obtained by curve fitting.
[d] $K_{D,2}$ of the inclusion complex of CD and Orange II/(mol L^{-1})^2.

Table 4
K_D of the inclusion complex of α-, β-CD and Orange II

	$[CD]_0$ (10^{-5} mol L^{-1})	$[L_O]_0$ (10^{-4} mol L^{-1})	$\sum CD$	$\sum CD - L_O$	$\sum CD - 2L_O$	a_1	a_2	$K_{D,1}$ (10^{-4} mol L^{-1})	$K_{D,2}$ (10^{-7} (mol L^{-1})^2)
α-CD	4.00	0.20	100.00	6.52	–	0.061	–	2.70	–
	4.00	0.40	100.00	10.81	0.54	0.097	0.0048	3.31	2.39
	4.00	0.80	100.00	17.61	1.19	0.148	0.0100	4.17	4.52
	4.00	1.20	100.00	19.76	2.50	0.162	0.0204	5.65	5.02
	4.00	2.00	100.00	41.89	5.33	0.285	0.0362	4.42	6.47
Average								4.05	4.60
S.D.								1.13	1.69
R.S.D. (%)								27.90	36.74
β-CD	6.67	0.667	77.93	100	1.00	0.559	0.0056	2.23	0.639
	6.67	1.33	36.23	100	2.64	0.72	0.0190	2.99	0.934
	6.67	2.00	27.79	100	2.87	0.765	0.0220	4.07	2.06
	6.67	2.67	19.57	100	5.26	0.801	0.0421	4.07	1.61
	6.67	3.33	16.26	100	5.28	0.823	0.0434	4.42	2.28
	6.67	4.00	13.72	100	7.52	0.825	0.0620	4.61	2.07
	6.67	4.67	12.92	100	8.56	0.823	0.0705	5.21	2.45
Average								3.94	1.72
S.D.								1.01	0.693
R.S.D. (%)								25.63	40.29

2:1 complex with CD, whereas others produce 1:1 complex. The intermolecular acting force existed in the systems of CD-sulphonated azo dyes are electrostatic force and hydrophobic interaction force.

The Orange II molecule is smaller and contains less polar substituent groups, so electrostatic force between cyclodextrin and Orange II is weaker and the main intermolecular acting force is hydrophobic interaction force. The α-CD molecule is smaller than β-CD, so hydrophobic interaction force is stronger for Orange II and α-CD than that and β-CD. It is maybe the reason that Orange II binds more strongly to α-CD versus β-CD. For other three ligands, the molecular structures exhibit similarity. The molecules contain more polar substituent groups, and the intermolecular acting force is mainly electrostatic force. It is maybe the other difference between Orange II and other three ligands.

4.3.5. Curve fitting

For the systems of one ligand interacting with one CD, the K_D values can be obtained with the nonlinear least squares regression method according to the formula in the literature [28], and the obtained K_D values of the complexes were consistent with the K_D values obtained with one ligand and one binding site system in this study. The results are shown at Table 3.

Table 5
K_D of the inclusion complex of α-CD, Ponceau SX and Allura red AC

$[L_P]_0$ (10^{-5} mol L^{-1})	$[L_A]_0$ (10^{-5} mol L^{-1})	$[CD]_0$ (10^{-5} mol L^{-1})	$\sum$α-CD	$\sum$α-CD − L_P	$\sum$α-CD − L_A	b_1	b_2	$K_{D,P}$ (10^{-6} mol L^{-1})	$K_{D,A}$ (10^{-5} mol L^{-1})
0.40	0.20	1.00	100.00	29.92	6.18	0.220	0.045	6.01	2.53
0.40	0.40	1.00	100.00	30.79	12.6	0.215	0.088	6.00	2.47
0.40	0.60	1.00	100.00	30.05	18.67	0.202	0.126	6.59	2.53
0.40	0.80	1.00	100.00	30.57	26.50	0.195	0.169	6.69	2.37
0.40	1.00	1.00	100.00	32.98	33.05	0.199	0.199	6.08	2.42
0.40	1.20	1.00	100.00	33.26	41.92	0.190	0.239	6.31	2.30
0.40	1.40	1.00	100.00	30.28	43.81	0.174	0.252	7.46	2.61
0.40	1.60	1.00	100.00	28.64	48.73	0.161	0.275	8.37	2.72
0.40	1.80	1.00	100.00	33.79	60.34	0.174	0.311	6.69	2.47
0	1.00	2.00	100.00	–	31.36	–	0.239	–	1.66
0.60	1.00	2.00	100.00	38.39	25.92	0.234	0.158	3.43	2.63
0.80	1.00	2.00	100.00	53.63	26.07	0.298	0.145	3.81	2.73
1.00	1.00	2.00	100.00	76.73	29.84	0.371	0.144	3.37	2.40
1.20	1.00	2.00	100.00	90.08	27.42	0.414	0.126	4.13	2.73
1.40	1.00	2.00	77.35	100.00	24.36	0.496	0.121	3.15	2.40
1.60	1.00	2.00	73.00	100.00	21.39	0.514	0.110	4.18	2.67
Average								5.48	2.49
S.D.								1.66	0.250
R.S.D. (%)								30.20	10.06

Table 6
K_D of the inclusion complex of α-CD, Orange II and Tartrazine[a]

$[L_T]_0$ (10^{-4} mol L^{-1})	$\sum$α-CD	$\sum$α-CD − L_A	$\sum$α-CD − $2L_A$	$\sum$α-CD − L_T	a_1	a_2	b	$K_{D,O1}$ (10^{-3} mol L^{-1})	$K_{D,O2}$ (10^{-7} mol L^{-1})	$K_{D,T}$ (10^{-5} mol L^{-1})
0.60	100.00	8.28	0.65	36.88	0.0568	0.00446	0.253	1.18	1.47	1.35
0.80	100.00	7.73	0.64	43.27	0.0510	0.00422	0.285	1.26	1.50	1.59
1.00	100.00	7.61	0.59	48.94	0.0484	0.00375	0.311	1.29	1.64	1.79
1.20	100.00	7.06	0.57	47.97	0.0454	0.00366	0.308	1.39	1.68	2.25
1.40	100.00	6.63	0.56	49.39	0.0423	0.00358	0.315	1.48	1.72	2.58
1.60	100.00	5.80	0.48	52.30	0.0366	0.00303	0.330	1.69	2.00	2.80
1.80	100.00	5.53	0.59	51.88	0.0350	0.00373	0.328	1.78	1.63	3.22
Average								1.44	1.66	2.23
S.D.								0.225	0.174	0.684
R.S.D. (%)								15.63	10.48	30.67

[a] The concentrations of α-CD and Orange II were maintained constant at 4.00×10^{-5} mol L^{-1} and 1.00×10^{-4} mol L^{-1}.

5. Conclusions

In this work, we attempt to study the inclusion complexes of CD and some sulphonated azo dyes and calculate the dissociation constants using the data obtained by ESI-MS. It is demonstrated that the inclusion complexes of CD and ligands can be characterized by ESI-MS. The dissociation constants can also be obtained by calculating directly without curve fitting. From the mass spectra, the stoichiometric relation of the host and guest can be obtained. Once the total concentrations of host and guest have been known, even if there are many ligands and many binding sites, the dissociation constant can be obtained expediently according to the mode mentioned above. For several ligands, each K_D can be obtained without prior knowledge of one of the K_D value. This calculation method can also be applied to other systems of other hosts and guests.

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