

## Inclusion Complexes of Folic Acid with Polyamine-Modified $\beta$ -Cyclodextrins: Characterization, Solubilization and Inclusion Mode

KUN LIU<sup>1</sup>, MANSHUO LIU<sup>1</sup>, HUDIE XIE<sup>1</sup>, BIN HAN<sup>1</sup>, YULIN ZHAO<sup>2</sup> and BO YANG<sup>1,\*</sup>

<sup>1</sup>Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 650500, P.R. China

<sup>2</sup>Faculty of Chemical Engineering, Kunming University of Science and Technology, Kunming 650500, P.R. China

\*Corresponding author: E-mail: yangb6910@sina.com

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The formation of inclusion complexes between folic acid with three types of polyamine-modified  $\beta$ -cyclodextrins (1-3) was studied in solution and steady state by means of fluorescence spectroscopy, NMR, XRD and DSC. The stoichiometric ratio and stability constant was determined by Job's plot and fluorescence titrations method, respectively. The results showed that 1:2 complexes are formed for folic acid with hosts  $\beta$ -cyclodextrins (1-3). From the ROESY data, benzene ring and pteridine ring of folic acid was found to be inserted to the cavity of  $\beta$ -cyclodextrin. Due to excellent enhancement of water solubility and thermal stability of folic acid with addition of hosts (1-3), this study will provide useful information for its application as herbal medicine.

**Keywords:** Folic acid, Polyamine-modified  $\beta$ -cyclodextrins, Inclusion complex, Solubilization.

### INTRODUCTION

Folic acid (pteroylmonoglutamic acid, Fig. 1) is a member of the vitamin B family and necessary for the function of a variety of bodily processes. Folic acid participates in one-carbon metabolism and important cellular pathways such as biosynthesis of purine, thymidylate and methionine and also acts as part of the coenzyme system for synthesis of DNA, amino acid and nucleoprotein [1-4]. It is usually used in food fortification, but different from the predominant form of naturally occurring folates in our diet. Because it exist as the oxidized state and contains only one conjugated glutamate residue [5]. Folic acid plays an important role in the prevention neutral tube defects (NTDs), so it is also investigated to prevent some other congenital anomalies or low birth weight [6], beyond that including chronic diseases such as cardiovascular disease, cerebral stroke, cancer of various sites, depression [7], dementia [8] and osteoporosis [9,10].

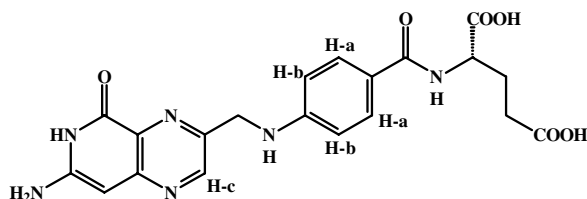


Fig. 1. Structure of folic acid

Apart from its use as a dietary supplement, folic acid is now used in the drug development process as well. It is being prepared in various prodrugs, mainly developed for cancer treatment. Folic acid in the human body works through binding to folate receptor, which is usually over expressed in tumor cells of the brain, kidney, lung and breast [11]. Folic acid as prodrugs are synthesized through using an easily cleave linker to combine folic acid with various drugs, such as cytotoxic drugs (*e.g.* camptothecin [12], taxol [13] and folate-tethered protein toxins (momordin)) [14]. Recently folic acid has also been incorporated with  $\gamma$ -cyclodextrin-derived ZnSe and CdSe quantum dots for drug application [15]. The wide range of the biological effects about folic acid, both alone and conjugates with various active compounds, cause growing interest in this molecule, in terms of functionalization, synthesis of prodrugs and the drug delivery. Recently, there are increasing reports about folic acid-appended drug carriers like nanoparticle [16], micelle [17] and liposome [18], in order to enhance bioavailability of folic acid. One of the possibilities of improving the bioavailability of folic acid is formation of an inclusion compound with the host molecule which works as a carrier for an active compound. Cyclodextrins could be a suitable choice for the host compounds [19].

Cyclodextrins (including  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin and  $\gamma$ -cyclodextrin) are truncated-one cyclic polysaccharides that are composed of six to eight D-glucose units, which have

numerous modifiable hydroxyl groups and a hydrophobic cavity [20]. The unique structure enables cyclodextrins to possess good aqueous solubility to include various drug compounds so they are commonly used as drug delivery carriers [21-24].

However, the aqueous solubility of the native  $\beta$ -cyclodextrin is relatively low and leads to the inclusion complex of  $\beta$ -cyclodextrin and drug dissociates before the drug reaches to the organs or tissues where the drug is to be delivered [25]. Possessing specific functional groups compared with the parent  $\beta$ -cyclodextrin, the chemically modified  $\beta$ -cyclodextrins can alter the original molecular-binding ability by stereochemical complements of the functional branch located in the cyclodextrin cavity [26-29].

Therefore, we wish to study the preparation and characterization of the inclusion complexes formation by folic acid and polyamine modified cyclodextrins (1-3) (Fig. 2) with different tethered chain lengths. It aims to explore the binding behaviour of highly water-soluble polyamine-modified cyclodextrins with folic acid, which will provide a better approach to achieve novel folic acid-based healthcare products with high water solubility. To our best of knowledge, this is the first investigation of the inclusion behaviour of folic acid and polyamine-modified cyclodextrins complexes.

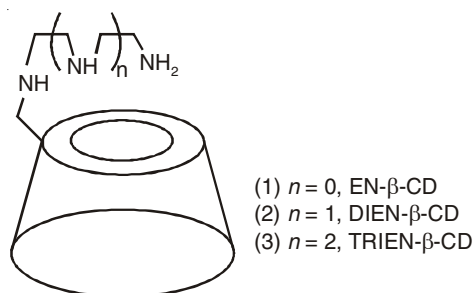


Fig. 2. Structures of polyamine-modified  $\beta$ -CDs (1-3)

## EXPERIMENTAL

Folic acid (FA, m.w. = 441.40, PC > 97 %) was purchased from Aladdin. Mono-6-deoxy-6-enthylenediamino- $\beta$ -cyclodextrin (CD-1, EN- $\beta$ -CD, m.w. = 1178), mono-6-deoxy-6-diethylenetriamino- $\beta$ -cyclodextrin (CD-2, DIEN- $\beta$ -CD, m.w. = 1222) and mono-6-deoxy-6-triethylenetetramino- $\beta$ -cyclodextrin (CD-3, TRIEN- $\beta$ -CD, m.w. = 1266) were synthesized according to the reported procedures [30]. Other reagents and chemicals were of analytical grade and all the experiments were carried out using ultrapure water.

**Modified  $\beta$ -CDs/FA complexes:** To obtain the cyclodextrin/drug complex, folic acid (0.05 mmol, 22.07 mg) and  $\beta$ -CD-1 (0.0125 mmol, 14.73 mg) were added to 25 mL of distilled water in a round flask and stirred for 4 days at room temperature in the dark environment, then filtered. The filtrate was evaporated under reduced pressure to remove the solvent and dried under vacuum to give  $\beta$ -CD-1/FA complexes. Complex  $\beta$ -CD-2/FA and complex  $\beta$ -CD-3/FA were prepared similarly as  $\beta$ -CD-1/FA.

Physical mixtures (to test for possible inclusion) were prepared grinding together a 1:1 molar mixture of folic acid and modified  $\beta$ -cyclodextrins (1-3) in an agate mortar for 0.5 h.

**Job's plot:** Stoichiometry of inclusion complex was studied by the continuous variation method called Job's plot [31]. The Job's plots were determined with fluorescence spectra data obtained in the pH = 8.0  $\text{Na}_2\text{HPO}_4$ - $\text{NaH}_2\text{PO}_4$  buffer solution. The sum of concentrations was kept constant ( $[\text{FA}] + [\text{CD}] = 4.0 \times 10^{-4}$  M) and the molar fraction of folic acid (FA) ( $r = [\text{FA}]/([\text{FA}] + [\text{CD}])$ ) varied from 0.1 to 0.9. In order to calculate the stoichiometry, the fluorescence emission intensity variations ( $\Delta F$ ) of folic acid were plotted *versus* the molar fraction ( $r$ ).

**Spectral titration:** The experimental procedure was carried out as follows: cyclodextrins (1 mM) and folic acid ( $1 \times 10^{-5}$  M) solution were conducted in the  $\text{Na}_2\text{HPO}_4$ - $\text{NaH}_2\text{PO}_4$  (pH = 8.0) buffer solution. In a 10 mL colorimetric tube, 1.0 mL folic acid solution and the varied amounts of cyclodextrins (0.00, 0.05, 0.15, 0.30, 0.45, 0.60, 0.70, 0.80 mM) were added in order. The mixed solution was diluted to the mark with buffer, ultrasonically oscillated for 0.5 h at room temperature. The fluorescence spectra were measured at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 350$  nm/456 nm.

**$^1\text{H}$  NMR and 2D NMR:** All NMR experiments were carried out in  $\text{D}_2\text{O}$  and tetramethylsilane (TMS) was used as a reference. Samples were dissolved in 99.98 %  $\text{D}_2\text{O}$  and filtered before use.  $^1\text{H}$  NMR and 2D NMR spectra were acquired on a Bruker Avance DRX spectrometer at 500 MHz and 298 K.

**Powder X-ray diffraction:** The powder X-ray diffraction (XRD) patterns were collected on a D/Max-3B diffractometer with  $\text{Cu-K}\alpha$  radiation ( $\kappa = 1.5460$  Å, 40 kV, 100 mA) at a scanning rate of  $5^\circ \text{min}^{-1}$ . The samples were mounted on a vitreous sample holder and scanned with a step size of  $2\theta = 0.02^\circ$  between  $2\theta = 5^\circ$  and  $70^\circ$  and radiation was measured with a proportional detector. All samples were analyzed in triplicate.

**Differential scanning calorimetry:** Differential scanning calorimetry measurements were performed using a NETZSCH STA449F3 instrument. All samples were placed in sealed aluminum pans and at the heating rate of  $10^\circ \text{C min}^{-1}$  from room temperature to  $450^\circ \text{C}$  in a dynamic nitrogen atmosphere (flow rate =  $100 \text{ mL min}^{-1}$ ). An empty pan sealed in the same way was used as reference.

**Solubilization test:** Excessive amounts of complexes were placed in 5 mL of water (pH 7.0), respectively and the mixture were stirred for 3 h at room temperature ( $25 \pm 2^\circ \text{C}$ ) in the dark. The solution were filtered by a  $0.45 \mu\text{m}$  cellulose acetate membrane and the filtrates were evaporated under reduced pressure to dryness, then the residues were dosed by appropriate weighing method.

## RESULTS AND DISCUSSION

**Stoichiometry:** Job's method was performed in order to confirm the stoichiometry of the complex [31]. Job's plot was plotted between the difference in fluorescence intensity and the mole fraction of folic acid and it is depicted in Fig. 3. The plot showed the curve at maximum of 0.33 in the mole fraction of folic acid, indicating that the stoichiometry of complex FA/ $\beta$ -CD-1 was 1:2, the same results were acquired from the complexes of folic acid with host  $\beta$ -CD-2, host  $\beta$ -CD-3, respectively.

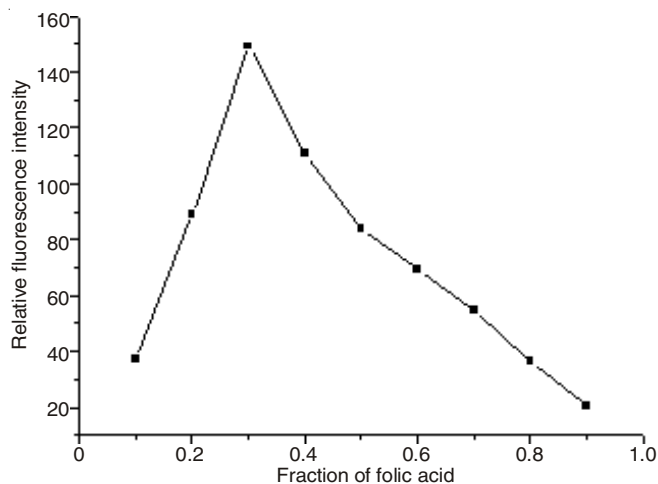


Fig. 3. Job plot for the complex of  $\beta$ -CD-2/FA ( $\lambda_{\text{ex}}/\lambda_{\text{em}} = 350 \text{ nm}/456 \text{ nm}$ ,  $[\text{FA}] + [\text{CD}] = 4.0 \times 10^{-4} \text{ M}$ ,  $\text{pH} = 8.0$ )

**Spectral titration:** Quantitative investigation of the binding behaviour of folic acid with host cyclodextrins were examined in phosphate buffer using fluorescence spectroscopy. As we all know the binding constant ( $K_s$ ) is an important parameter to reflect the inclusion ability, which can be determined by the Hildebrand-Benesi equation [32]. As the Job's plot showed the stoichiometry of inclusion complex was 1 : 2, thus the binding constants ( $K_s$ ) were calculated from the eqn. 1:

$$\frac{1}{\Delta F} = \frac{1}{F - F_0} = \frac{1}{(F' - F_0)K[\text{CD}]^2} + \frac{1}{F' - F_0} \quad (1)$$

where  $F$  and  $F_0$  are the fluorescence intensities of folic acid in the presence and absence of cyclodextrins, respectively;  $F'$  is the intensity at the maximum concentration of cyclodextrins;  $K$  is the binding constant;  $[\text{CD}]$  is the concentration of modified  $\beta$ -cyclodextrins (1-3). When making a plot of  $1/(F - F_0)$  versus  $1/[\text{CD}]^2$ , a lineal relationship was obtained in Fig. 4(a and b), indicated that the stoichiometry of the inclusion complex was 1:2 as well.

As illustrated in Fig. 5(a and b), the fluorescence intensity of folic acid was increased with the stepwise addition of cyclodextrins. These curves suggested that a stable complex was formed between  $\beta$ -cyclodextrins (1-3) and folic acid. As the cavity of cyclodextrin behaves similarly to the organic solvent, it affords an apolar surrounding for the included chromophore. This altered microenvironment can provide favourable polarity and acid/base equilibrium for enhanced quantum efficiencies and the intensities of fluorescence [33]. In repeated measurements, the  $K$  values of obtained are listed in Table-1, with the free energy changes of complex formation ( $-\Delta G_0$ ) obtained upon addition of large excess of the host  $\beta$ -cyclodextrins 1-3.

TABLE-1

STABILITY CONSTANT ( $K_s$ ) AND GIBBS FREE ENERGY CHANGE ( $-\Delta G_0$ ) FOR INCLUSION COMPLEXATIONS OF MODIFIED  $\beta$ -CD (1-3) WITH FOLIC ACID ( $\text{pH} = 8.0$ ) AT  $25^\circ \text{C}$

| Host               | $K_s \times 10^7$<br>( $\text{L mol}^{-2}$ ) | $\log K_s$     | $-\Delta G_0$<br>( $\text{kJ mol}^{-2}$ ) |
|--------------------|--|----------------|---|
| EN- $\beta$ -CD    | $1.4 \pm 0.06$                               | $7.1 \pm 0.04$ | $40.5 \pm 0.04$                           |
| DIEN- $\beta$ -CD  | $1.9 \pm 0.02$                               | $7.3 \pm 0.03$ | $41.6 \pm 0.00$                           |
| TRIEN- $\beta$ -CD | $1.1 \pm 0.01$                               | $7.0 \pm 0.04$ | $39.9 \pm 0.01$                           |

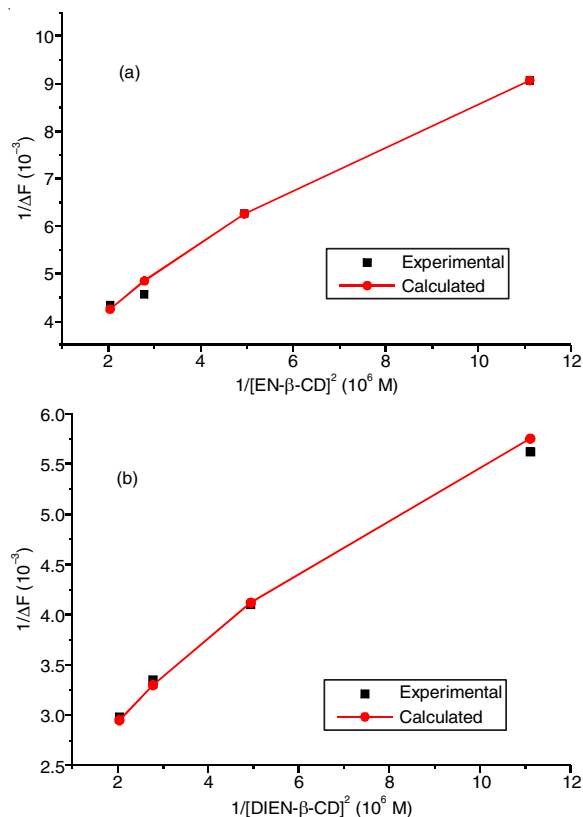


Fig. 4. Benesi-Hildebrand plots obtained from (a)  $1/(F - F_0)$  versus  $1/[\text{EN-}\beta\text{-CD}]^2$ ; (b)  $1/(F - F_0)$  versus  $1/[\text{DIEN-}\beta\text{-CD}]^2$

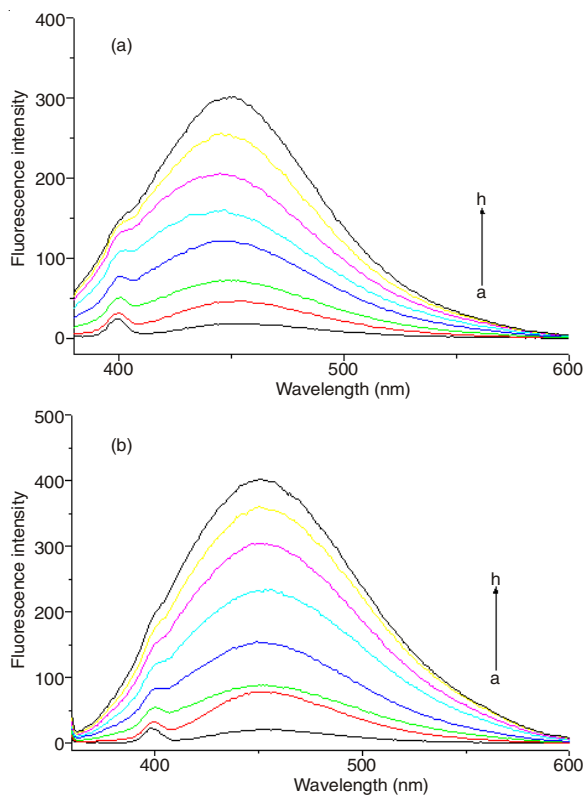


Fig 5. (a) Fluorescence emission spectra of folic acid ( $1.0 \times 10^{-5} \text{ M}$ ) containing various concentrations of EN- $\beta$ -CD (from a to h : 0.00, 0.05, 0.15, 0.30, 0.45, 0.60, 0.70, 0.80 mM),  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 350 \text{ nm}/456 \text{ nm}$ ; (b) Fluorescence emission spectra of folic acid ( $1.0 \times 10^{-5} \text{ M}$ ) containing various concentrations of DIEN- $\beta$ -CD (from a to h: 0.00, 0.05, 0.15, 0.30, 0.45, 0.60, 0.70, 0.80 mM),  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 350 \text{ nm}/456 \text{ nm}$

**Binding ability:** Many studies have revealed that the size/shape-fit concept plays a key role in the formation of inclusion complexes of host cyclodextrins and guest molecules with various structures. Several weak intermolecular forces such as ion-dipole, hydrogen bond, dipole-dipole, van der Waals, electrostatic and hydrophobic interactions are known to cooperatively contribute to inclusion complexation.  $\beta$ -Cyclodextrin possesses a cyclic truncated cone cavity with a height of 0.79 nm, an inner diameter of 0.62-0.78 nm and a cavity volume of 0.262 nm<sup>3</sup> [34]. The host-guest size match may dominate the stability of the complexes formed between modified  $\beta$ -CDs (1-3) and folic acid. From Table-1, it is noted that the binding constants ( $K_s$ ) for the complexation of folic acid by hosts increase in the order:  $\beta$ -CD-3 <  $\beta$ -CD-1 <  $\beta$ -CD-2. In other words, host  $\beta$ -CD-2 with a moderate-length chain is the most suitable for the inclusion complexation of folic acid. This result is reasonable for the cavity of  $\beta$ -cyclodextrin could not encapsulate the folic acid tightly, however, native  $\beta$ -cyclodextrin could slip onto the guest molecular chain like a bead [35]. The introduction of self-introduction of self-included substances at the rim of cyclodextrins may reduce the effective cavity of the cyclodextrins and then hold folic acid much more tightly. The moderate-length side chain of host  $\beta$ -CD-2 is partially included in its own hydrophobic cavity, increasing the binding ability of host  $\beta$ -CD-2 toward folic acid to some extent. Owing to its strict size fit between host  $\beta$ -CD-2 and guest, consequently exhibits the strongest binding ability between  $\beta$ -CD-2 and folic acid. The shortest side chain present in host 1 is shallowly self-included in the cyclodextrin cavity. It leads to a lower binding ability between  $\beta$ -CD-1 and folic acid. For the longest-chain host  $\beta$ -CD-3, the lowest binding ability is the result of the size unfit between the cavity of  $\beta$ -CD-3 and the guest, due to the longest chain being deeply included in its own cavity, increasing the steric hindrance and reducing the host-guest interaction.

**NMR analysis:** In order to explore the possible inclusion mode of FA/CDs complexes, the <sup>1</sup>H NMR spectra of folic acid in the presence of host modified  $\beta$ -CDs (1-3) were compared (Fig. 6) where the <sup>1</sup>H resonances of modified  $\beta$ -CDs (1-3) were assigned according to the reported method [36]. Owing to its poor water solubility, folic acid is transparent to <sup>1</sup>H NMR under most conditions when D<sub>2</sub>O is used as a solvent. Assessment of the FA/CDs complexes by <sup>1</sup>H NMR clearly demonstrated the presence of the framework protons of the folic acid molecule, which implied a significant solubility increase of FA/CDs compared with native folic acid. As illustrated in Fig. 6B, the majority of the folic acid protons appear at  $\delta$  6.50-8.50 ppm, so can well separated from those of the cyclodextrin H-atoms (chemical shifts usually at  $\delta$  3.0-5.0 ppm).

It is well known that 2D NMR spectroscopy provides important information about the spatial proximity between host and guest molecules *via* observations of the intermolecular dipolar cross-correlations. Two protons which are closely located in space can produce a NOE cross-correlation between the relevant protons in NOESY or ROESY spectrum. The presence of NOE cross-peaks between protons of two species indicates spatial contacts within 0.4 nm [37]. To further explore the inclusion mode, ROESY of the inclusion complex of folic

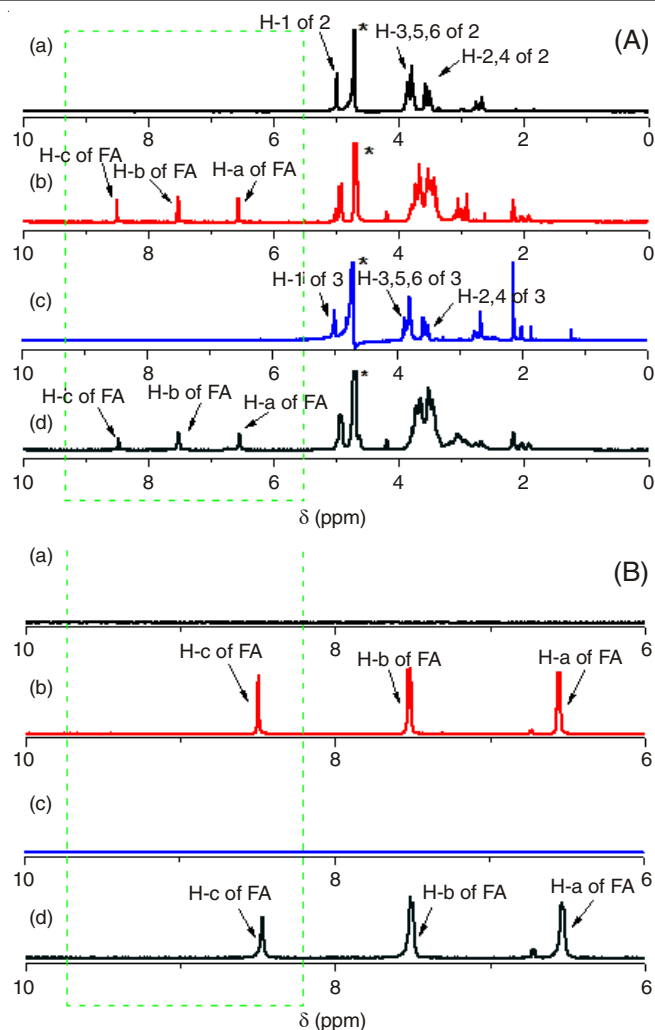


Fig. 6. (A) <sup>1</sup>H NMR spectra of 2 and 3 in the absence and presence of folic acid in D<sub>2</sub>O: (a) 2, DIEN- $\beta$ -CD; (b) FA/2 complex; (c) 3, TRIEN- $\beta$ -CD; (d) FA/3 complex (asterisk highlights the water peak); (B) The enlarged NMR spectrum from approximately 6.0-10.0 ppm

acid with cyclodextrins (Fig. 7) were obtained, with a partial contour plot. The ROESY spectrum of FA/CDs complexes (Fig. 7) showed appreciable correlation of H-a, H-b, H-c protons of folic acid with H-3 and H-5 protons of cyclodextrins, indicating that the benzene ring and pteridine ring of folic acid are included in the cyclodextrins' cavities. Based on above mentioned observations and the stoichiometric ratio 1:2, the possible inclusion mode of folic acid with cyclodextrins was deduced (Fig. 8).

**Powder X-ray diffraction analysis:** The powder X-ray diffraction (XRD) patterns of folic acid,  $\beta$ -CD-1 as well as their inclusion complexes and physical mixtures (FA/ $\beta$ -CD-1) are listed in Fig. 9. As indicated in Fig. 9, folic acid (Fig. 9a) and  $\beta$ -CD-1 (Fig. 9b) showed sharp peaks that prove the crystalline nature of the compound in the crystalline forms. On the contrary, the XRD of the FA/ $\beta$ -CD-1 complex was amorphous and showed halo patterns, which were different from the superimposition of FA/ $\beta$ -CD-1 physical mixture, indicating the formation of the inclusion complex between cyclodextrin and folic acid. The same results obtained from the complex of FA/ $\beta$ -CD-2 as well as FA/ $\beta$ -CD-3.

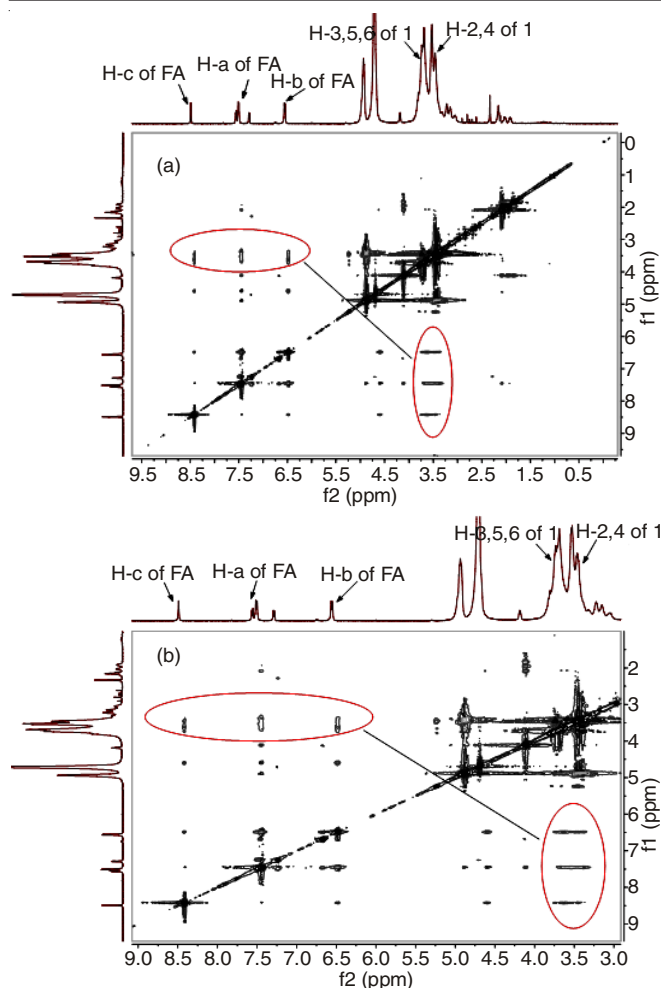


Fig. 7. (a) ROESY spectrum of FA/I complex in  $D_2O$ ; (b) Partial contour plot of ROESY spectrum

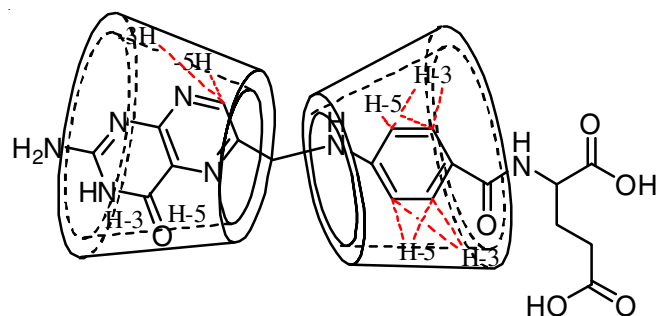


Fig. 8. Possible inclusion mode of FA/CDs

**Thermal analysis:** Thermal properties of the FA/CDs complexes were investigated by thermogravimetric analysis. Folic acid had different thermal stability with a melting point of about  $150\text{ }^\circ\text{C}$  (Fig. 10a). The TG curves showed that the FA/ $\beta$ -CD-1 complex decomposed at about  $300\text{ }^\circ\text{C}$  (Fig. 10d). A similar phenomenon for FA/ $\beta$ -CD-2 and FA/ $\beta$ -CD-3 complexes were found. These results indicated that folic acid's native thermal property was changed after inclusion complexation and the FA/CDs complexes had higher decomposition temperatures.

**Solubilization:** The results showed that the water solubility of FA/ $\beta$ -CD-1, FA/ $\beta$ -CD-2 and FA/ $\beta$ -CD-3, compared with native folic acid ( $0.0016\text{ mg mL}^{-1}$ ) [38], was remarkably

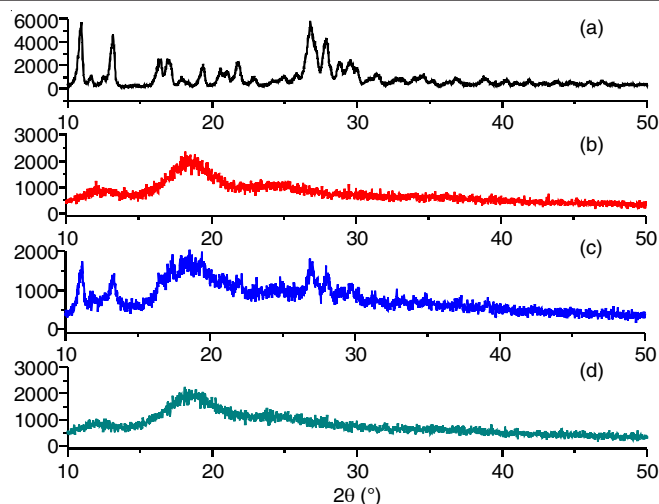


Fig. 9. XRD patterns: (a) folic acid; (b) 1, EN- $\beta$ -CD; (c) FA/I physical mixture (molar proportion 1:1); (d) FA/I complex

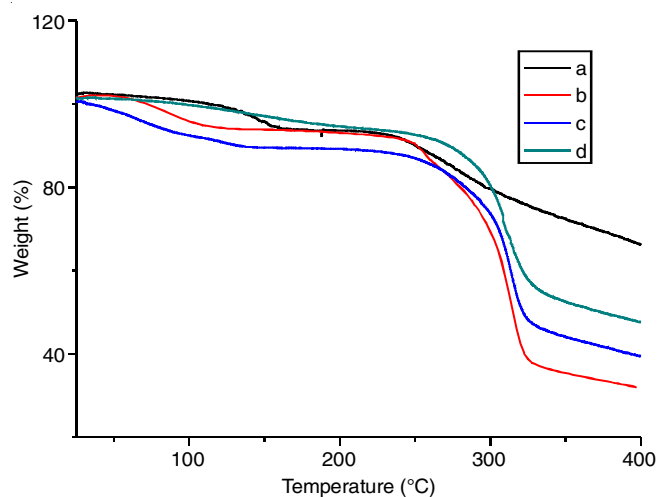


Fig. 10. TG curves: (a) folic acid; (b) 1, EN- $\beta$ -CD; (c) FA/I physical mixtures (1:1 molar ratio); (d) FA/I complexes

increased to approximately  $19.4$ ,  $25.1$  and  $20.6\text{ mg mL}^{-1}$ , respectively. In the control experiment, a clear solution was obtained after dissolving the FA/ $\beta$ -CD-1 ( $347.4\text{ mg}$ ), FA/ $\beta$ -CD-2 ( $378.8\text{ mg}$ ), FA/ $\beta$ -CD-3 ( $341.3\text{ mg}$ ) complexes, which was equivalent to  $19.4$ ,  $25.1$  and  $20.6\text{ mg}$  of folic acid, respectively, in  $1\text{ mL}$  of water at room temperature. The results confirmed the accuracy of obtained satisfactory water solubility of the FA/CDs complexes, which would be beneficial for the pharmaceutical applications of folic acid.

## Conclusion

Characterization, inclusion behaviour and binding ability of the inclusion complexes between folic acid and modified  $\beta$ -cyclodextrins (1-3) were investigated in this work. The stoichiometry of the host-guest inclusion complex is found to be 1:2 in the case of folic acid as the guest molecule, which suggested that two cavities of cyclodextrins could encapsulate the folic acid molecule fully. The host 2 with moderate-length chain among these modified cyclodextrins studied was the most suitable for inclusion complexation of folic acid. The experimental data displayed that modified  $\beta$ -cyclodextrins enhanced

the water solubility and thermal stability of folic acid. Considering that all the cyclodextrins employed in this work were easily prepared, they should be regarded as a useful choice in the design of carriers for medicinal drugs.

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