Synthesis of a water-soluble cyclodextrin modified hypocrellin and ESR study of its photodynamic therapy properties

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A water-soluble cyclodextrin modified hypocrellin B (HBCD) was designed and synthesized. HBCD retained the phototherapeutic properties and exhibited much stronger photoinduced damage to calf thymus DNA (CT DNA) than hypocrellin B and mercaptoacetic acid substituted hypocrellin B (MAHB). The mechanism of electron transfer from CT DNA to the triplet state of HBCD was confirmed by steady-state electron spin resonance (ESR) and a time-resolved ESR study.

Hypocrellins, including hypocrellin A (HA) and hypocrellin B (HB), are new photodynamic agents isolated from the fungus of *Hypocrella bambuase*. They have been successfully employed in the photodynamic therapy (PDT) treatment of certain skin diseases.[1] But their poor solubility in water limits their further use in photodynamic therapy. Many efforts have been made to enhance the water solubility of hypocrellins: Zou and coworkers used liposomes as a delivery system,[2] He *et al.* introduced mercaptoacetic acid into HB,[3] and Ma *et al.* used cysteine as hydrophilic groups.[4] In recent years, cyclodextrins, as traditional drug delivery agents,[5] have been successfully applied to improve the water solubility and biocompatibility of some phototherapeutic agents, porphyrins,[6] fullerenes,[7] *etc.*, while maintaining their therapeutic activities. However, there are still no reports utilizing cyclodextrin to improve the water solubility of hypocrellins. Herein, we report on the synthesis of a cyclodextrin modified hypocrellin derivative and its PDT properties.

It has been reported that hypocrellins and their derivatives exhibited photosensitized cleavage and damage to isolated and cellular DNA, whether under aerobic or anaerobic conditions.[8–10] In the presence of oxygen, the photoinduced cleavage involves reactive oxygen species,[8,9] while He *et al.* discovered that the electron transfer between the triplet state of hypocrellin and CT DNA, studied by UV-Visible spectroscopy, could initiate the photodamage of CT DNA in anaerobic conditions.[10] Clearly, ESR is a more powerful technique for investigating photoreaction intermediates. In this paper, steady state ESR (SS-ESR) and time-resolved ESR (TR-ESR) were employed to detect the radical intermediates responsible for the photodamage of CT DNA by our cyclodextrin modified hypocrellin under anaerobic and aerobic conditions.

Experimental

Chemicals

HB was prepared by dehydration of HA and purified by recrystallization twice from acetone.[11] β-Cyclodextrin (β-CD) of reagent grade was recrystallized twice from water and dried over night *in vacuo* at 110 °C. *p*-Tosyl chloride of reagent grade was recrystallized from petroleum ether (60–90 °C). *N*,*N*-Dimethylformamide (DMF) was dried over K_2CO_3 and distilled in the presence of calcium hydride *in vacuo*. Pyridine of analytical grade was dried over calcium hydride and was distilled in the presence of fresh calcium hydride just before use. All the other solvents were redistilled before use.

Calf thymus DNA (CT DNA) and cytochrome c were purchased from Sigma Chemical Company. 2,2,6,6-Tetraethyl-4-piperidone (TEMP), dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole hydrate (HOBT), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), superoxide dismutase (SOD), ethidium bromide (EB) and 9,10-diphenylanthracene (9,10-DPA) were all purchased from Aldrich Chemical Company, USA.

All experiments involving CT DNA were performed in buffer solution (10 mM acetic acid ammonium salt, 100 mM sodium chloride, pH 7), unless otherwise noted. DNA solutions were prepared by dispersing the desired amount of DNA in buffer solution and stirring overnight at a temperature below 4 °C. In the experiments where titration with DNA was required, the DNA solution was sonicated at 0 °C for 10 min using a Brason probe ultrasonicator. This operation significantly reduced the viscosity of the DNA solutions and permitted more accurate and precise titrations. The concentration of CT DNA is expressed as the concentration of nucleotide and was calculated using an average molecular weight of 338 for a nucleotide and an extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm.

Synthesis of cyclodextrin modified hypocrellin (Scheme 1)

Compound 2 (MAHB). Compound **2** was synthesized by modification of a literature procedure.[3] Compound **1** (HB) (2.8 mM) and mercaptoacetic acid (0.2 M) were put into a three-necked round-bottomed flask with 500 ml of methanol–buffer (1:3 v/v, pH 11) solution. The reaction mixture was bubbled with air (5 ml min^{-1}) and irradiated (>470 nm) with a medium pressure sodium lamp for 10 h. The mixture was isolated by TLC, and 0.63 g of monosubstituted HB was obtained (yield: 67%). ^{1}HNMR (400 MHz, $CHCl_3$-d_1, δ): 15.97 (s, 2H, exchangeable with D_2O, phenolic OH), 6.56 (s, 1H), 3.75–4.2 (m, 14H), 2.38 (s, 3H), 1.93 (s, 3H). FAB-MS ($[M - H]^-$): 617.

Compound 4. A solution of *p*-tosyl chloride (6.1 g, 32.0 mmol) in dry pyridine (200 ml) was added into a solution of β-CD (47.0 g, 41.4 mmol) and DCC (8.54 g, 41.4 mmol) in pyridine (600 ml) cooled below 0 °C. After stirring for 8 h at 0 °C

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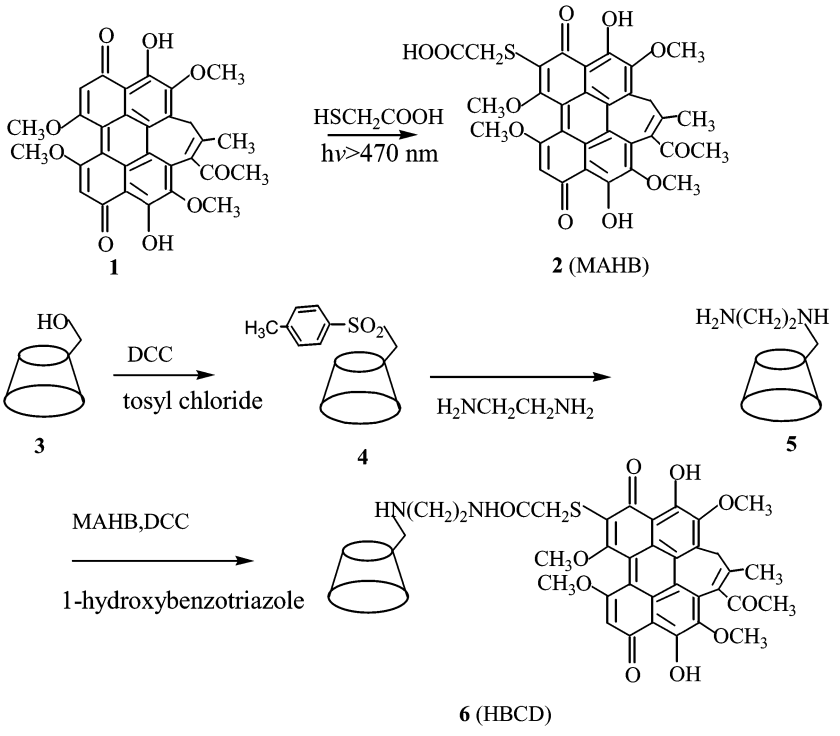


Scheme 1 Synthesis of cyclodextrin modified hypocrellin.

and 24 h at room temperature, the reaction was stopped by adding 2 ml of water to the solution and the mixture was evaporated *in vacuo* (10 mmHg) at 40 °C to dryness. The crude product was precipitated two times from DMF–acetone (100 ml:1000 ml). The precipitate was collected and recrystallized three times from water (42% yield). ^{1}HNMR (DMSO-d_6, δ): 7.7 (d, 2H), 7.4 (d, 2H), 5.6 (s, 14H), 4.85 (s, 7H), 4.5–3.3 (m, 47H), 2.42 (s, 3H).

Compound 5. Two grams of compound **4** were dissolved in 30 ml of ethylenediamine. After stirring for 20 h under reflux, 300 ml of acetone was added to the solution. The mixture was filtered and the precipitate was recrystallized from 95% ethanol. ^{1}HNMR (DMSO-d_6, δ): 6.0–5.6 (br m, 14H), 4.90 (s, 7H), 4.2–3.6 (br m, 47H), 2.70 (m, 4H), 2.5 (s, 1H), 2.3 (s, 2H).

Compound 6 (HBCD). Compound **2** (10 mmol) and compound **5** (15 mmol) were dissolved in dry DMF (20 ml), and then DCC (10 mmol) and HOBT (14 mmol) was added. After stirring for 48 h at room temperature, 200 ml of acetone was poured into the reaction mixture. The mixture was filtered. The precipitate was dissolved in 10 ml distilled water and this solution was directly loaded onto a Sephadex C-25 column (3 cm × 40 cm). The product was eluted from the column with distilled water. Fractions, each of 10 ml, containing the product were analyzed by TLC (eluant: acetic acid–ethyl acetate–water 8:1:2, $R_f = 0.50$). The fractions containing the product were combined and evaporated to dryness at 40 °C *in vacuo* (10 mm Hg). The final product (30% yield), 6-deoxy-6-*N*-[*N*′-(5-monomercapto acid hypocrellin B)diaminoethyl]-β-cyclodextrin (HBCD), was characterized by MALDI-TOF and ^{1}HNMR. ^{1}HNMR: 8.04 (s, 1H), 6.42 (s, 1H), 5.74 (s, 14H), 4.81 (s, 7H), 4.55 (s, 6H), 3.0–4.1 (62H), 2.77 (s, 1H), 2.07 (s, 3H), 1.89 (s, 3H). MALDI-TOF: 1780 [M – Na^+].

ESR measurements

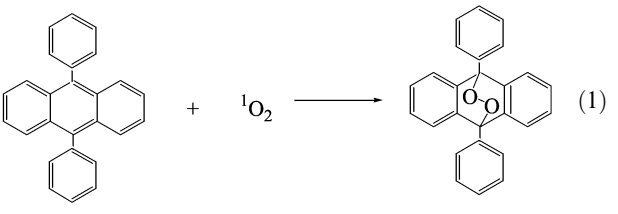
The ESR spectra were recorded at 25 °C on a Bruker ESP-300E spectrometer at 9.8 GHz, X-band with 100 Hz field modulation. Samples were injected quantitatively into specially made quartz capillaries for ESR analysis, and purged with argon, air or oxygen for 30 min in the dark, depending on the experimental requirements, and illuminated directly in the cavity of the ESR spectrometer with a Nd:YAG laser (532 nm, 5–6 ns pulse width, 10 Hz repetition frequency, 10 mJ pulse energy).

The superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}$OH) and singlet oxygen ($^1O_2$) were detected by ESR using DMPO or TEMP as the spin traps, respectively.

TR-ESR measurements were also performed on a Bruker ESP-300E spectrometer, while a boxcar integrator (Stanford SR 250) and a digital oscilloscope (LeCrory 9350A) were added to monitor transient signals. The data from a SR250 Computer Interface Module were transferred to a PC, in which data was stored and processed using the SR272 Data Acquisition Program.

Quantum yield of $^1O_2$ generation

The photobleaching of 9,10-diphenylanthracene (DPA) [eqn. (1)] was used to determine the quantum yields of $^1O_2$ generation by HBCD, MAHB and HB.[12]



(1)

An oxygen-saturated solution of DPA and these three kinds of hypocrellins in DMSO were irradiated with light of 436 nm separately.The quantum yields of singlet oxygen ($^1O_2$) sensitized by HBCD and MAHB were determined by using HB as a reference whose singlet oxygen quantum yield has been measured (0.76).[12]

Reduction of cytochrome c

The efficiency of superoxide anion ($O_2^{\bullet -}$) generation is often determined by the reduction of cytochrome c.[13,14] After reduction by $O_2^{\bullet -}$, the ferric cytochrome c (Fe^{3+}-cyt c) is transformed into ferrous cytochrome c (Fe^{2+}-cyt c). UV-Vis absorption of cytochrome c changes remarkably at 550 nm where the molar extinction coefficient is 8900 M^{-1} cm^{-1} for Fe^{3+}-cyt c and 29500 M^{-1} cm^{-1} for Fe^{2+}-cyt c. By monitoring the optical density change of the photoreaction system at 550 nm, the percentage of reduced cytocrome c can be determined according to eqn. (2):

$$\%\mathrm{cyt}_{\mathrm{red}} = 100 \times \frac{A(550)_t - A(550)_0}{A(550)_0 \times \frac{\varepsilon(550)_{\mathrm{red}}}{\varepsilon(550)_{\mathrm{ox}}} - A(550)_0} \qquad (2)$$

where $A(550)_0$ and $A(550)_t$ are the absorbance at 550 nm initially and after irradiation for time t, respectively. The $O_2^{\bullet -}$ generation efficiency can thus be estimated.

Ethidium bromide (EB) assay for DNA cleavage

A simple assay for DNA cleavage was applied based on the *ca.* 20-fold enhancement of the fluorescence intensity exhibited by EB upon intercalation into DNA.[15–17] When the concentration of EB is more than twofold that of the DNA base pair, the fluorescence intensity of EB is linearly proportional to the concentration of DNA base pair. Any process in which the potential EB binding site is destroyed results in a decrease in fluorescence intensity.

After preparation of solutions of EB–DNA buffer solution (80 μM EB, 40 μM CT DNA), HB, MAHB or HBCD was added into 8 ml of EB–DNA buffer solution at a final concentration of these hypocrellins of 10 μM. These mixture solutions were divided into 4 aliquots and irradiated in a 'merry-go-round' apparatus with a medium pressure sodium lamp ($h\nu > 470$ nm).[10] The aliquots were removed at various times and analyzed with the fluorescence emission spectrum excited at 510 nm and measured from 525–800 nm.

The percentage of binding site remaining (%BSR) at a given time t was calculated from the following equation:

$$\%\mathrm{BSR} = 100 \times \left(1 - \frac{I_0 - I_t}{I_0 - I_{\mathrm{buffer}}}\right) \qquad (3)$$

where I_0, I_t, and I_{buffer} denote the integrated fluorescence intensities before irradiation, after irradiation for time t, and of DNA-free buffer, respectively.

Results and discussion

Synthesis

Compound **2** (MAHB) was once prepared with a yield of only 12% by photoreaction of mercaptoacetic acid with Hypocrellin B (HB) in the presence of O_2.[3] The mechanism for this photoreaction has been proven to be nucleophilic addition of mercaptoacetic acid anion to triplet HB followed by oxidation. The role of O_2 in this reaction, however, is complex. On the one hand, O_2 is indispensable; on the other hand, too high a concentration of O_2 will ether quench the HB triplet or speed up the oxidation of mercaptoacetic acid anion, and consequently reduce the yield of mono- or bimercaptoacetic acid substituted HB. Therefore, it is necessary to optimize the concentration of O_2 to achieve high yields. In our experiments, this factor was controlled carefully, and a collection yield as high as 67% was obtained when O_2 was bubbled into the reaction system at the rate of 5 ml min^{-1} and the molar ratio of mercaptoacetic acid to HB was enhanced from 50 to 100 (Scheme 1). The mixture of 5- or 8-monomercaptoacetic acid substituted hypocrellin B (MAHB) is able to meet the requirements for PDT because both isomers have the same photochemical and photophysical properties.

Compound **4** was prepared by reaction of β-CD with tosyl chloride in dry pyridine. A low yield (*ca.* 31%)[18] was often obtained in this reaction because it is difficult to completely dehydrate the β-cyclodextrin. When we added an equimolar amount of dicyclohexylcarbodiimide (DCC) to serve as a dehydrating agent, the yield of compound **4** increased to 42%.

The UV-visible spectra of compound **6** (HBCD) and HB are similar (Fig. 1). The maximum concentration of HBCD in the buffer solution can reach 10 mM, while that of HB is less than 0.05 mM, which means that the water solubility of HBCD is enhanced significantly.

Electron transfer between HBCD and CT DNA under anaerobic conditions

After illuminating an argon-saturated DMSO–buffer solution (1:1 v/v, pH 7) of HBCD (0.1 mM) for 15 min with the laser at 532 nm, an ESR spectrum (Fig. 2, spectrum A) was recorded. This ESR signal increased in intensity when increasing the concentration of HBCD, suggesting that the self-electron transfer between the ground and the excited states of HBCD:

$$\mathrm{HBCD}^* + \mathrm{HBCD} \rightarrow \mathrm{HBCD}^{\bullet -} + \mathrm{HBCD}^{\bullet +} \qquad (4)$$

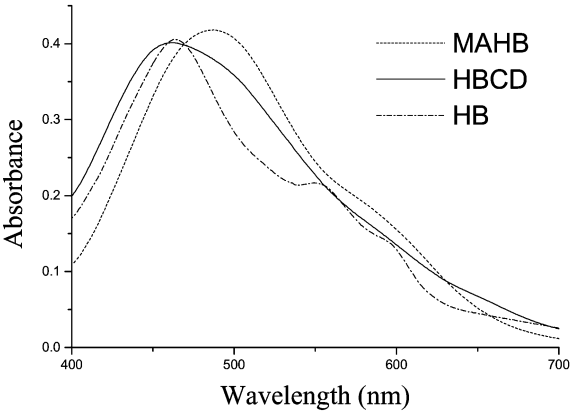


Fig. 1 Absorption spectra of HB, HBCD and MAHB in DMSO–buffer solution (1:1 v/v, pH 7).

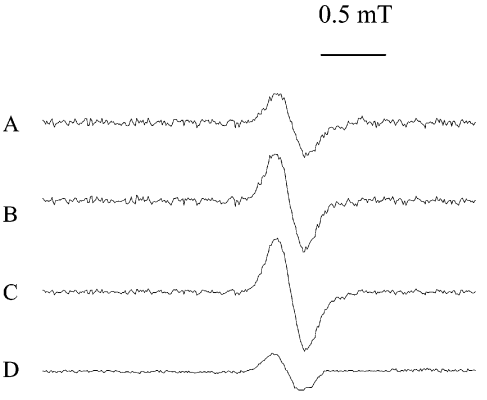


Fig. 2 Spectrum A, photoinduced ESR spectrum of a deaerated solution of HBCD (0.1 mM) in DMSO–buffer (1:1 v/v, pH 7), illuminated with 532 nm laser light for 15 min. Spectrum B, similar to spectrum A but in the presence of calf thymus DNA (2.85 mM). Spectrum C, similar to spectrum A, but in the presence of aniline (2.85 mM). Spectrum D, similar to spectrum B but with the addition of methylene blue (0.1 mM). Spectral parameter settings: X-band, microwave power, 8.0 mW; time constant, 20.48 ms; modulation amplitude, 2.0 G; receiver gain, 1×10^5.

might be responsible for the origin of the ESR signal. Due to their strong oxidation ability, quinone radical cations can only be detected in solutions with high ionization potential, such as $CFCl_3$,[19] Freon-133[20] and trifluoacetic acid[21] at low temperature. Hence this ESR signal is attributed to the HBCD radical anion. The g value of 2.002 is in good agreement with the literature.[22] The quenching of this ESR signal by O_2 supports such an assignment. Similar results were also obtained in the case of HA or HB.[23]

It is expected that the ESR signal intensity of HBCD radical anion will increase if a good reductant is present in the irradiated system, such that the electron transfer from the reductant to the excited state of HBCD:

$$HBCD^* + Reductant \rightarrow HBCD^{\bullet -} + Reductant^{\bullet +} \quad (5)$$

becomes more favorable than the self-electron transfer process of HBCD [eqn. (4)]. A remarkable increase in intensity of ESR signal was indeed observed (Fig. 2, spectrum C) when aniline, a strong electron donor, was added, and this observation in turn further proves the assignment of this ESR signal to HBCD radical anion. The intensity of the ESR signal also increased when CT DNA was added (Fig. 2, spectrum B), though the increase is smaller than in the case of aniline (see Table 1) owing to the weaker reductive ability of CT DNA relative to aniline. This suggests that CT DNA interacts with the excited state of HBCD as an electron donor:

$$HBCD^* + CT\,DNA \rightarrow HBCD^{\bullet -} + CT\,DNA^{\bullet +} \quad (6)$$

and, accordingly, gives rise to a stronger ESR signal of HBCD radical anion.

Photoreactions often occur in a non-thermal equilibrium state, and as a result, the created radicals usually possess a non-Boltzmann distribution of the spin state, which leads to chemically induced dynamic electron polarization (CIDEP).[24] As a powerful technique for the study of CIDEP, transient ESR often provides valuable information about the details of the initial stages of reactions that cannot be obtained by other methods. The short-lived intermediate radicals involved in the reaction can be detected and identified and thus the reaction mechanism can be clarified. Whether the reaction is taking place from an excited singlet state or a triplet state can only be easily deduced from the pattern of the spectrum in the form of enhanced absorption and/or stimulated emission peaks in the ESR spectrum.[25] Therefore, TR-ESR was applied in this work to gain insight into the photodamage mechanism of CT DNA by HBCD under anaerobic conditions.

A pure emissive TR-ESR signal (Fig. 3, spectrum B) was observed 420 ns after illuminating a DMSO–buffer solution (1:1 v/v, pH 7) of CT DNA and HBCD with a 532 nm pulsed laser. This signal is located at the same position as that of the HBCD anion radical generated in SS-ESR (Fig. 2, spectrum B) and therefore was attributed to the polarized ESR signal of HBCD radical anion. This assignment is supported by the fact that the signal can be quenched efficiently by O_2. This completely pure emissive TR-ESR signal can be attributed to the triplet mechanism (TM) of the photoreaction between CT DNA and the triplet HBCD. After pulsed laser illumination of HBCD, the triplet state T_1 was generated by efficient intersystem crossing (ISC) from the excited singlet state S_1. The ISC process is spin selective and creates spin polarized triplet states. In the presence of CT DNA, a precursor complex between CT DNA and triplet HBCD is formed in which electron transfer takes place with CT DNA serving as the electron donor. Accompanying the electron transfer reaction, the spin polarization of the HBCD triplet state is transferred to the produced radical anion. Thus, polarized HBCD anion radicals are formed. The precursor, triplets of HBCD, may have population in the T_+ or T_- spin states that deviate from the Boltzmann equilibrium at the time of reaction. An excess population in the T_+ level produces a stimulated emission ESR signal. Similar to the SS-ESR, in the presence of aniline, the TR-ESR signal (Fig. 3, spectrum C) increased in intensity, while only a very weak emissive TR-ESR signal was obtained at the absence of CT DNA or aniline (Fig. 3, spectrum A). The trend in the changes of polarized ESR signal intensity for the three solutions (see Table 1) agrees with the assignment that the polarized signals originate from the HBCD radical anions.

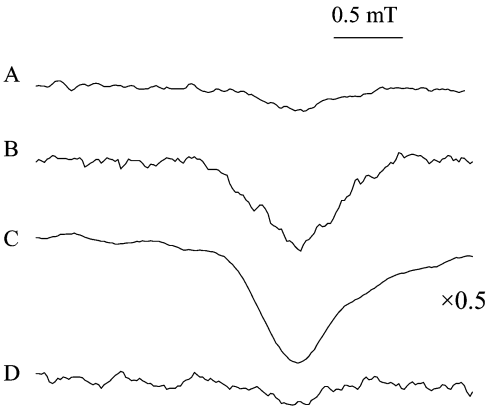


Fig. 3 Spectrum A, TR-ESR spectrum of argon-saturated HBCD (1 mM) DMSO–buffer solution (1:1 v/v, pH 7) measured 420 ns after the laser flash. Spectrum B, similar to spectrum A but in the presence of calf thymus DNA (2.85 mM). Spectrum C, similar to spectrum A but in the presence of aniline (2.85 mM). Spectrum D, similar to spectrum B, but with added methylene blue (1 mM). Spectral parameter settings: microwave power, 8.0 mW; time constant, 20.48 ms; modulation amplitude, 2.0 G; receiver gain, 1×10^5.

Table 1 Relative intensity of ESR signal obtained with HBCD, HBCD + CT DNA, HBCD + aniline, HBCD + CT DNA + MB

	Relative Intensity	
Sample	SS-ESR	TR-ESR
HBCD	1.00	1.00
HBCD + DNA	1.54	4.13
HBCD + aniline	1.77	7.43
HBCD + DNA + MB	0.58	1.09

In our TR-ESR experiment, only HBCD anion radical was observed. There were some reports on electron transfer from pyrimidine to triplet states of anthraqunone derivatives,[26–28] where the pyridine cation radicals were detected by Fourier Transform ESR (FT-ESR), which has a high resolution on the nanosecond time scale. An alternative approach to observe ESR signals of cation radicals of DNA bases is to carry out measurements at very low temperature (77 K).[29] However, ,the CT DNA cation radical signal was not observed in our experiments, probably because of the limitation of our ESR instrument.

When methylene blue was added into the solution mixture of CT DNA and HBCD (spectra D in Figs. 2 and 3), both the SS-ESR and the TR-ESR signal dramatically weakened. This is because methylene blue quenchs the triplet of HBCD and inhibits the electron transfer from CT DNA to triplet HBCD. From all of these results, electron transfer *via* CT DNA and the triplet HBCD can be definitely concluded.

Generation of superoxide anion radical ($O_2^{\bullet -}$)

The photoinduced SS-ESR spectrum of $HBCD^{\bullet -}$ disappeared when oxygen was bubbled through the argon-saturated DMSO solution containing HBCD (0.1 mM) after irradiation.

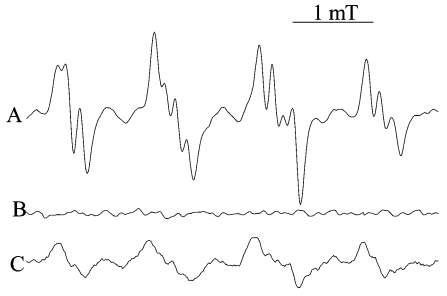


Fig. 4 Spectrum A, ESR spectrum of DMPO–superoxide radical adduct produced from irradiation of an oxygen-saturated DMSO solution of HBCD (0.2 mM) and DMPO (50 mM) for 2 min. Spectrum B, in the absence of HBCD, oxygen or light. Spectrum C, similar to spectrum A but in the presence of SOD (40 μg ml^{-1}). Spectral parameter settings: microwave power, 1.0 mW; time constant, 20.48 ms; modulation amplitude, 1.0 G; receiver gain, 1×10^5.

One possible explanation for this observation is that electron transfer from $HBCD^{\bullet -}$ to oxygen takes place and $O_2^{\bullet -}$ is produced:

$$HBCD^{\bullet -} + O_2 \rightarrow HBCD + O_2^{\bullet -} \quad (7)$$

The following experiments were carried out to examine this possibility. When an oxygen-saturated DMSO solution containing HBCD (0.1 mM) and DMPO (50 mM) was irradiated, a typical spectrum of the DMPO–$O_2^{\bullet -}$ adduct was immediately seen(Fig. 4, curve A). The hyperfine coupling constants and the position is in good agreement with the literature ($\alpha^N = 0.13$ mT, $\alpha_\beta^H = 0.99$ mT, $\alpha_\gamma^H = 0.015$ mT).[30] Control experiments confirmed that oxygen, HBCD, and light were all necessary to produce DMPO–$O_2^{\bullet -}$ (Fig. 4, curve B). The ESR spectrum could be suppressed by the addition of SOD (40 μg ml^{-1}), an efficient scavenger of superoxide anion (Fig. 4, curve C), which further confirms the assignment of the ESR spectrum to DMPO–$O_2^{\bullet -}$ adduct.

Although the spin trapping method is more sensitive than the reduction of cytochrome c for the measurement of $O_2^{\bullet -}$, the cytochrome c method is a more traditional method to quantitatively analyze $O_2^{\bullet -}$.[31] When oxygen and cytochrome c (cyt Fe^{3+}) were present in the buffer solution containing HBCD, irradiation resulted in an increase in absorption intensity at 550 nm, which is characteristic of the reduction of cytochrome c (cyt $Fe^{3+} \rightarrow$ cyt Fe^{2+}). If SOD is also included in the reaction solution, the reduction of cytochrome c is inhibited, suggesting that the cytochrome is reduced by $O_2^{\bullet -}$ rather than by direct electron transfer from hypocrellin anion radicals to the cytochrome c. Attempts were also made to compare the $O_2^{\bullet -}$ generation efficiency of HBCD with that of HB and MAHB. Fig. 5 suggests that HBCD generates $O_2^{\bullet -}$ with a lower efficiency than that of MAHB, but higher than that of HB. The higher $O_2^{\bullet -}$ generation efficiency of MAHB can be attributed to its stronger electron donating properties due to the presence of the mercapto group. When cyclodextrin is introduced, the bulkiness of cyclodextrin weakens the electron transfer between the exited state of HBCD and ground state HBCD and reduces the generation of $HBCD^{\bullet -}$, from which $O_2^{\bullet -}$ can be generated *via* eqn. (7). As a result, the $O_2^{\bullet -}$ generation efficiency of HBCD is lower than that of MAHB.

Generation of hydroxyl radical (•OH)

After addition of DMPO (50 mM) to an oxygenated buffer solution of HBCD (0.6 mM, pH 7), irradiation with the laser at 532 nm gave rise to an ESR spectrum of the spin adduct DMPO–•OH (Fig. 6, spectrum A), which is characterized by two coupling constants connected with the non-zero nuclear

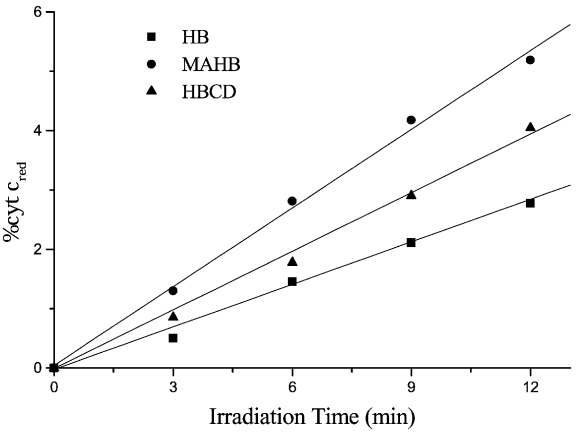


Fig. 5 Reduction of cytochrome c (58 μM) in oxygen-saturated buffer solution (pH 7) by hypocrellins (10 μM) as a function of irradiation time.

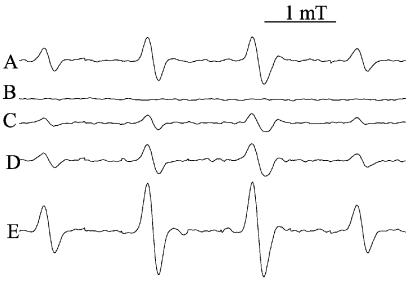


Fig. 6 Spectrum A, ESR spectrum of DMPO–hydroxyl radical adduct resulting from irradiation of an oxygen-saturated buffer solution of HBCD (0.4 mM, pH 7) and DMPO (50 mM). Spectrum B, in the absence of HBCD, oxygen or light. Spectrum C, similar to spectrum A, but in the presence of sodium benzoate (10 mM). Spectrum D, similar to spectrum A, but with HB instead of HBCD. Spectrum E, similar to spectrum A, but MAHB was used instead of HBCD. Spectral parameter settings: microwave power, 1.0 mW; time constant, 20.48 ms; modulation amplitude, 2.0 G; receiver gain, 1×10^5.

spins of the nitrogen atom and the hydrogen atom in the β-position: $\alpha^N = \alpha^H = 14.9$ G. The two identical coupling constants from β-N and β-H give a four-line ESR spectrum with an intensity ratio of 1:2:2:1. The values of the coupling constants are in good agreement with the literature.[32]

Control experiments ensured that no signal was obtained without light, oxygen, or HBCD (Fig. 6, spectrum B). The presence of sodium benzoate, a scavenger of hydroxyl radical, decreases the ESR signal significantly (Fig. 6, spectrum C). These observations confirm the assignment of the spectrum shown in Fig. 6 (spectrum A) to the DMPO–•OH radical adduct.

In addition, we compared the •OH generation efficiency of HBCD with HB and MAHB at the same concentration (Fig. 6, spectra D and E). It can be seen that HBCD generates •OH with lower efficiency than MAHB, but higher than that of HB. Interestingly, HBCD, MAHB and HB generate $O_2^{\bullet -}$ with the same trend (*c.f.* Fig. 4).

Generally speaking, there are two pathways to generate hydroxyl radical in the hypocrellin photosensitization system.[33,34] One is *via* the superoxide-driven Fenton reaction [eqns. (8) and (9)]:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \quad (8)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + HO^{\bullet} \quad (9)$$

in which H_2O_2 originates from dismutation of the superoxide radical:

$$O_2^{\bullet -} + O_2^{\bullet -} \rightarrow H_2O_2 + O_2 \quad (10)$$

while Fe^{3+}, Fe^{2+} or other transition metal ions with multiple oxidation states are often present in trace amounts in aqueous solution.

The other pathway is *via* the reaction of semiquinone radical anion with H_2O_2:

$$HBCD^{\bullet -} + H_2O_2 \rightarrow HBCD + {}^{-}OH + HO^{\bullet} \quad (11)$$

Both pathways involve the generation of superoxide anion radical; this is why the hypocrellin derivative with higher $O_2^{\bullet -}$ generation efficiency can produce $^{\bullet}OH$ more efficiently.

Generation of singlet oxygen

It has been observed that $^1O_2$ was involved in many photooxidation reactions sensitized by hypocrellins.[35] $^1O_2$ is generated *via* energy transfer from the triplet of hypocrellins to the ground state oxygen. Moan and Wold[36] have reported that TEMP can easily react with $^1O_2$ to yield a nitroxide, TEMPO, which is long-lived and hence can be detected readily by SS-ESR spectroscopy. Irradiation of oxygen-saturated DMSO solutions containing HBCD (0.1 mM), SOD (40 μg ml^{-1}), HCOONa (0.1 M) and TEMP (20 mM) afforded a typical three-line ESR spectrum (Fig. 7, spectrum A). The coupling constants $\alpha^N = 16.0$ G and $g = 2.0056$ are in good agreement with those of TEMPO in the literature.[37] Control experiments confirmed that oxygen, HBCD, and light are all necessary to produce this ESR spectrum (Fig. 7, spectrum B). The addition of NaN_3 (1 mM), a traditional singlet oxygen scavenger, diminishes the ESR signals significantly (Fig. 7, spectrum C).

The photooxidation of DPA to its *endo*-peroxide derivative by singlet oxygen is usually used to detect singlet oxygen formed from hypocrellin.[12] In order to compare the quantum yield of HBCD with that of HB and MAHB, the DPA bleaching method was adopted by using HB as reference.

Control experiments indicated that the presence of O_2, hypocrellins, and light are all essential for DPA bleaching. Moreover, the addition of NaN_3 (1 mM) almost completely inhibited DPA bleaching. These results confirmed that the bleaching of DPA resulted from the reaction of DPA with $^1O_2$ formed by hypocrellin photosensitization. The $^1O_2$ quantum yields for both HBCD and MAHB in DMSO are estimated to be 0.22 by using HB (0.76) as reference (Fig. 8).

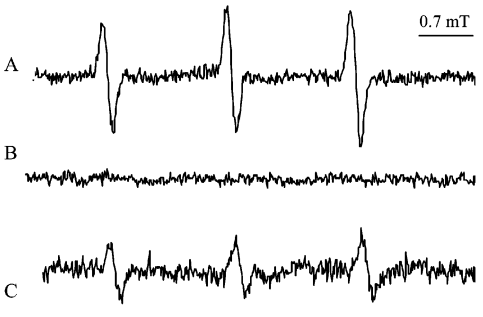


Fig. 7 Spectrum A, ESR spectrum produced by irradiation of an oxygenated DMSO–buffer solution (1:1 v/v, pH 7) containing HBCD (0.1 mM), SOD (40 μg ml^{-1}), HCOONa (0.1 M) and TEMP (10 mM) for 2 min. Spectrum B, in the absence of HBCD, oxygen or light. Spectrum C, similar to spectrum A but in the presence of NaN_3 (1 mM). Spectral parameter settings: microwave power, 3.17 mW; time constant, 10.24 ms; modulation amplitude, 1.0 G; receiver gain, 1×10^4.

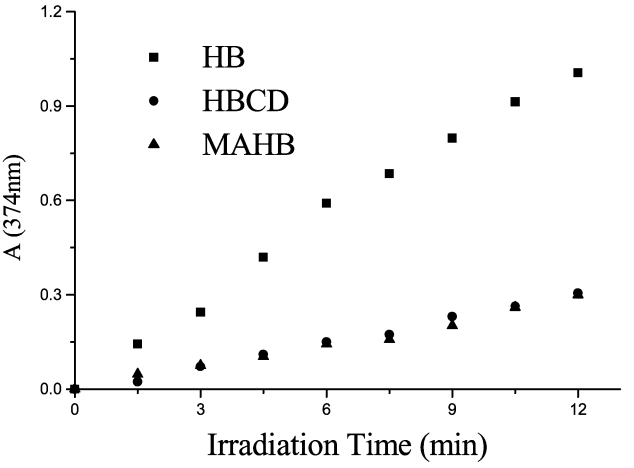


Fig. 8 DPA (0.6 mM) bleaching in oxygen-saturated DMSO photosensitized using hypocrellins ([HB] = 11.2 μM, [HBCD] = 10 μM, [MAHB] = 13.5 μM) by measuring the absorbance decrease (ΔA) at 374 nm for different irradiation times.

The generation of $O_2^{\bullet -}$, $^{\bullet}OH$ and $^1O_2$ by HBCD photosensitization indicates that it maintains the photodynamic activity in terms of type I and type II mechanisms.

Photoinduced damage to CT DNA

It has been reported that the combining of cyclodextrin with the potent DNA intercalating agent anthrylamine provided for a superior water solubility as well as an increased affinity for the negatively charged groove of double-strand DNA.[38] So it could be expected that this cyclodextrin modified hypocrellin derivative (HBCD) also possesses a higher affinity for the negatively charged groove of CT DNA than HB and MAHB, and therefore induces greater photodamage to the DNA.

When HB was added into the anaerobic buffer solution of EB–DNA (80 μM EB, 40 μM CT DNA), 20% of binding sites were destroyed during a 30 min irradiation as calculated fromeqn. (3) (Table 2). Under the same conditions, MAHB and HBCD exhibited similar destruction ability, a little higher than HB. Under anaerobic conditions, the photodamage of CT DNA results from the electron transfer between CT DNA and triplet HB, MAHB or HBCD. But DNA is not a strong reductant and therefore the poor photoinduced electron

Table 2 Photocleavage of CT DNA by the hypocrellins (10 μM) detected by remaining binding site (BSR%) of ethidium bromide to the damaged CT DNA under different conditions [CT DNA] = 40 μM, [EB] = 80 μM.

	Irradiation time/min		
Sample	10	20	30
Control experiment[a]	97.6	95.6	94.1
HBCD + N_2	90	82.4	77.1
HB + N_2	89.6	85.3	80.8
MAHB + N_2	89.1	84.2	77.6
HBCD + O_2	64.6	48	40.6
HB + O_2	79.5	74.1	68.5
MAHB + O_2	75.8	61.9	55.9
HBCD + O_2 + SOD[b]	80.3	68.8	59.5
HBCD + O_2 + NaN_3[c]	73.6	61.4	55.9
HBCD + O_2 + C_6H_5COONa[d]	76.2	65.6	53.7

[a] In the absence of hypocrellins. [b] [SOD] = 40 μg ml^{-1}. [c] [NaN_3] = 1 mM. [d] [$C_6H_5CO_2Na$] = 10 mM

transfer efficiency perhaps accounts for the none observation of higher photodamage with HBCD, contrary to expectation.

In the presence of oxygen, the photosensitized cleavage efficiency of HB, MAHB, and HBCD to CT DNA all increased (Table 2). However, HBCD inflicts stronger photoinduced damage to CT DNA than HB and MAHB. Besides the electron transfer damage pathway, the reactive oxygen species ($O_2^{\bullet -}$, $^1O_2$, $^{\bullet}OH$) are included in the process of hypocrellin-photosensitized cleavage of CT DNA, which is confirmed by the addition of SOD, NaN_3 or sodium benzoate into the solution (Table 2).

Although HBCD generates $O_2^{\bullet -}$, $^{\bullet}OH$ less efficiently than MAHB and generates $^1O_2$ less efficiently than HB, the water solubility and affinity for DNA are improved significantly relative to HB and MAHB, and as a resultant, HBCD exhibits more efficient photodamage to CT DNA.

Conclusions

By strictly controlling the reaction conditions of HB photoreaction with the mercaptoacetic acid, the collection yield of monosubstituted HB increased from 12% to 67%. The yield from the tosylation of the β-cyclodextrin was likewise increased from 31% to 42% when DCC was used as dehydrant. Profitting from the above improvements, the cyclodextrin modified HB (HBCD) was successfully synthesized. The water solubility of HBCD was enhanced significantly because of the introduction of the β-cyclodextrin group.

The electron transfer between triplet HBCD and CT DNA was confirmed by SS-ESR and TR-ESR studies. A pure emissive CIDEP signal of the hypocrellin, generated *via* the triplet mechanism, is first reported in this paper. When CT DNA is added to the HBCD solution, this CIDEP signal increases in an obvious fashion. The increase of the CIDEP signal intensity results from the electron transfer from CT DNA to the triplet HBCD, which was proved by various control experiments. The electron transfer between CT DNA and HBCD initiates the photodamage of CT DNA under anaerobic conditions.

HBCD also preserves the photodynamic properties of HB and can generate active oxygen species such as oxygen anion radical, hydroxyl radical and singlet oxygen. All these active oxygen species can inflict damage on CT DNA. The β-cyclodextrin group reinforces the affinity of HBCD for CT DNA, and allows the active oxygen species formed by HBCD to damage CT DNA more efficiently. As a result, HBCD exhibits better photodynamic therapy properties than HB and MAHB. In a word, the introduction of the β-cyclodextrin group enhances both water-solubility and the PDT suitability of HBCD.

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References

1 N. W. Fu, Y. X. Chu and J. Y. An, *Acta Pharm. Sin.*, 1989, **10**, 371.
2 W. Zou, J. Y. An and L. J. Jiang, *Sci.China, Ser. B*, 1996, **26**, 206.
3 Y. Y. He, J. Y. An, W. Zou and L. J. Jiang, *J. Photochem. Photobiol., B*, 1998, **44**, 45.
4 L. Ma, M. H. Zhang and L. J. Jiang, *Kexue Tongbao*, 1991, **36**, 1551.
5 D. Duchen, *Cyclodextrins and their Industrial Use*, Edition de Sante, Paris, 1987.
6 J. G. Moser, A. Ruebner, A. Vervoots and B. Wangner, in *Proceedings of the Eighth International Symposium on Cyclodextrins*, ed. J. Szetli, Kluwer, Boston, 1996, p. 71.
7 S. Samal and K. E. Geckler, *Chem.Commun.*, 2000, 1101.
8 E. H. En, S. M. Xin and L. S. Cheng, *Int. J. Radiat. Biol.*, 1992, **61**, 213.
9 W. Zou, J. Y. An and L. J. Jiang, *J. Photochem. Photobiol., B*, 1996, **33**, 73.
10 Y. Y. He and L. J. Jiang, *Biochem. Biophys. Acta*, 2000, **1523**, 29.
11 K. H. Zhao and L. J. Jiang, *Youji Huaxue*, 1989, **9**, 252.
12 Z. J. Diwu and J. W. Lown, *J. Photochem. Photobiol., A*, 1992, **64**, 273.
13 I. Fridovich, *J. Biol. Chem.*, 1970, **245**, 4053.
14 C. Hadjur, A. Jeunet and P. Jardonj, *J. Photochem. Photobiol., B*, 1994, **26**, 67.
15 W. A. Pruetz, *Radiat. Environ. Biophys.*, 1984, **23**, 1.
16 W. A. Pruetz, *Radiat. Environ. Biophys.*, 1984, **23**, 7.
17 H. C. Birnboim and J. J. Jevcak, *Cancer Res.*, 1981, **41**, 889.
18 Y. Matsui and A. Okimoto, *Bull. Chem. Soc. Jpn.*, 1978, **51**, 3030.
19 H. Chandra, L. Portwood and M. C. R. Symons, *J. Chem. Soc., Faraday Trans. 1*, 1989, **85**, 1799.
20 J. Mayer and R. Krasluklanls, *J. Chem. Soc., Faraday Trans. 1*, 1991, **87**, 2943.
21 M. C. Depew, Z. L. Liu and J. K. S. Wan, *J. Am. Chem. Soc.*, 1983, **105**, 2480.
22 L. Y. Zang, B. R. Misra and H. P. Misra, *Photochem. Photobiol.*, 1992, **56**, 453.
23 Y. Z. Hu, J. Y. An, L. J. Jiang and D. W. Chen, *J. Photochem. Photobiol., A*, 1995, **89**, 45.
24 Y. N. Molin, *Spin Polarization and Magnetic Effects in Radical Reactions*, Elsevier, Amsterdam, 1984.
25 K. A. McLauchlan, *Modern Pulsed and Continious-Wave Electron Spin Resonance*, John Wiley and Sons Inc., New York, 1990.
26 J. Geimer and D. Beckert, *J. Phys. Chem.*, 1999, **103**, 3991.
27 J. Geimer, O. Brede and D. Beckert, *Chem. Phys. Lett.*, 1997, **276**, 411.
28 J. Geimer and D. Beckert, *Chem. Phys. Lett.*, 1998, **288**, 449.
29 M. D. Sevilla, *J. Phys. Chem.*, 1976, **80**, 1898.
30 J. R. Harbour and M. L Hair, *J. Phys. Chem.*, 1978, **82**, 1397.
31 S. P. Sanders, S. J. Harrison, P. Kuppusamy, J. T. Sylvester and J. L. Zweier, *Free Radical Biol. Med.*, 1994, **16**, 753.
32 K. Lang, M. Wagnerova, P. Stopka and W. Dameran, *J. Photochem. Photobiol., A*, 1992, **67**, 187.
33 Y. Y. He, J. Y. An and L. J. Jiang, *Free Radical Biol. Med.*, 1999, **27**, 203.
34 Y. Z. Hu and L. J. Jiang, *J. Photochem. Photobiol., B*, 1996, **33**, 51.
35 L. J. Jiang, *Kexue Tongbao*, 1990, **35**, 1608.
36 J. Moan and E. Wold, *Nature*, 1979, **279**, 450.
37 Y. Lion, M. Delmelle and A. V. Vorst, *Nature*, 1976, **263**, 442.
38 T. Ikeda, K. Yoshida and H. J. Schneider, *J. Am. Chem. Soc.*, 1995, **117**, 1453.