Spectrophotometric Study of Fluorescence Sensing and Selective Binding of Biochemical Substrates by 2,2′-Bridged Bis(*β*-cyclodextrin) and Its Water-Soluble Fullerene Conjugate

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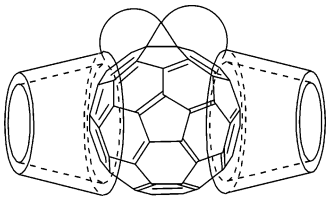
A bis(*β*-cyclodextrin)−fullerene conjugate (**3**) linked at the secondary hydroxyl side was prepared in a good yield from its precursor *N*,*N*′-bis(2-(2-aminoethylamino)ethyl)malonamide-bridged bis(*β*-cyclodextrin) (**2**). Spectrophotomeric studies on the conformation and the inclusion complexation behavior of **3** with a variety of organic and biochemical substrates by means of UV−vis, FT-IR, NMR, fluorescence, and circular dichroism spectroscopy showed that the bis(*β*-cyclodextrin)−fullerene conjugate displayed an intramolecular capsule-type conformation in aqueous solution. Because of the multiple binding of bis(*β*-cyclodextrin) with substrates, **2** can act as an efficient fluorescence sensor for biochemical substrates, while its fullerene conjugate **3** displays a capability of cleaving DNA under visible-light irradiation.

Introduction

Known to be highly water-soluble and essentially nontoxic[1] and to have the capability to include various guest molecules in aqueous solution or the solid state,[2−5] cyclodextrins (CDs), a family of cyclic oligosaccharides composed of six to eight α-D-glucose units, are generally regarded as good molecular receptors for many inorganic, organic, and biological substrates and are widely applied in various fields, such as analytical chemistry, enzymology,[6] pharmaceuticals,[7] the food industry,[8] and so forth. The functionality and solubility of fullerenes by CDs have attracted much attention with significant work reported, especially by Geckeler et al. and Andersson et al.[9−13] Recently, Rassat et al. reported the syntheses and the spectral investigations of several C_{60}-bridged bis(*β*-CD)s,[14] which led to the prediction that the bis(*β*-CD)−C_{60} conjugates linked at the secondary hydroxyl side of CD (Chart 1) would be more useful for biochemical studies because of their possible self-included conformation in aqueous media. However, the secondary-linked bis(*β*-CD)−C_{60} conjugates prepared by Zhang et al.[15] were unable to form this kind of capsule-type species. In this work, we prepared a bis(*β*-CD) (**2**) with a short linker via the secondary rim of CD and investigated its fluorescence sensing and selective binding capabilities for various biochemical substrates. A further reaction of **2** with C_{60} yields a bis(*β*-CD)−C_{60} conjugate **3** (Scheme 1). For **3**, the hydrophilic protection from the bis(2-(2-aminoethyl-amino)ethyl)malonamide linker and CD cavities can efficiently avoid the micelle-like aggregation of fullerene and enable bis(*β*-CD)−C_{60} conjugate **3** to exist as a self-included conformer in aqueous solution. To the best of our knowledge, this is the first example of a covalently linked capsule-type CD−fullerene conjugate. An inherent advantage of this kind of capsule-type CD−fullerene conjugate is that the CD cavity can selectively bind certain fragments of the biological substrates, enabling the site-specific interaction between the fullerene and target substrate. Characteristics of **3** such as its satisfactory water-solubility at a physiological temperature and DNA-cleaving ability make this molecule pharmaceutically relevant.

CHART 1



Experimental Section

General. *N*,*N*-Dimerthylformamide (DMF) was dried over calcium hydride for 2 days and then distilled under reduced pressure prior to use. Elemental analyses were performed on a Perkin-Elmer-2400C instrument. NMR spectra were recorded on a Varian Mercury VX300 instrument. UV−vis and circular dichroism spectra were performed on a Shimadzu UV 2401 spectrophotometer and a JASCO J-715S spectropolarimeter, respectively. Fluorescence spectra were measured in a conventional rectangular quartz cell (10 × 10 × 45 mm) at 25 °C on a JASCO FP-750 spectrometer equipped with a constant-temperature water bath, with the excitation and emission slits at a width of 5 nm. The EPR spectrum is measured with a BRUKER EMX-6/1 spectrometer and recorded for 167.772 s in a phosphate buffer (pH 7.2) at 298 K. The magnitude of the modulation was chosen to optimize the resolution and the signal-to-noise ratio of the observed spectra.

Synthesis of Mono[2-(2-aminoethylamino)-2-deoxy]-*β*-CD (1). A solution of mono-2-tolylsulfonyl-2-deoxy-*β*-CD[16] (4.0 g, 3 mmol) in 150 mL of water was added dropwise under nitrogen to a solution of diethylenetriamine (3.2 mL, 30 mmol) in 10 mL of water. After the reaction mixture was stirred at 70 °C for 3 h, most of the solvent was removed under reduced pressure and 200 mL of acetone/methanol (40:1) was added to produce a white precipitate. The crude product obtained was dried in vacuo to give pure **1** as a white solid. ^{1}H NMR (300 MHz, D_2O): *δ* 2.4−2.8 (m 8H), 3.3−3.8 (m 42H), 4.8−5.0

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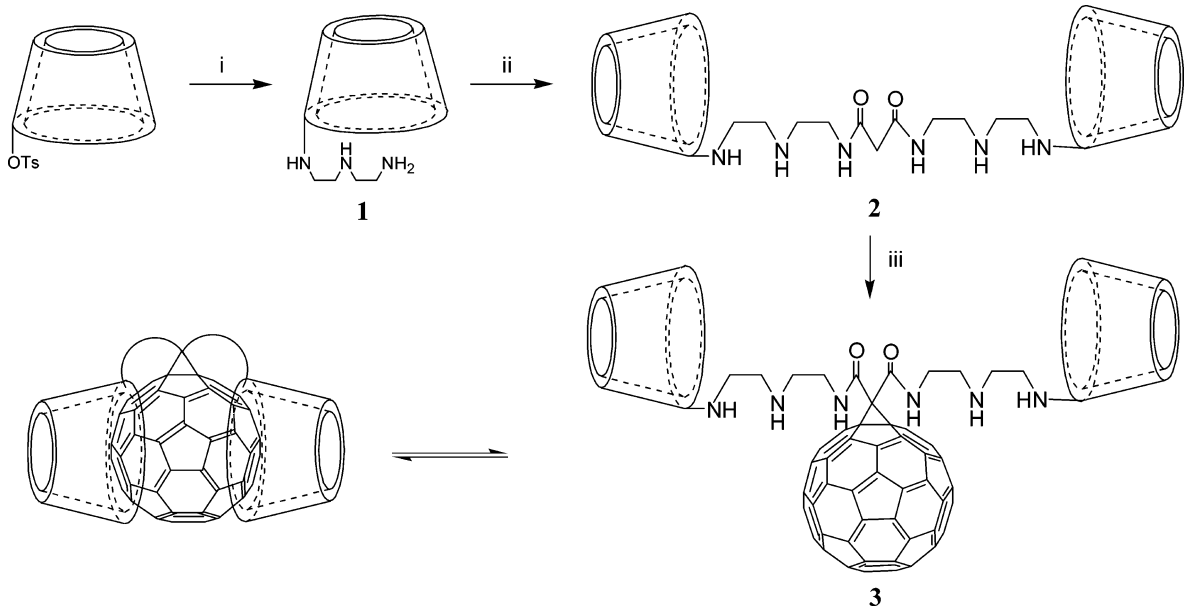
SCHEME 1[a]



[a] (i) Diethylenetriamine, 70 °C. (ii) Malonic acid, DCC, DMF, 0 °C to room temperature. (iii) C_{60}, CBr_4, DBU, DMF/toluene, room temperature.

(m 7H). Anal. Calcd for $C_{46}H_{81}O_{34}N_3 \cdot 6H_2O$: C, 41.60; H, 7.06; N, 3.16. Found: C, 41.47; H, 7.16; N, 3.13.

Synthesis of *N,N′*-Bis(2-(2-aminoethylamino)ethyl)malonamide-Bridged Bis(*β*-CD) (2). To a mixture containing **1** (1220 mg, 1 mmol) and dicyclohexylcarbodiimide (DCC, 1030 mg, 5 mmol) in 40 mL of DMF, 52 mg (0.5 mmol) of malonic acid in 20 mL of DMF was added dropwise under nitrogen. The resultant mixture was stirred for 20 h on ice and stirred an additional 2 days at room temperature. The precipitate that formed was removed by filtration and evaporated under reduced pressure to dryness. The residue was dissolved in the minimum amount of water and poured into acetone (200 mL), giving a yellow precipitate. The crude product obtained was dried and purified by column chromatography over Sephadex G-25 with distilled deionized water as an eluent to give pure **2** as a yellow solid. ^{1}H NMR (300 MHz, D_2O): δ 2.4−2.9 (m 16H), 3.1−3.8 (m 86H), 4.8−5.0 (m 14H).^{13}C NMR (75 MHz, DMSO-d_6): δ 30.5, 31.0, 32.4, 35.2, 37.2, 60.4, 69.3, 72.0, 73.2, 76.7, 80.9, 99.6, 102.0, 164.2. IR (KBr) ν/cm^{-1}: 3345, 2931, 1650, 1559, 1541, 1454, 1434, 1362, 1338, 1243, 1153, 1079, 1030, 927, 851, 756, 706, 576. Anal. Calcd for $C_{95}H_{162}O_{70}N_6 \cdot 6H_2O$: C, 43.61; H, 6.70; N, 3.21. Found: C, 43.50; H, 6.65; N, 3.26.

Synthesis of 1,1-Fullerene-*N,N′*-bis(2-(2-aminoethylamino)ethyl)malonamide-Bridged Bis(*β*-CD) (3). A solution of **2** (262 mg, 0.1 mmol) in 30 mL of DMF was added to a solution containing C_{60} (72 mg, 0.1 mmol) and CBr_4 (33.2 mg, 0.1 mmol) in 80 mL of toluene. The reaction mixture was stirred at room temperature for 24 h. Then, 15 mg (0.1 mmol) of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in 10 mL of toluene was added dropwise to the mixture under nitrogen. After the mixture was stirred for an additional 2 days at room temperature, the deep-purple homogeneous solution turned turbid deep-brown. After collection via centrifugation, the solid was dissolved in a minimal amount of water and added to acetone (200 mL) to give a brown precipitate. The crude product was dried in vacuo to give a deep-brown pure **3** solid. ^{1}H NMR (300 MHz, D_2O): δ 2.4−2.9 (m 16H), 3.3−3.8 (m 84H), 4.8−5.0 (m 14H). ^{13}C NMR (75 MHz, D_2O): δ 24.1, 26.8, 29.2, 30.8, 32.5, 39.0, 39.3, 39.6, 39.9, 40.2, 40.4, 40.7, 45.1, 49.2, 50.7, 54.2, 57.0, 60.5, 69.7, 72.5, 72.7, 78.0, 81.1, 102.5, 140.7, 143.6, 144.3, 145.7, 147.0, 147.9, 150.0, 162.7. IR (KBr) ν/cm^{-1}: 3324, 2930, 1747, 1660, 1438, 1360, 1324, 1149, 1079, 1032, 843, 765, 708, 662, 525, 418. UV−vis (water) λ_{max}/nm: 260, 336. Anal. Calcd for $C_{155}H_{160}O_{70}N_6 \cdot 5H_2O$: C, 56.13; H, 5.17; N, 2.53. Found: C, 55.83; H, 5.27; N, 2.83.

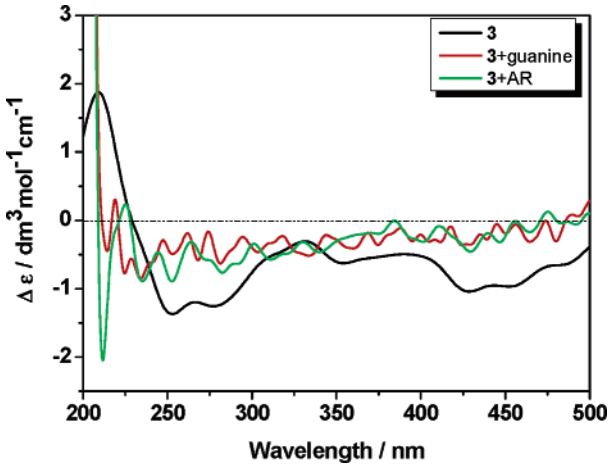


Figure 1. Circular dichroism spectra of **3** (8.827×10^{-5} M), **3** (8.827×10^{-5} M) + guanine (5.823×10^{-6} M), and **3** (8.827×10^{-5} M) + acridine red (5.147×10^{-6} M) in a phosphate buffer solution (pH 7.20) at 298 K.

Results and Discussion

Spectroscopic Study on the Conformation of 3. The bis(*β*-CD)−C_{60} conjugate **3** is obtained in 64% yield by the reaction of C_{60} and *N,N′*-bis(2-(2-aminoethylamino)ethyl)malonamide-bridged bis(*β*-CD) (**2**), which was prepared from mono[2-(2-aminoethylamino)-2-deoxy]-*β*-CD (**1**) (Scheme 1). On top of the elemental analysis and the NMR evidences, the FT-IR spectrum of **3** shows a typical vibration band at 525 cm^{-1}, and the UV−vis spectrum of **3** displays two absorption peaks at 260 and 336 nm. These data jointly confirm the presence of C_{60} in the resultant conjugate. The intramolecular capsule-type conformation of **3** is verified by the circular dichroism spectrum (Figure 1). Seen from Figure 1, bis(*β*-CD)−C_{60} conjugate **3** displays the appreciable circular dichroism signals in the absorption band of C_{60}, like the case of *γ*-CD/C_{60} inclusion

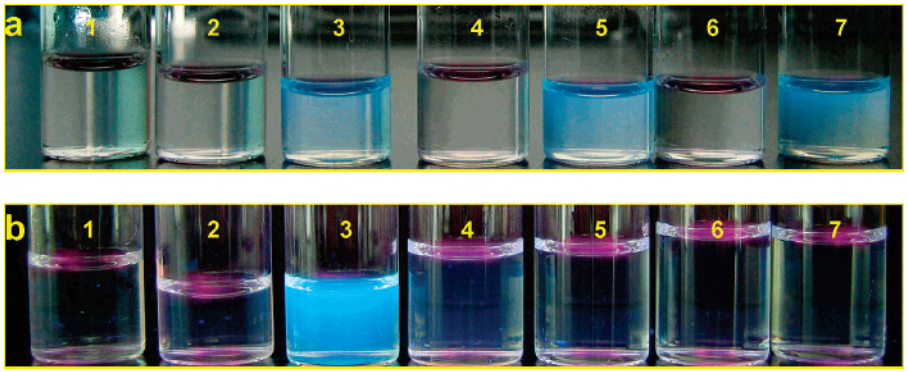
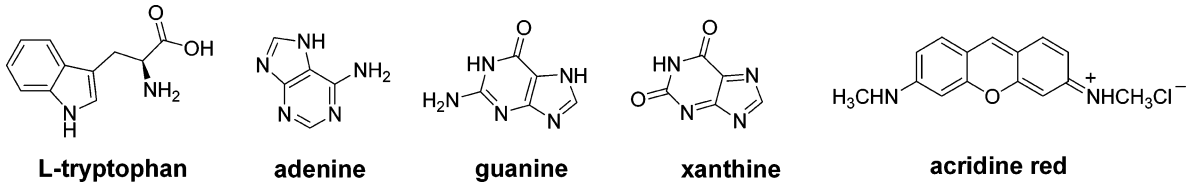


Figure 2. Visible emission observed from samples of **2** and various biochemical molecules: (a) From 1 to 7: **2**, adenine, **2** + adenine, guanine, **2** + guanine, xanthine, and **2** + xanthine. (b) From 1 to 7: **2**, L-tryptophan, **2** + L-tryptophan, **2** + L-alanine, **2** + L-cysteine, **2** + L-glutamic acid, and **2** + L-aspartic acid.

CHART 2



complexes.[17] Since C_{60} is chromophoric but achiral and CDs are chiral but nonchromophoric, we can deduce that the C_{60} unit in **3** is accommodated in the CD cavities because the inclusion of an achiral chromophoric guest/moiety in the CD cavity always leads to the induced circular dichroism. This self-included conformation of the bis(β-CD)−C_{60} conjugate will efficiently prevent the micelle-like aggregation of C_{60} in aqueous solution and consequently increase its water solubility, especially at a relatively high temperature. In this work, the water solubility of **3** is measured to be 11 mg/mL (25 °C) and remains unchanged until the temperature reaches 42 °C. It should be emphasized that the conjugate **3** has similar water solubility to that of β-CD, which is potentially advantageous for its application in the biological systems.

Fluorescence Sensing. Molecular sensing based on the guest-involved response of the receptor is a very significant topic in organic chemistry. Herein, biochemical substrates such as L-tryptophan, adenine, guanine, and xanthine (Chart 2) were found to fluoresce in the presence of bis(β-CD) **2**. Significantly, the intensity of the emittance is strong enough to be readily distinguished by the eye (Figure 2), even at a low concentration (5×10^{-6} M).

Generally, the fluorescence intensities of L-tryptophan, adenine, guanine, and xanthine are sensitive to change in their microenvironment. That is, they barely fluoresce in a hydrophilic microenvironment but emit strong fluorescence in a highly hydrophobic one. Therefore, the significant fluorescence mentioned above suggests that the fluorophores in these biochemical substrates are located in a highly hydrophobic microenvironment. Considering the structural feature of bis(β-CD) **2**, we deduce that the biochemical substrates are embedded in the hydrophobic CD cavities to form the host−guest inclusion complexes.

To quantitatively assess the inclusion complex of bis(β-CD) **2** with the biochemical substrates, fluorescence titration experiments were performed at 25 °C in a phosphate buffer (pH 7.20). In the spectral titration experiments, the concentration of the biochemical substrate (guest) was kept constant, while the concentrations of **2** (host) varied. The spectral changes depended critically on the formation of the new species, that is, the host−guest inclusion complex. As shown in Figure 3, the fluorescence intensity of guanine gradually increased with the addition of bis(β-CD) **2**.

After validating the 1:2 complex stoichiometry between the host and guest by the continuous variation method, the complex stability constant (K_s) for the inclusion complexation of bis(β-CD) **2** with the biochemical substrates can be determined to be 2.828×10^8 M^{-2} for the **2**/L-tryptophan system, 2.485×10^8 M^{-2} for the **2**/adenine system, 2.203×10^8 M^{-2} for the **2**/guanine system, and 2.156×10^8 M^{-2} for the **2**/xanthine system (see the Supporting Information for the derivation of K_s). By examining the apparent binding constant (K_{app}) of each CD cavity in **2** upon complexation, we can conclude that bis(β-CD) **2** can form stable inclusion complexes with these biochemical substrates. For example, bis(β-CD) **2** gives a K_s value upon complexation with two L-tryptophan molecules up to 2.828×10^8 M^{-2}. When it is assumed that two β-CD cavities in **2** have equal binding abilities toward guest molecules, the K_{app} value for the inclusion complexation of each β-CD cavity with an L-tryptophan molecule can be calculated to be 1.68×10^4 M^{-1} ($K_{app} = K_s^{1/2}$), which is higher than the K_s values for the inclusion complexation of an L-tryptophan molecule with a native β-CD or a mono-modified β-CD ($K_s \leq 6000$ M^{-1}).[18]

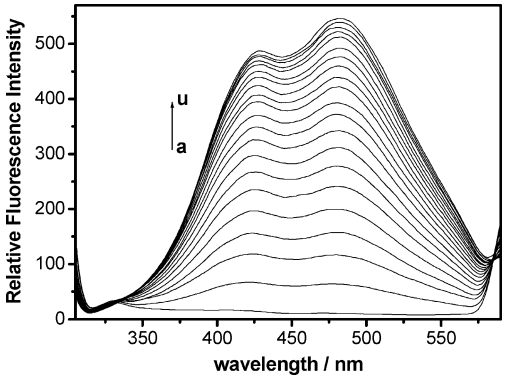


Figure 3. Fluorescence spectral changes of guanine (5.823×10^{-6} M) upon addition of **2** ($0-207.6 \times 10^{-6}$ M from a to u) in a phosphate buffer solution (pH 7.20); excitation wavelength = 297 nm, excitation and emission slit = 10 nm.

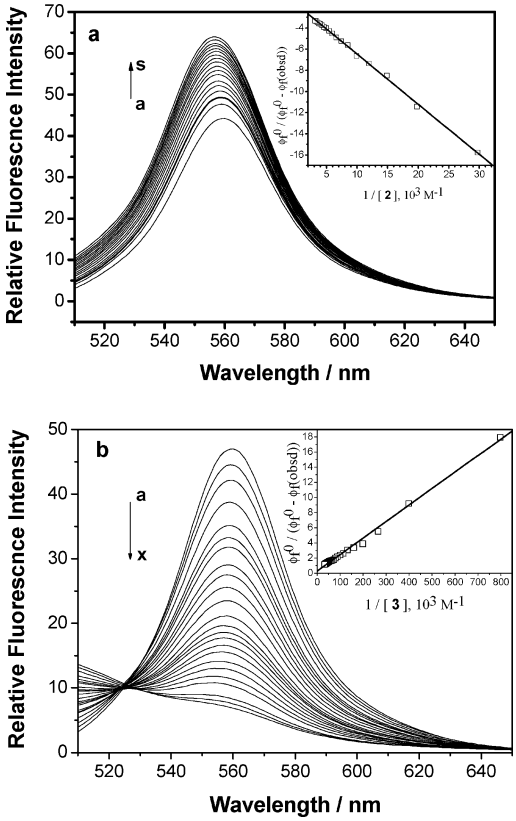


Figure 4. Fluorescence spectra of (a) **2** + AR ([AR] = 5.2×10^{-6} M, [**2**] = $0-3.2 \times 10^{-4}$ M from a to s) and (b) **3** + AR ([AR] = 5.2×10^{-6} M, [**3**] = $0-3.01 \times 10^{-5}$ M from a to x) in a phosphate buffer solution (pH 7.20) at 25 °C; excitation wavelength = 490 nm, excitation and emission slit = 5 nm.

Similar results are also observed in the inclusion complexation of **2** with other biochemical substrates. The satisfactory binding abilities of **2** may be attributed to the good size fit between the guest molecule and host CD cavity, giving the strong hydrophobic interactions. Moreover, the hydrogen-bonding interactions between the N atoms of the guest molecules and the hydroxyl groups of the CD cavity as well as the −NH− groups in the bis(2-(2-aminoethylamino)ethyl)malonamide linker may also contribute to the formation of the stable host−guest inclusion complexes.

Cooperative Binding of Bis(β-CD)−C_{60} Conjugate 3. After validating the efficient binding of bis(β-CD) **2** with the guest molecules, we further compare the molecular recognition behaviors of bis(β-CD) **2** and its C_{60} conjugate **3**. We investigated the cooperative contributions of CD and C_{60} units upon complexation with guest molecules. Herein, acridine red (AR) was selected as a typical guest, and the contrasting fluorescence behavior of AR was observed. Under our experimental conditions, the fluorescence intensity of AR slowly increased by 4−42% when gradually adding 3−62 equiv of bis(β-CD) **2** (Figure 4a). This phenomenon is ascribed to the cooperative binding of AR with the dual CD cavities, leading to increased microenvironmental hydrophobicity around the AR fluorophore. In sharp contrast, the gradual addition of 0.25−4.8 equiv of **3** significantly quenched 6−77% of the AR fluorescence under comparable conditions (Figure 4b).

Such contrasting fluorescence behavior of AR may point to a mechanism concerning the cooperative binding of two CD cavities and a C_{60} unit in **3** toward the guest AR. Upon inclusion complexation, the AR molecule is simultaneously bound by two CD cavities to give a sandwich-type complex, where the C_{60} unit in **3** and the guest AR are closely located in space. Therefore, a photoinduced electron transfer process from the electron-donor AR to C_{60} decreases the fluorescence quantum yield of AR and results in the quenched fluorescence. The additional evidences for the sandwich complexation between **3** and AR come from the 2D NMR experiments and the Corey−Pauling−Kultun (CPK) model examinations. In the nuclear Overhauser effect spectrometry (NOESY) of the **3**/AR system (Figure 5), we can find clear NOE correlations (peak A) between the aromatic protons of AR and the H-2 protons of CD. Because the H-2 protons are located at the secondary hydroxyl side (wide side) of the CD cavity, these NOE correlations indicate that the AR molecule is accommodated in the CD cavities from the wide side. Moreover, the examinations on the CPK models indicate that the flexible bis(2-(2-aminoethylamino)ethyl)-malonamide linker in **3** can align the two CD cavities and the C_{60} unit in the right position and distance to enable the formation of the sandwich complex. In addition, the intensity of the circular dichroism signals of **3** in the absorption band of C_{60} obviously decreases with the addition of AR (Figure 1), indicating that the inclusion of AR leads to the expulsion of C_{60} from the CD cavities. Hence, the results of the NOESY, CPK, and circular dichroism experiments are in good agreement with the proposed sandwich-type conformation and strongly support the operation of the cooperative binding mode in the complexation of the model substrate by the bis(β-CD)−C_{60} conjugate.

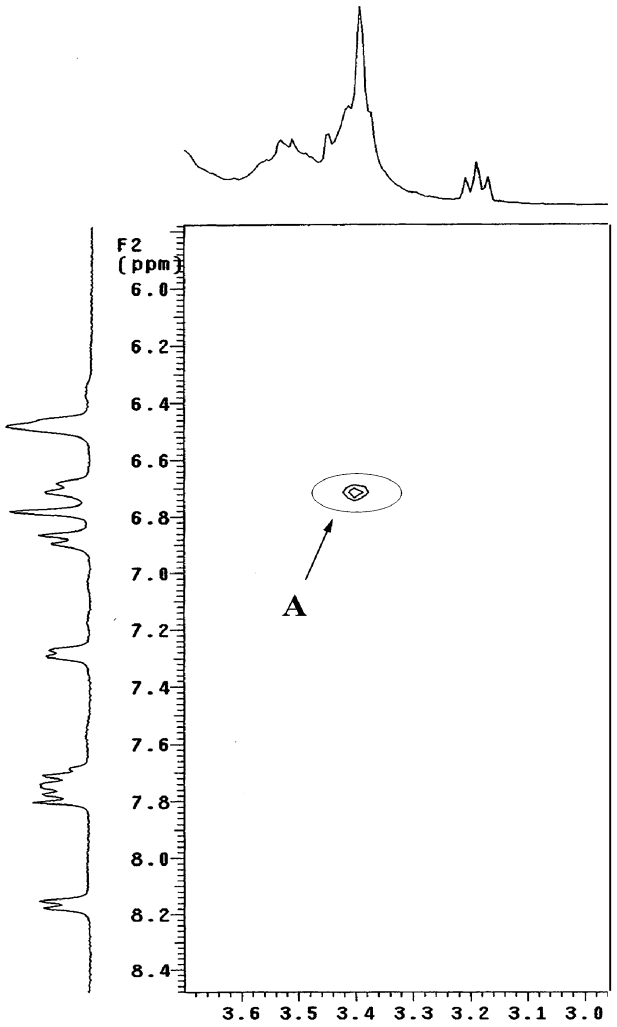


Figure 5. ^{1}H NOESY spectrum (300 MHz) of **3** with AR at 298 K with a mixing time of 400 ms.

Figure 6. Agarose gel electrophoretic patterns of DNA nicked by **3**. The reaction samples contained 0.2 μg/μL of pBR322 plasmid. Line 1: no reagent in a Tris−HCl buffer (pH 7.2). Line 2: 20 μmol dm^{-3} of **3** incubated under visible light irradiation at 25 °C for 6 h. Electrophoresis was performed by using 1% agarose gel containing ethidium bromide (0.5 μmol/μL).

The K_s values also support this conclusion. The Job's experiments demonstrate the 1:1 stoichiometry for the inclusion complexation of AR with hosts **2** and **3**. Therefore, we can calculate the K_s values using eq 1[19]

$$\frac{1}{\phi_f^{\,0} - \phi_f(\text{obsd})} = \frac{1}{\phi_f^{\,0} - \phi_f'} + \frac{1}{K_s(\phi_f^{\,0} - \phi_f')[\text{H}]} \qquad (1)$$

where ϕ_f^0 is the initial fluorescence quantum yield of AR and ϕ_f(obsd) is the observed fluorescence quantum yield of AR in the presence of the host with the various concentrations. The obtained K_s value for **3** (12 600 M^{-1}) is much larger than that for **2** (3670 M^{-1}). This result concurs with the preferential complexation of AR by **3** through the cooperative binding by two CD cavities and the C_{60} unit. That is to say, besides two CD cavities, the C_{60} unit also acts as a positive binding site upon inclusion complexation with the guest molecules.

After validating the advantage of the bis(β-CD)−C_{60} conjugate on binding with the model substrate, we started to investigate its interaction potential with the biological molecules by examining the photodriven cleavage ability of **3** toward the pBR322 supercoiled DNA. Under dark conditions, DNA is not cleaved in the presence of native β-CD, mono-β-CD **1**, bis(β-CD) **2**, or bis(β-CD)−C_{60} conjugate **3**. Under visible-light irradiation, β-CD, **1**, and **2** still show no DNA-cleavage ability. However, bis(β-CD)−C_{60} conjugate **3** displays appreciable DNA-cleaving ability. Seen from Figure 6, about 50% of the closed supercoiled DNA (form I) is converted to the nicked DNA (form II; lane 2) after 6 h under visible-light irradiation in the presence of **3**, which is close to the values reported (about 50−90%) by Yamakoshi et al.[20] To explore the DNA-cleavage mechanism, the EPR spectrum of **3** with 2,2,6,6-tetramethyl-4-piperidone (TEMP) is performed under visible-light irradiation at 298 K. It is well-documented that, when the singlet oxygen ($^1O_2$) is sensitized by the photoexcitation of fullerene, it can be detected by EPR spin-trapping using a $^1O_2$-trapping agent such as TEMP,[21−23] because TEMP can react with $^1O_2$ to give a $^1O_2$ adduct, TEMPO. In the present case, three characteristic EPR signals assigned to TEMPO are observed in a phosphate buffer solution (pH 7.20) of the **3**/O_2 system under visible-light irradiation, as shown in Figure 7, which indicates that $^1O_2$ is generated. In the control experiments, neither **3** nor TEMP exhibits the appreciable EPR signals in the same condition. Moreover, bis(β-CD)−C_{60} conjugate **3** shows no DNA-cleaving ability in the absence of O_2. Therefore, we deduce that a singlet oxygen mechanism should be responsible for the DNA-cleavage reaction. That is, the C_{60} moiety in **3** is located close to the guanosine position of DNA. Under visible-light irradiation, the singlet oxygen ($^1O_2$) is sensitized by the photoexcitation of C_{60}. Then, the sensitized singlet oxygen reacts with the guanosines in the DNA by either the [4+2] or [2+2] cycloaddition to the five-membered imidazole ring of the purine base, thus cleaving the DNA.

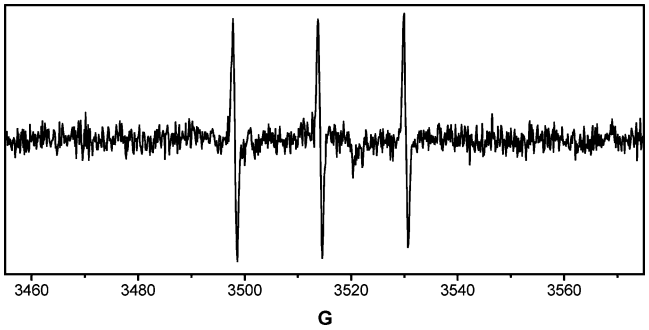


Figure 7. EPR spectrum of a mixture of **3** (0.06 mM) with TEMP (80 mM) after visible-light irradiation in a phosphate buffer solution (pH 7.20) at 298 K.

To investigate the relationship between the inclusion complexation ability of **3** and its DNA photocleavage property, we performed fluorescence titration experiments to determine the binding ability of **3** toward guanine. The results show that **3** gives a K_s value up to 2.58×10^8 M^{-2} upon complexation with two guanine molecules. That means the K_{app} value for the complexation of each β-CD cavity in **3** with one guanine molecule is as high as 1.61×10^4 M^{-1}. Moreover, the circular dichroism spectrum of **3** in the presence of guanine shows that the intensity of the circular dichroism signals of **3** in the absorption band of C_{60} obviously decreases with the addition of guanine (Figure 1), indicating the expulsion of C_{60} from the β-CD cavities when complexing **3** with guanine. These results jointly indicate that the β-CD cavity in **3** can efficiently bind to the guanosine position of DNA, which may significantly favor the interactions between the C_{60} unit in **3** and the guanosines of DNA through a singlet oxygen mechanism.

In conclusion, we successfully prepared a bridged bis(β-CD) as a fluorescence sensor for a variety of biochemical substrates and its water-soluble C_{60} conjugate as a DNA-cleaving reagent. Owing to their satisfactory water solubility and good biochemical properties, they could potentially be used in biological systems and pharmaceutical chemistry.

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Supporting Information Available: Equations to calculate the K_s values for the 1:2 inclusion complexation between host and guest. This material is available free of charge via the Internet at http://pubs.acs.org.

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