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Bioorganic & Medicinal Chemistry 14 (2006) 131–137
Bioorganic & Medicinal Chemistry

Interactions of some modified mono- and bis-β-cyclodextrins with bovine serum albumin

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Received 5 June 2005; revised 31 July 2005; accepted 1 August 2005
Available online 23 September 2005

Abstract—Two mono-substituted β-cyclodextrins and two bridged bis-β-cyclodextrins, that is, mono(6-(2-aminoethylamino)-6-deoxy)-β-cyclodextrin (**1**), mono(6-(2-(2-aminoethylamino)ethylamino)-6-deoxy)-β-cyclodextrin (**2**), ethylene-1,2-diamino bis-6-(6-deoxy-β-cyclodextrin) (**3**), and iminodiethylene-2,2′-diamino bis-6-(6-deoxy-β-cyclodextrin) (**4**), were prepared from β-cyclodextrin. Their binding ability with bovine serum albumin as a model protein was investigated through proton magnetic resonance (^{1}H NMR), ultraviolet visible spectroscopy (UV–vis), circular dichroism (CD), and fluorescence spectroscopy. In the ^{1}H NMR spectra of the modified cyclodextrins, the resolution of proton signals decreases after the addition of BSA. From the UV and CD spectra, it is found that both the UV absorption and the α-helix content of BSA increase with the concentration of the modified cyclodextrins. The protein–ligand interactions cause a fluorescence quenching. The quenching constants are determined using the Stern–Volmer equation to provide an observation of the binding affinity between modified cyclodextrins and BSA. All these results indicate that the modified cyclodextrins can interact with BSA and the bridged bis(β-cyclodextrin)s (**3** and **4**) have much stronger interactions than the mono-substituted β-cyclodextrins (**1** and **2**). The strong binding stability of bis-cyclodextrins should be attributed to the cooperative effect of two adjacent cyclodextrin moieties. Job's plot shows that the complex stoichiometries of BSA to the modified cyclodextrins were 1:4 for **1** and **2**, as well as 1:3 for **3** and **4**, respectively.

1. Introduction

Numerous problems have to be solved before the practical use of therapeutic peptides and proteins on account of their chemical and enzymatic instabilities, poor absorption through biological membranes, rapid plasma clearance, peculiar dose–response curves, and immunogenicity.[1] Among the various inorganic and organic compounds, cyclodextrins (CyDs) and their derivatives may serve as carriers for therapeutically important peptides, proteins, and oligonucleotides.[2] CyDs are a class of cyclic oligosaccharides with six to eight α-glucose units linked by α-1,4-glucose bonds. They have well-defined chemical structures with many sites for chemical modification or conjugation, availability of different cavity sizes, low toxicity, certain hydrophilicity, and protection effect of the included/conjugated drugs from biodegradation.[2,3] In recent years, many researches were focused on amino acid and oligopeptide recognition by CyDs and their derivatives.[4–7] However, there have been only a few studies due to about protein recognition by CyDs or modified CyDs as ligands[8,9] of the complexity of protein secondary and ternary structures.[10] Bovine serum albumin (BSA), an easily obtained protein with well-known properties, is often used as a model protein.[11] Binding of several different categories of small molecules to BSA has been studied using spectroscopy techniques to elucidate the details of protein structures and binding mechanisms.[12–15]

Most proteins, including BSA, have hydrophobic residues, such as alkyl and aromatic groups, ionic groups, such as $-NH_3^+$ and $-COO^-$, hydrophilic polar groups such as –OH and $-NH_2$, etc. Those groups can act as binding sites with ligand molecules that have similar groups. In this study, $-NH_3^+$ as the ionic group and $-NH_2$ as the polar group different from –OH on CyDs molecules were introduced to β-cyclodextrin (β-CyD). Additionally, two β-CyD molecules were coupled together with an amino-containing spacer to obtain some β-CyD derivatives with high binding ability to protein. In our laboratory, two mono-substituted

Keywords: β-Cyclodextrin; Bovine serum albumin; Protein; Cooperative interaction; Inclusion complex; Stoichiometry.
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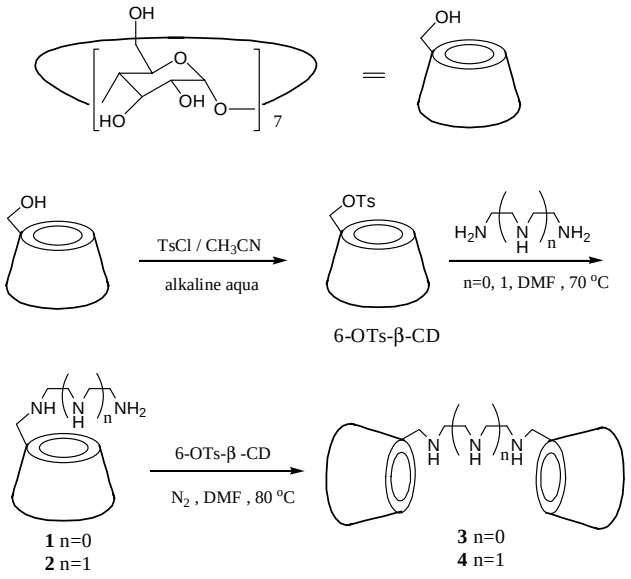
doi:10.1016/j.bmc.2005.08.002



Scheme 1. Syntheses of the modified β-CyDs **1**–**4**.

β-CyDs and two bridged bis-β-CyDs, that is, mono (6-(2-aminoethylamino)-6-deoxy)-β-cyclodextrin (**1**), mono-(6-(2-(2-aminoethylamino)ethylamino)-6-deoxy)-β-cyclodextrin (**2**), ethylene-1,2-diamino bis-6-(6-deoxy-β-cyclodextrin) (**3**), and iminodiethylene-2,2′-diamino bis-6-(6-deoxy-β-cyclodextrin) (**4**), were prepared from β-CyD, as shown in Scheme 1.[16,17] And their binding ability with bovine serum albumin as a model protein was investigated using proton magnetic resonance (^{1}H NMR), ultraviolet visible spectroscopy (UV–vis), circular dichroism (CD), and fluorescence spectroscopy.

2. Materials and methods

2.1. Materials

BSA in electrophoresis purity (fatty acid free) was purchased from Institute of Hematology & Hospital of Blood Diseases, Chinese Academy of Medical Sciences, and used without further purification. β-CyD of reagent grade (Shanghai Reagent Factory, China) was recrystallized twice from water and dried in vacuo at 95 °C for 24 h prior to use. *N*,*N*′-Dimethylformamide (DMF) was dried over calcium hydride for 2 days and then distilled under a reduced pressure before use. D_2O is of the isotopic purity with 99.9%, Cambridge Isotope Laboratories, Inc.

2.2. Synthesis of β-CyD derivatives

2.2.1. Preparation of amino β-CyD derivatives 1 and 2. Mono(6-*O*-(*p*-toluenesulfonyl))-β-cyclodextrin (6-OTs-β-CyD) was prepared by the reaction of *p*-tosyl chloride with β-CyD in alkaline aqueous solution.[18,19] 6-OTs-β-CyD reacted with freshly distilled ethyldiamine and diethylenetriamine[16] to afford the amino derivatives of β-CyD **1** and **2** in ca. 70% yield. Their purities were detected by thin-layer chromatography (TLC) and their structures were elucidated by ^{1}H NMR spectroscopy in D_2O on the Varian UNITY-plus 400 spectrometer at 400 MHz.

2.2.2. Preparation of bis-β-CyD derivatives 3 and 4. Amino β-CyD derivatives **1** and **2** were allowed to react with excess 6-OTs-β-CyD in DMF when stirring in nitrogen atmosphere at 80 °C for 3 days to obtain bis-β-CyD derivatives **3** and **4**.[17] The crude products were purified by precipitation from a mass of acetone and then by chromatography on a CM Sephadex C-25 ionic column, as well as Sephadex G-25 column. The yield of **3** and **4** was 21% and 25%, respectively. Their structures were validated by ^{1}H NMR spectra in D_2O and elemental analyses.

2.3. Measurements of the interactions of β-CyD derivatives with BSA

2.3.1. ^{1}H NMR measurements. ^{1}H NMR spectra of CyD derivatives were recorded from a Varian UNITY-plus 400 spectrometer at 400 MHz in the presence and absence of BSA. Water suppression was achieved with a presaturation pulse, prior to acquisition. The stock BSA solutions were freshly prepared by dissolving BSA in D_2O. The molecular weight of BSA, 66 700, was used to estimate the molar ratios of the CyD derivatives to BSA. The concentration of the modified CyDs was 2.30 mM and that of BSA was varied from 0.12 to 0.46 mM.

2.3.2. UV–vis measurements. The UV absorption spectra were recorded at 25 ± 0.1 °C on the U-3010 spectrophotometer (HITACHI, Japan) with $1 \times 1 \times 4$ cm quartz cell. The concentration of BSA was kept at 5.25×10^{-5} M and that of **1**–**4** was 0, 2×10^{-5}, 4×10^{-5}, 6×10^{-5}, 8×10^{-5}, 1×10^{-4}, and 2×10^{-4} M in phosphate buffer solutions (PBS, pH 7.4, 0.1 M), respectively.

2.3.3. CD measurements. The far-UV CD measurements of the samples in PBS were performed at 25 ± 0.1 °C in the range of 190–260 nm on the Jasco J-715 spectropolarimeter (JASCO, Tokyo, Japan) with a 1 cm light path length quartz cylindrical cuvette. The following parameters were set: resolution = 0.2 nm; speed = 100 nm/min; bandwidth = 1 nm; response = 1 s; and sensitivity = 20 mdeg. The average of three scans was calculated. The concentration of BSA was 0.3 mg/mL and the ratio of modified CyDs to BSA was 5:1, 25:1, and 50:1, respectively.

2.3.4. Fluorescence measurements. 0.25 mL of modified CyDs solutions was added to 3.0 mL of 10 μM BSA solution in PBS to reach final concentration of 0, 10, 25, 50, 100, and 200 μM, respectively. After 10 min, fluorescence measurements were carried out at 25 ± 0.1 °C on a fluorescence instrument (Jobin Yvon, Fluromax-P) with a 1 cm cell. The excitation wavelength was 283 nm and the emissions were scanned from 303 to 440 nm for all samples. The slits of excitation and emission monochromators were adjusted to 3 nm.

To establish the stoichiometry of the complexes, Job's experiments were also performed by means of fluores-

cence spectroscopy. The method of continuous variations[6,20,21] was employed as follows: the total mole concentrations of modified CyDs and BSA were held constant (10^{-5} mol), while the mole fraction of modified CyDs varied from 0 to 1.0. After being incubated for 1 h at 25 °C, its fluorescence emission intensity was then measured as described above. Duplicate fluorescence intensity readings were taken to ensure reproducibility. A Job's plot of fluorescence emission intensity difference versus the mole fraction of modified CyDs was used to determine the stoichiometry of binding from the ratio of mole fraction of modified CyDs to BSA.

3. Results and discussion

NMR spectroscopy is increasingly used to elucidate interactions of CyDs with biopolymers.[8] Although the current resolution of one-dimensional NMR spectra is not high enough to locate the protons of BSA due to the complicated structure of BSA, the ^{1}H NMR signal changes of the modified CyDs in the absence and presence of BSA were studied to monitor their interactions. The modified β-CyDs possess the outer-surface (H-2 and H-4) and inner-surface (H-3 and H-5) protons, as well as the H-6 protons situated at the primary side of the CyDs cavity.[8,22] The adsorption of proton signals is illustrated in Figure 1. The H-1 proton signal at δ 4.9 ppm is of no significance because it is influenced by the water suppression at δ 4.8 ppm. On addition of BSA, the chemical shift of every proton signal in the NMR spectra of compounds **1**–**4** almost remains unchanged. The resolution loss of proton signals of modified CyDs is observed and its loss extent is enhanced with the increased BSA concentration. When the concentration of BSA reaches up to 0.46 mM, the broadened effect may be attributed to the external viscosity of protein solution.[23] Instead, at the concentrations of BSA less than 0.23 mM, as studied by Sułkowska et al.,[24] the protons of the small molecule stabilized by the interactions with the protein can be identified if it has a longer correlation time and gives a much-broadened spectral resonance signal. Therefore, there must be some interactions between the modified CyDs and BSA. These interactions, including hydrophobic interactions, hydrogen bonds, and electrostatic interactions, considerably restrict the protons and change the proton signals in their NMR spectra. The high loss of signal resolution for the NMR spectra of the compounds **3** and **4** takes place as compared with that of **1** and **2** in the presence of BSA. The reason is undoubtedly the cooperative effect of their two hydrophobic cavities located near each other in the compounds **3** and **4** as the Bis-CyDs.

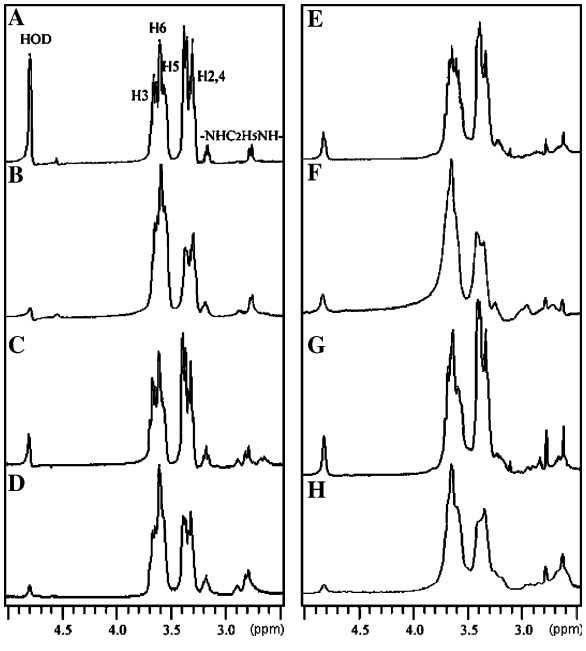


Figure 1. The ^{1}H NMR spectra of the CyD derivatives **1**–**4** (2.3 mM) in the absence (**1**–**4**, from A to D) of and the presence (**1**–**4**, from E to H) of BSA (0.23 mM).

BSA has a maximum UV/Vis absorption at ca. 280 nm and this property can be used to determine BSA concentration in a proper concentration range. The typical UV spectra changes of **1**–**4** are shown in Figure 2. The UV absorbance in the spectrum of BSA increases gradually with the increased concentration of CyDs **1**–**4**, and the degree of absorption intensity change is influenced with the modified CyDs in the order of **4** > **3** > **2** > **1**. The results indicate that the solvation shell and the intramolecular or intermolecular association of the chromophoric groups on BSA macromolecule may be partly or totally destroyed in the presence of the modified CyDs. The inclusion and other complex interactions of the modified CyDs with BSA lead to an altered solute–environment interaction. When the concentrations of **1**–**4** are more than 2×10^{-4} M (approximately 4 times of the BSA concentration) in a phosphate buffer (PBS), the change of the BSA adsorption spectra can hardly be observed again, indicating that the highest equivalence is achieved at the molar ratio of 1:4 for BSA to the modified CyDs.

CD spectra may provide some information on the secondary structures of proteins, nucleic acids and the binding of ligands to these types of macromolecules.[25] The binding of the modified CyDs to those biopolymers was also confirmed by CD spectra. Final spectra may be presented in mean residue ellipticity, [θ]:

$$[\theta] = \frac{\theta M}{10CLN_{\mathrm{r}}}.$$

In the formula, θ is the measured ellipticity (mdeg), M is the molecular weight of the protein, C is the BSA concentration (mg/mL), L is the pathlength of the cell (cm), and N_{r} is the number of amino acids of the protein molecules.[26]

At first, CD spectra were analyzed using a curve-fitting program software, CDPro, as described by Sreerama and Woody[27] to obtain the secondary structural contents of the proteins. However, the low signal-to-noise ratio below 195 nm implies a poor quality of the spectra recorded in the region and the results are not reliable. So, the α-helix content of proteins may be estimated according to the following equation:[28]

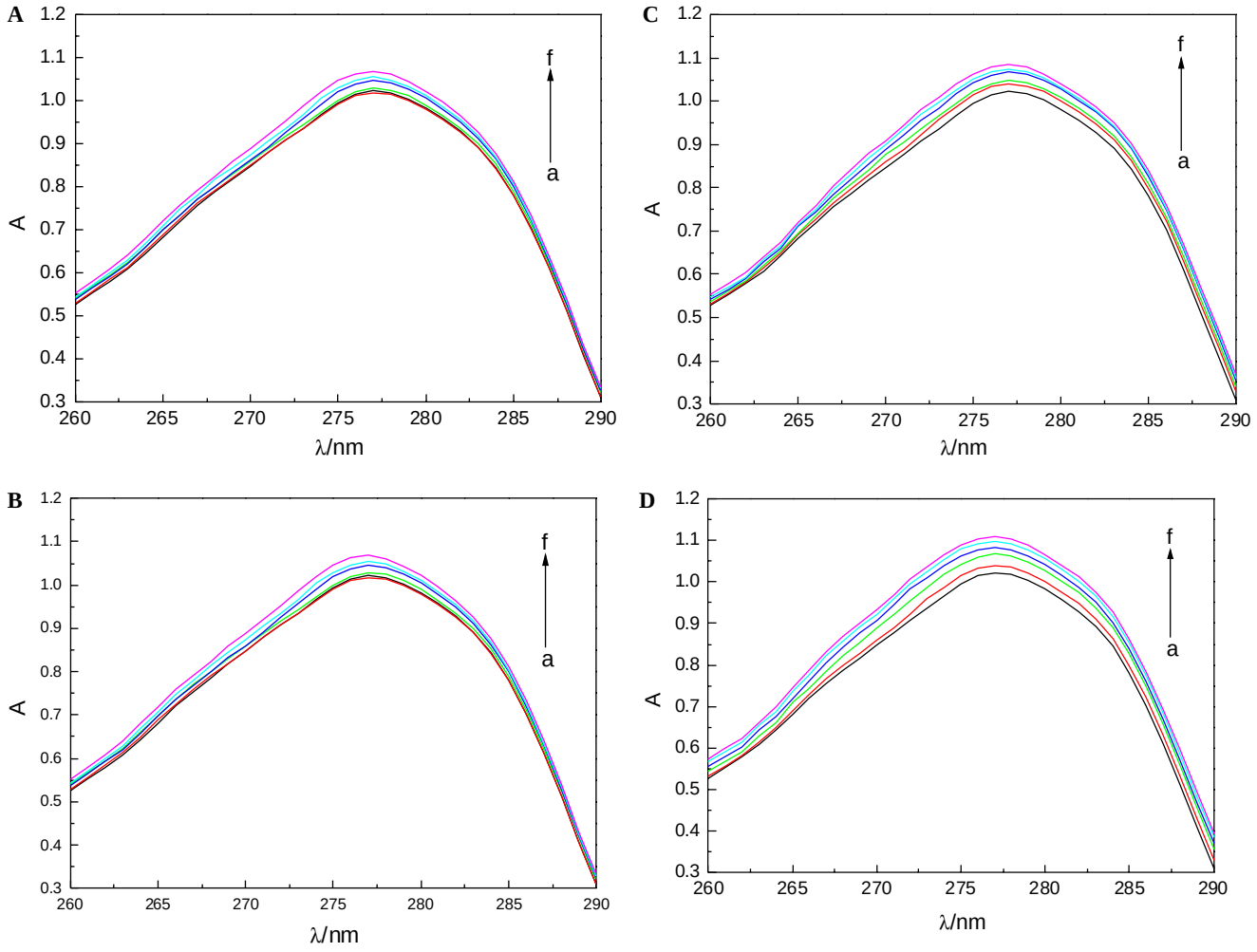


Figure 2. The UV/Vis spectral changes of BSA (5.25×10^{-5} M) in the presence of **1** (A), **2** (B), **3** (C), and **4** (D) with concentrations of 0, 2×10^{-5}, 4×10^{-5}, 6×10^{-5}, 8×10^{-5}, 1×10^{-4}, and 2×10^{-4} M (from a to f) in the PBS buffer.

$$\%\alpha\text{-helix content} = \frac{[\theta]\text{mrd} - 4000}{33000 - 4000},$$

where $[\theta]$mrd is the mean molar ellipticity per residue at 208 nm (deg cm^2 dmol^{-1}).

Typical CD spectra of BSA in the presence of the representative modified CyD, **4**, are shown in Figure 3. As expected, the strong double minimums, respectively, at 220 and 209 nm suggest that the α-helix structure exists in BSA[25] and no apparent peak shift occurs in the presence of the modified CyDs. The intensity of the double minimums reflects more than 50% of α-helical structure in BSA. The binding of the modified CyDs to BSA results in a deepening of the trough at 208 nm, which is due to an increase in the helix content of BSA with the conformational changes. The estimated percentages of α-helix contents from CD spectra are listed in Table 1. It is measured that the native BSA at pH 7.4 in the PBS buffer has 60.5% of α-helix, close to the value of 59 ± 2% reported previously.[29,30] In parallel to the UV absorbance change trend, the percentage of α-helix content also increases in the order of **4** > **3** > **2** > **1** when these modified CyDs are added. The interactions of

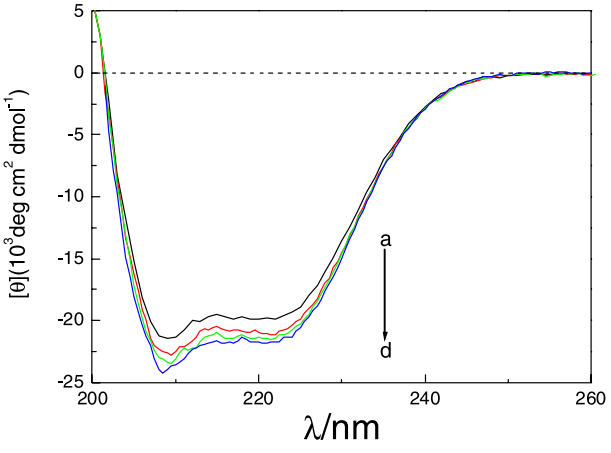


Figure 3. The CD spectra of BSA (0.3 mg/mL) upon addition of compounds **4** (a: 0, b: 0.15, c: 0.75, and d: 1.5 mg/mL) in the PBS buffer.

the modified CyDs with BSA can weaken the BSA solvation with the water molecules and the self-association within the chain of BSA, so that a much tighter secondary structure of α-helix becomes dominant in BSA.

Table 1. The α-helix percentages in BSA (0.03 mg/mL) upon addition of compounds **1**–**4** with concentrations of 0.15 mg/mL (a), 0.75 mg/mL (b), and 1.5 mg/mL (c)

Compound	Helix
BSA	60.5
BSA-**1** (a)	60.7
BSA-**1** (b)	59.4
BSA-**1** (c)	61.0
BSA-**2** (a)	61.1
BSA-**2** (b)	62.6
BSA-**2** (c)	63.4
BSA-**3** (a)	61.9
BSA-**3** (b)	64.1
BSA-**3** (c)	66.9
BSA-**4** (a)	63.4
BSA-**4** (b)	65.3
BSA-**4** (c)	68.4

However, the CD spectrum is not due solely to the polypeptide backbone and its conformation. Other UV-active chromophores, such as the aromatic groups of tyrosine and phenylalanine, may contribute to CD of proteins and polypeptides.[31] Aromatic amino acids are known to complex with CyDs. Such an interaction possibly affects the overall three-dimensional structure of peptides and proteins.[1] In the studies of a 17-mer helical model peptide, Chakrabartty et al.[32] assessed the aromatic side-chain contribution to far-UV CD by substituting an alanine at the N-terminus of the helix with aromatic residues. Nevertheless, the complicated compositions of BSA make the substitution method unfeasible. As a whole, it is conceivable that besides the aromatic contribution, CD changes contribute to the backbone conformations of BSA.

Meanwhile, protein fluorescence has been used as a sensitive detector for the conformational change studies in the ternary protein structures. BSA is a single-chain transporting protein of 585 amino acids with 17 disulfide bridges, two tryptophanes (Trp 135 and Trp 214), and one free thiol at Cys 34. It is known that the fluorescence of aqueous tryptophan-containing proteins arises almost from their tryptophanyl residues.[24] The fluorescence emission spectrum of BSA at an excitation of 283 nm shows λ_{max} at 349 nm. Addition of each CyD derivative induces a loss in fluorescence intensity due to the quenching effect at various degrees. Typical fluorescence quenching spectra of BSA in the presence of compounds

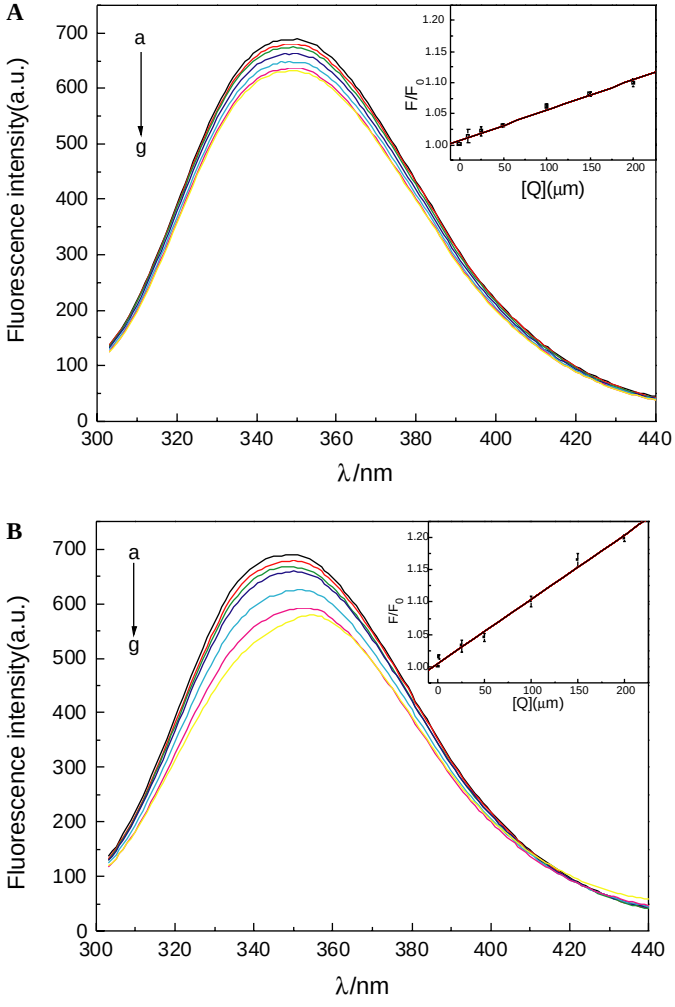


Figure 4. Fluorescence spectral changes of BSA (10 μM) with an excitation wavelength of 283 nm upon addition of compounds **2** (A), **4** (B) (0–200 μM, from a to g) in the PBS buffer. The inset is the corresponding Stern–Volmer plot ($n = 5$).

2 and **4** are shown in Figure 4. The fluorescence intensity decreases with an increase in the concentration of the added compounds. As a conclusion, the interactions between the CyD derivatives and BSA contribute to the alteration of BSA ternary structure near the binding site.

Due to the interactions of compounds **1**–**4** with BSA, a quenching of fluorescence takes place and the Stern–Volmer analysis allows us to estimate their quenching constants. Fluorescence quenching was carried out by measuring the fluorescence intensities at 349 nm as a function of the concentration of each compound:

$$F_0/F = 1 + \mathrm{K}_q[Q],$$

where F_0 and F are, respectively, fluorescence intensities in the absence and presence of quencher, K_q is the Stern–Volmer quenching constant, and $[Q]$ is the concentration of the quencher. The equation assumes a linear plot of F_0/F versus $[Q]$ and the slope equals K_q.[12] K_q values of the tryptophan quenching, expressing tryptophan accessibility to the quencher,[33] are listed in Table 2. The extent of quenching is higher for the bridged CyDs than for the mono-substituted CyDs. The cooperative effects should play an important role in the interactions of BSA with the modified CyDs as mentioned above. The K_q of diethylenetriamino-substituted CyDs (**2** or **4**) was a little more than the corresponding ethylenediamino-substituted ones (**1** or **3**). This may be caused by the electrostatic interactions owing to the more amino groups in compounds **2** and **4** than in **1** and **2**.

A slight redshift in emission maximum wavelength is also observed in BSA fluorescence when the solution of **4** is added, suggesting that the binding of **4** is accompanied by changes in the dielectric environment of at least one of the two indole rings in BSA.[34,35] It is known that a shift of maximum emission wavelength corresponded to a polarity change around the chromophore residues. A redshift always indicates that tryptophan residues are, on average, more exposed to the solvent, whereas a blueshift is a consequence of transferring tryptophan residues into a more hydrophobic environment. In this study, the redshift can be explained as the result of change in the higher structures of BSA. Since the interaction of BSA with the modified CyDs results in a microenvironment change of BSA backbone and its residues, the increased α-helix content (as shown in Table 1) indicates a tightening secondary structure and hence the ternary structure of BSA should be broken to some extent. While the ternary structure of BSA is changed, the tryptophan residue may be exposed to the outside, owing to the affinity of compound **4**. The redshift in maximum emission wavelength is then aroused as a result of the change in the local tryptophan microenvironment. In case **4** is sticky to BSA, then it is imperative to consequently rearrange the tryptophan microenvironment in BSA.[33]

Table 2. The Stern–Volmer quenching constants of tryptophan residue fluorescence in the presence of compounds **1**–**4**

Compound	Stern–Volmer constant, $K_q[M^{-1}]$
1	305 ± 14
2	485 ± 27
3	893 ± 26
4	994 ± 44

To establish a stoichiometry, a double reciprocal curve is first plotted[36] for $F/\Delta F$ (where $\Delta F = F_0 - F$) versus 1/[modified CyD] employing the data in Figure 4. Nonlinearity from the double reciprocal plots indicates that higher-order complexes are involved.

Finally, in the concentration range examined, Job's plot shows the maximum at a molar fraction of 0.75 (**1** and **2**) and 0.8 (**3** and **4**), indicating that the complex stoichiometries of BSA to the modified CyDs are 1:4 for both **1** and **2**, as well as 1:3 for both **3** and **4**, respectively. Representative Job's plot for the complex of **2**/BSA and **4**/BSA are shown in Figure 5. When considering the molecular dimension, several CyDs derivatives may be involved in forming a complex with BSA. The size of the bridged CyDs is larger than that of mono-substituted CyDs. Therefore, four molecules of the mono-substituted CyDs (**1** and **2**) can bind with one molecule of

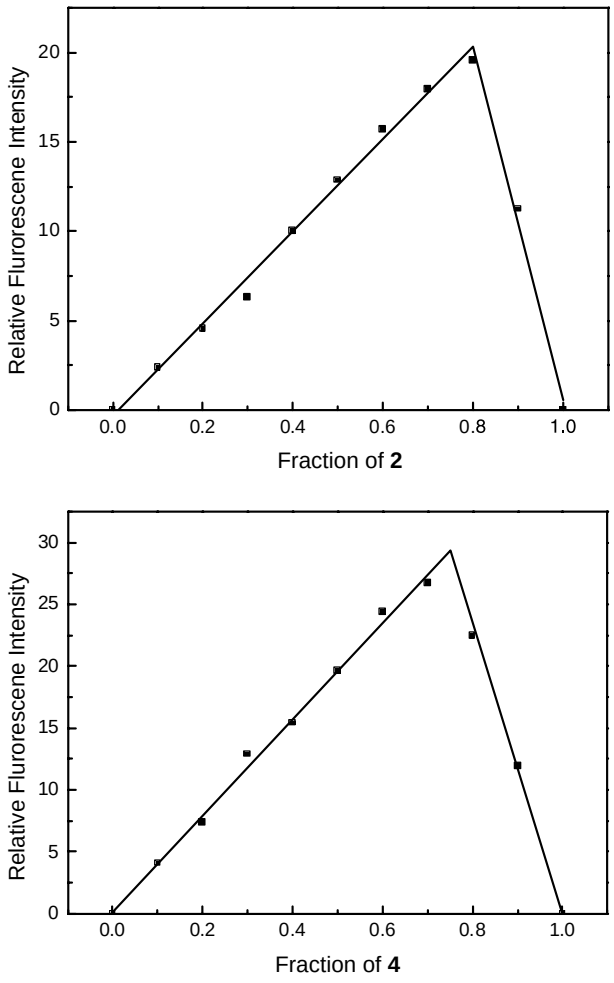


Figure 5. Continuous variation plot of the **2**/BSA ([2] + [BSA] = 10^{-5} M) and **4**/BSA system ([4] + [BSA] = 10^{-5} M) in PBS.

BSA, while three bridged CyDs (**3** and **4**) can complex with one BSA.

4. Conclusion

The interactions of BSA with the modified CyDs were observed, owing to the formation of complex on the basis of ^{1}H NMR, UV, CD, and emission fluorescence spectroscopy. Analysis of the helix content and Stern–Volmer quenching constant indicates that the complexation abilities of compounds **1**–**4** were in the order of **4** > **3** > **2** > **1**. For the stoichiometry, BSA can complex with the modified CyDs at a ratio of 1:4 (for **1** and **2**) and 1:3 (for **3** and **4**).

Acknowledgments

Authors acknowledge the National Natural Science Foundation of China for the financial support (Project No.: 20174018). We thank Prof. Ping-Chuan Sun and Hai Lin for their measurement of ^{1}H NMR spectra.

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