Free Radical Research, Vol. 35, pp. 607–617
Reprints available directly from the publisher
Photocopying permitted by license only

Photosensitization Mechanism of Active Species by the Complex of Hypocrellin B with Aluminum Ion

JIANG-HUA MA, JING-QUAN ZHAO* and LI-JIN JIANG

Center for Molecular Science, Institute of Chemistry, The Chinese Academy of Sciences, Beijing 100080, China

Accepted by Professor B. Kalyanaraman

(Received 4 December 2000; In revised form 2 March 2001)

To improve the water solubility and red absorption of the parent hypocrellin B (HB), the complex of HB with aluminum ion has been first synthesized in high yield. The photodynamic action of Al^{3+}-HB, especially the generation mechanism of active species, $([Al^{3+}\text{-}HB]^{\cdot-}$, $O_2^{\cdot-}$ and $^1O_2$) was studied using electron paramagnetic resonance (EPR) and spectrophotometric methods. In the deoxygenated DMSO solution of Al^{3+}-HB, the semiquinone anion radical of Al^{3+}-HB is photogenerated via the self-electron transfer between the excited and ground state species. The presence of electron donor significantly promotes the reduction of Al^{3+}-HB. When oxygen is present, superoxide anion radical ($O_2^{\cdot-}$) is formed via the electron transfer from $[Al^{3+}\text{-}HB]^{\cdot-}$ to the ground state molecular oxygen. Singlet oxygen ($^1O_2$) can be produced via the energy transfer from triplet Al^{3+}-HB to ground state oxygen molecules. Furthermore, it is very significant that the accumulation of $[Al^{3+}\text{-}HB]^{\cdot-}$ would replace that of $O_2^{\cdot-}$ or $^1O_2$ with the consumption of oxygen in the sealed system.

Keywords: Aluminum ion-complexed hypocrellin B; Semiquinone anion radical; Superoxide anion radical; Singlet oxygen; Electron paramagnetic resonance

Abbreviations: PDT, photodynamic therapy; HA, hypocrellin A; HB, hypocrellin B; DMPO, 5,5-Dimethyl-1-pyrroline-*N*-oxide; SOD, superoxide dismutase; DPA, 9,10-diphenylanthracene; DABCO, 1,4-Diazabicyclo[2.2.2]octane; TEMP, 2,2,6,6-tetramethy1-4-piperidone; TEMPO, 2,2,6,6-tetramethy1-4-piperidone-*N*-oxyl radical

INTRODUCTION

Hypocrellins, including hypocrellin A (HA) and hypocrellin B (HB), are new types of naturally photosensitive pigments and medicines which, derived their names from the natural fungus *Hypocrella bambusae* (B et Br) sacs, growing abundantly in Yunan province of China.[1,2] Recent investigations demonstrated that hypocrellins and their derivatives could induce the photo-damage to viruses,[3–5] tumor cells,[6,7] mixed phospholipids,[8] and pBR322 DNA.[9] Hypocrellins are efficient singlet oxygen generators during photochemical reaction[10]

*Corresponding author. Present address: Institute of Photographic Chemistry, The Chinese Academy of Sciences, Beijing 100101, China.

and may also exert photosensitization via radical mechanism,[11] which may offer an alternative to classical oxygen-dependent photochemical mechanisms. This feature is important in the context of impaired radiosensitivity and chemosensitivity of hypoxic human tumor cells. These findings provide compelling impetus for the development of hypocrellins and their derivatives as photodynamic therapy (PDT) photosensitizers.

Recently, people have paid much attention to develop photosensitizers towards better tumor selectivity and higher phototherapeutic efficiency. Although the mechanism of photosensitizer retention by tumors is not well understood, the balance between lipophilicity and hydrophilicity is recognized as an important factor for the photosensitizing efficiencies and cellular uptake.[12,13]

However, natural hypocrellins are lipophilic compounds that prevent their effective drug delivery *in vivo* and they do not show strong absorption in the domain of phototherapeutic windows (600–900 nm), which limits their application on the treatment of solid tumors. To be targeted at the requirements of suitable water solubility and strong red absorption, the complex of HB with aluminum ion (Al^{3+}-HB) (Fig. 1) has been first synthesized in a high yield. It is easily soluble in water or organic polar solvents. Preliminary study demonstrated that Al^{3+}-HB could generate superoxide anion radical ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) efficiently.[14] In order to clarify the generation mechanism of active species by Al^{3+}-HB, in this paper, we further studied the photogeneration processes of semiquinone anion radical and active oxygen species by this new photosensitizer using electron paramagnetic resonance (EPR) and spectrophotometric methods.

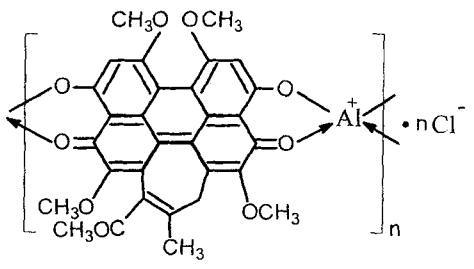

FIGURE 1 The chemical structure of Al^{3+}-HB.

EXPERIMENTAL

Chemicals

Al^{3+}-HB was synthesized according to our previous report.[14] Catalase, superoxide dismutase (SOD) and cytochrome c were purchased from Sigma Chemical Company. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,2,6,6-tetramethy1-4-piperidone (TEMP), 9,10-dipheny1-anthracene (DPA) were purchased from Aldrich Chemical Company. Cysteine and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Biochem. Technology Corporation, the Chinese Academy of Sciences. 1,4-Diazabicyclo[2.2.2]octane (DABCO) and dimethylsulfoxide (DMSO) were purchased from Merck Chemical Company. Sodium azide (NaN_3), deuterated solvents and other agents of analytical grades were purchased from Beijing Chemical Plant. Water was freshly distilled before use. The working stock solutions were prepared immediately before use.

Spectroscopic Measurements

The UV-Visible absorption spectra were recorded on a Shimadzu UV-1601 spectrophotometer. The variation of the absorption spectra with irradiation time in the presence of electron donor was used to monitor the photoinduced reduction of Al^{3+}-HB. A 500 W high-pressure mercury lamp with a 578 nm monochromatic filter was used as the light source. Fluorescence spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer.

EPR Measurements

The EPR measurements were performed at 9.80 GHz in a Bruker ESP 300E spectrometer at room temperature. Unless otherwise indicated,

the following instrumental settings were used: microwave power, 10 mW; modulation amplitude, 1 G; sweep width, 100 G; receiver gain, 1.25×10^4. Photoinduced EPR spectra were obtained from the samples (40 μl) injected into quartz capillaries designed specially for EPR analysis. Anaerobic samples were prepared in cuvettes that allowed purging the reactive volume with argon for 30 min in the dark. Samples were irradiated directly inside the microwave cavity of the spectrometer using a 532 nm YAG-900 Laser (Spectro-Physics Laser, Mountain View, CA, USA). EPR spectra were recorded and manipulated in an IBM/PC computer. The kinetics of spin adduct generation were studied by recording the peak height of an EPR spectrum every 30 s.

RESULTS AND DISCUSSION

The complexation of HB with Al^{3+} resulted in the change of 4,9-dihydroxyl-3,10-perylenequinonoid moiety in HB. The resulting product is easily soluble in organic polar solvents such as DMSO, DMF, CH_3CN, CH_3CH_2OH, CH_3OH, and even in H_2O. Furthermore, the longest absorption band shifts to 614 nm (Fig. 2). So it is necessary to investigate the effect of chemical modifications on the photodynamic action of the new photosensitizer. It is believed that not only the active oxygen species but also the semiquinone anion radical of hypocrellins participate in the photodynamic damage.[15–17] The generation efficiencies of active species by Al^{3+}-HB have been discussed elsewhere in detail;[14] herein, we will survey their generation mechanism during photosensitization.

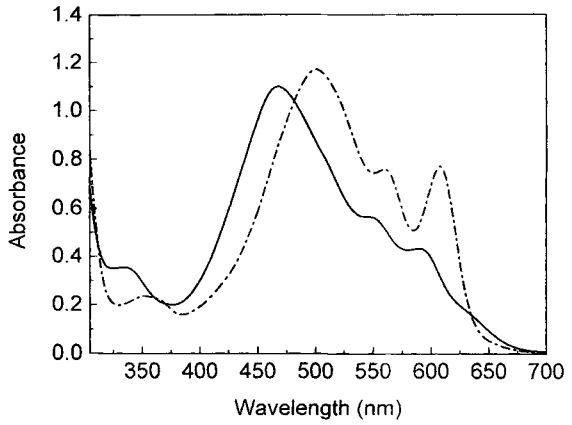

FIGURE 2 Absorption spectra of HB and Al^{3+}-HB measured in DMSO solution. —— HB (3.3×10^{-5} mol l^{-1}), —Al^{3+}-HB (0.22 mg ml^{-1}; ≤ 3.3×10^{-5} mol l^{-1}).

Generation of Al^{3+}-HB Semiquinone Anion Radical

EPR Measurements

Irradiation of Al^{3+}-HB (5 mg ml^{-1}) in deoxygenated DMSO solution for 1 min generated a strong hyperfine EPR spectrum shown in Fig. 3A, with $g = 2.0079$.

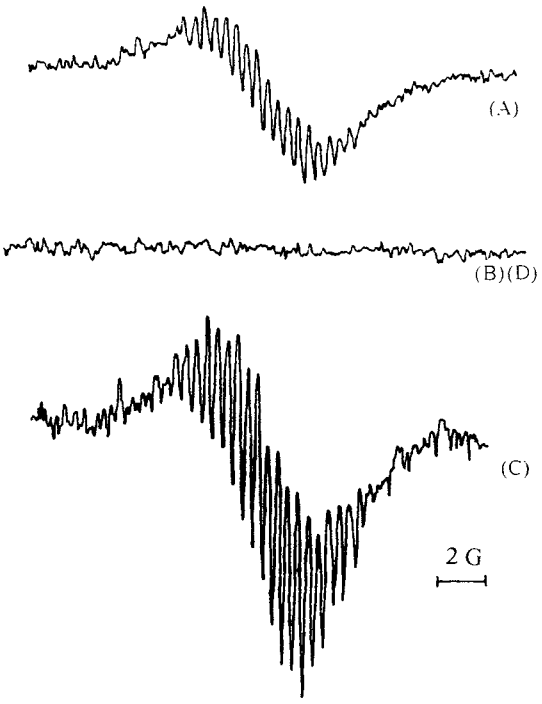

FIGURE 3 (A) Photoinduced EPR spectrum from a deoxygenated DMSO solution of Al^{3+}-HB (5 mg ml^{-1}) on illumination for 1 min. (B) Same as (A) except that Al^{3+}-HB, or light was omitted. (C) Same as (A) except that NADH (2 mM) was added. (D) Same as (A) except that oxygen was bubbled through the solution after illumination. Instrumental settings; microwave power, 5.05 mW; modulation amplitude: 0.1 G; scan width, 50 G; receive gain, 1.25×10^{-4}.

In general, the cation radical of quinone is difficult to detect in common organic solvent due to its strong oxidizing ability. The detection of cation radical should be performed in solvents with high ionization potential such as Freon-113 at low temperature.[18] Therefore, the detected EPR signal might be ascribed to the Al^{3+}-HB anionic radical. In order to identify the EPR signal further, the following experiments were carried out.

(1) No EPR signal could be detected in the absence of Al^{3+}-HB or illumination (Fig. 3B), which implied that the EPR signal was generated via photosensitization of Al^{3+}-HB.
(2) When NADH (2 mM), a typical electron donor, was added to the deoxygenated DMSO solution of Al^{3+}-HB and irradiated for 10 s, the EPR signal obtained (Fig. 3C) was similar to that in Fig. 3A, but the signal intensity was enhanced significantly. This indicates the anionic nature of Al^{3+}-HB radical.
(3) When oxygen was bubbled through the irradiated Al^{3+}-HB solution, the EPR signal of Al^{3+}-HB radical disappeared completely (Fig. 3D). Furthermore, when DMPO and oxygen were present in the irradiated Al^{3+}-HB solution, the EPR signal of DMPO-superoxide radical adduct would be detected immediately, accompanied by the significant decrease or disappearance of the Al^{3+}-HB radical signal (details discussed below).

In accordance with these observations, the EPR signal shown in Fig. 3A could be safely assigned to the semiquinone anion radical of Al^{3+}-HB ($[Al^{3+}\text{-HB}]^{\cdot -}$). No attempt was made to resolve the hyperfine splitting due to the complication of this structure.

Under our experimental conditions, the semquinone anion radical might be formed via self-electron transfer between the triplet and the ground state of Al^{3+}-HB in the absence of electron donors (Eqs. (1) and (2)). In the presence of electron donors, the electron transfer from electron donor (D) to triplet Al^{3+}-HB also generates the semiquinone anion radical of Al^{3+}-HB (Eq. (3)).

$$Al^{3+}\text{-HB} \xrightarrow{h\nu} {}^1[Al^{3+}\text{-HB}] \xrightarrow{isc} {}^3[Al^{3+}\text{-HB}] \quad (1)$$

$${}^3[Al^{3+}\text{-HB}] + Al^{3+}\text{-HB} \rightarrow [Al^{3+}\text{-HB}]^{\cdot -} + [Al^{3+}\text{-HB}]^{\cdot +} \quad (2)$$

$${}^3[Al^{3+}\text{-HB}] + D \rightarrow [Al^{3+}\text{-HB}]^{\cdot -} + D^{\cdot +} \quad (3)$$

Spectrophotometric Measurements

When a deoxygenated DMSO solution of Al^{3+}-HB (0.2 mg ml^{-1}) and cysteine (8 mM) was irradiated, the color of solution changed from red to green. Figure 4 showed the absorbance changes with a series of irradiation time.

It can be seen that the absorption bands of Al^{3+}-HB at 503, 568 and 614 nm decreased while new bands appeared at 627, 757 nm, accompanied by an isosbestic point at 621 nm within the spectral region examined (300–700 nm). The photoproduct was very stable in the absence of oxygen. Introduction of oxygen to the illuminated solution led to the disappearance of the absorption spectrum of the photoproduct

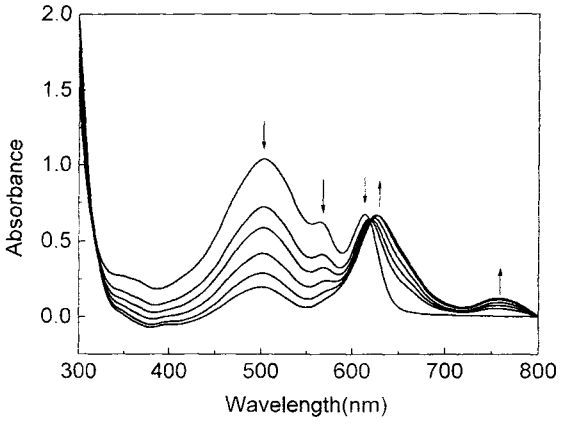

FIGURE 4 Absorption spectra in deoxygenated DMSO solution containing Al^{3+}-HB (0.2 mg ml^{-1}) and cysteine (8 mM) upon irradiation for 0, 30, 50, 60, 70 s. The arrows indicate the direction of changes.

and 85% recovery of the absorption spectrum of the Al^{3+}-HB complex.

Since strong reductive agent and argon gas are both necessary for the reaction, it is reasonable to assume that the intermediate is the reduction form of Al^{3+}-HB. EPR measurement indicated that the EPR signal of $[Al^{3+}\text{-HB}]^{\cdot -}$ could be observed.

The EPR and spectrophotometric results obtained showed that the Al^{3+}-HB complex has the ability to undergo one-electron reduction to generate a new complexed semiquinone radical anion.

Generation of Superoxide Anion Radical ($O_2^{\cdot -}$) By Al^{3+}-HB

EPR Spin Trapping

It was mentioned above that the photo-induced EPR spectrum of Al^{3+}-HB radical anion shown in Fig. 3A disappeared when oxygen was bubbled through the deoxygenated Al^{3+}-HB solution. When DMPO and oxygen were introduced into the reaction system, a new EPR signal appeared. This suggested the oxidation of $[Al^{3+}\text{-HB}]^{\cdot -}$ by dissolved oxygen and the formation of another radical that could be trapped by DMPO. When the air-saturated DMSO solution of Al^{3+}-HB ($0.5\,mg\,ml^{-1}$) and DMPO (50 mM) was irradiated, an EPR signal appeared immediately (inset in Fig. 5). This EPR spectrum was characterized by three coupling constants, which are due to the nitrogen and two hydrogen atoms at β and γ positions. The g factor and determined constants ($g = 2.0056$, $\alpha^N = 12.5\,G$, $\alpha_\beta^H = 10.2\,G$ and $\alpha_\gamma^H = 1.2\,G$) were in good agreement with the literature values of DMPO-superoxide radical adduct.[19]

Control experiments confirmed that Al^{3+}-HB, oxygen and light were all necessary for the production of EPR signal shown in inset of Fig. 6 (line 2, Fig. 5). The addition of SOD ($30\,\mu g\,ml^{-1}$) prior to illumination inhibited the EPR signal intensity (line 3, Fig. 5), whereas thermally denatured SOD has no effect on the EPR spectrum. These observations suggested the correct assignment of the EPR spectrum shown in inset of Fig. 5 to the DMPO-$O_2^{\cdot -}$ adduct.

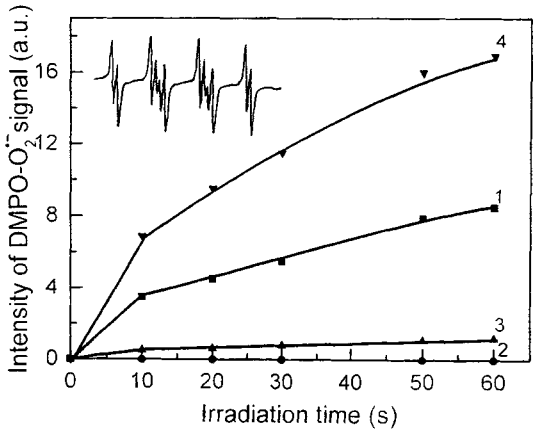

FIGURE 5 Dependence of the DMPO-superoxide radical adduct intensity on irradiation time derived from irradiation of an air-saturated DMSO solution containing Al^{3+}-HB ($0.5\,mg\,ml^{-1}$) and DMPO (50 mM) (line 1). Line 2: same as line 1 except that Al^{3+}-HB, oxygen or illumination was omitted. Line 3: same as line 1 except that SOD ($30\,\mu g\,ml^{-1}$) was added. Line 4: same as line 1 except that NADH was added. The inset: the EPR signal of DMPO – $O_2^{\cdot -}$ adduct generated under the conditions as line 1 (a.u., arbitrary unit).

The EPR signal intensity of DMPO-$O_2^{\cdot -}$ adduct dose not decrease by the addition of DABCO (10 mM), commonly used to inhibit $^1O_2$-dependent reactions,[20] indicating that $^1O_2$ is not involved in the formation of $O_2^{\cdot -}$ by Al^{3+}-HB. Moreover, catalase ($50\,\mu g\,ml^{-1}$) and hydrogen peroxide (10 mM) had a negligible effect, thus excluding a role of H_2O_2 in the formation of $O_2^{\cdot -}$ photosensitized by Al^{3+}-HB. The addition of electron donor NADH (2 mM) greatly enhanced the EPR signal intensity of DMPO-$O_2^{\cdot -}$ adduct (line 4, Fig. 5), which suggested that $[Al^{3+}\text{-HB}]^{\cdot -}$ could be the precursor for the formation of $O_2^{\cdot -}$ (Eq. (4)). Alternatively, the possibility that triplet Al^{3+}-HB might transfer one electron directly to oxygen molecule to form $O_2^{\cdot -}$ could not be excluded, although there were no data on this process (Eq. (5)).[21]

$$[Al^{3+}\text{-HB}]^{\cdot -} + O_2 \rightarrow O_2^{\cdot -} + Al^{3+}\text{-HB} \quad (4)$$

$$^3(Al^{3+}\text{-HB}) + O_2 \rightarrow O_2^{\cdot -} + [Al^{3+}\text{-HB}]^{\cdot +} \quad (5)$$

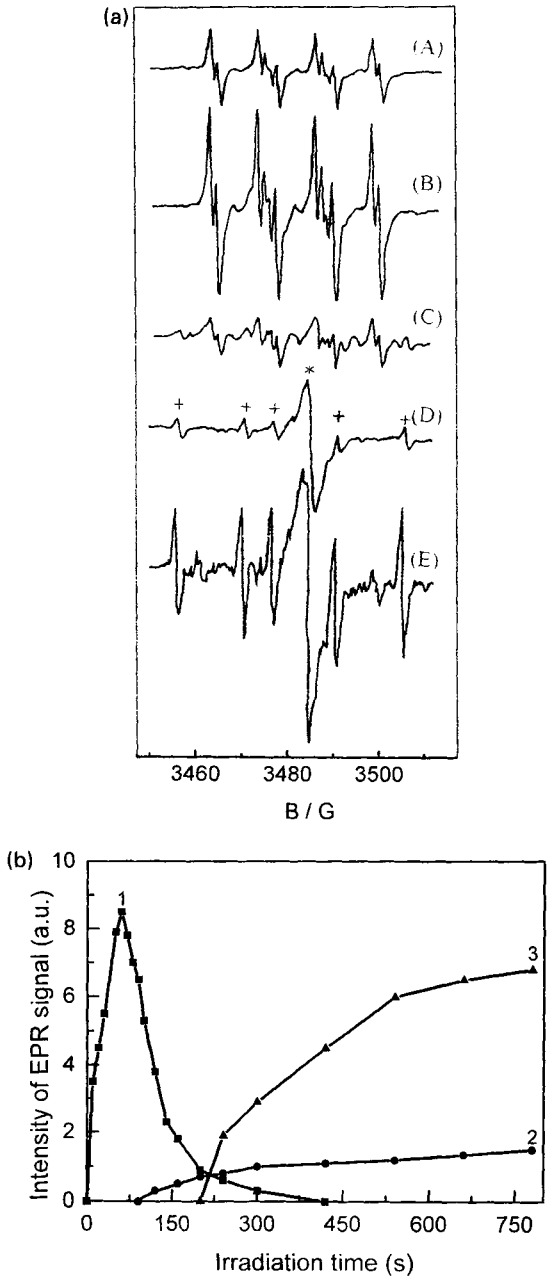

FIGURE 6 (A) The EPR spectra produced from the sealed air-saturated DMSO solution containing Al^{3+}-HB ($0.5\,mg\,ml^{-1}$) and DMPO (50 mM) with the different irradiation time. (B) The dependence of the EPR signal intensity on irradiation time in the above sealed system. Line 1: DMPO – $O_2^{\cdot-}$ radical adduct; Line 2: DMPO-carbon-centered radical adduct; Line 3: $[Al^{3+}\text{-HB}]^{\cdot-}$.

To our surprise, when the sealed air-saturated DMSO solution of Al^{3+}-HB and DMPO was irradiated, the EPR signal intensity of DMPO-$O_2^{\cdot-}$ adducts increased to the maximum within about 1 min. On further irradiation, the signal intensity of DMPO-$O_2^{\cdot-}$ adducts decreased and then disappeared accompanied by the appearance of a new spectrum (marked by asterisks in spectrum D of Fig. 6A). At the same time, the color of the solution changed from red to green. Apparently the spectrum D in Fig. 6A originates from tow species: one similar to the Al^{3+}-HB semiquinone anion radical signal in spectral shape and splitting parameter, marked by asterisks; the other six-line spectrum, partially overlapped by the semiquinone anion radical signal, marked by crosses. Furthermore, when NADH was added to the system, the intensities of the two unidentified species increased. Accordingly, one of these two new radicals can be assigned to Al^{3+}-HB semiquinone anion radical.

The dependence of the EPR signal intensities of different species on irradiation time in the sealed system was shown in Fig. 6B. The decreasing process of DMPO-$O_2^{\cdot-}$ signal may be due to the degradation of the DMPO-$O_2^{\cdot-}$ adduct.[22] With the exhaustion of oxygen in the sealed system, the above-mentioned reaction (Eq. (4)) cannot occur, i.e. $[Al^{3+}\text{-HB}]^{\cdot-}$ cannot be transformed to $O_2^{\cdot-}$. Thus the signal intensity of $[Al^{3+}\text{-HB}]^{\cdot-}$ is increased with prolonging irradiation time.

The six-line spectrum, characteristic of DMPO-carbon-centered adduct, was not observed by irradiation of an argon-saturated DMSO solution of Al^{3+}-HB ($0.5\,mg\,ml^{-1}$) and DMPO (50 mM), which indicated the DMPO-$^{\cdot}$C adduct might be generated by irradiation of aerated DMSO solution, but was detected after the sample became anaerobic. Because photolysis may lead to accumulation of superoxide and, possibly, H_2O_2, in the presence of DMSO, H_2O_2, and traces of iron, carbon-centered radicals ($^{\cdot}CH_3$) could be formed via a Fenton reaction (Eqs. (6)–(9)).[23] Moreover, the EPR splitting parameters of the six-line spectrum ($\alpha_N = 14.7\,G$, $\alpha_H = 20.9\,G$) are

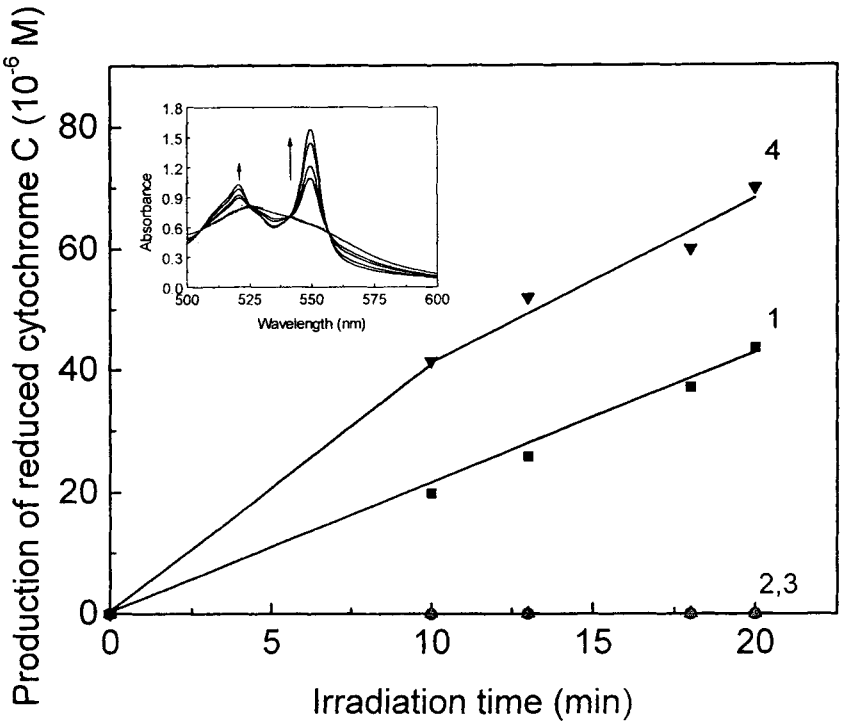

FIGURE 7 Reduction of cytochrome c mediated by Al^{3+}-HB (0.05 mg ml^{-1}) in aqueous solution (pH 7.4) with Cyt Fe^{3+} (85 μM) as a function of illumination time using 578 nm light (line 1). Line 2: same as line 1 except that Al^{3+}-HB or light was omitted. Line 3: same as line 1 except that SOD (60 μg ml^{-1}) was added. Line 4: same as line 1 except that NADH (2 nM) was added. The inset: absorbance spectra of an oxygen-saturated aqueous solution (pH 7.4) containing Al^{3+}-HB (0.05 mg ml^{-1}) and ferricytochrome c (85 μM) under different irradiation time 0, 10, 13, 18, 20 min.

in agreement with the literature values of DMPO-CH_3 adduct.[24] This further indicates the correct assignment of the six-line spectrum of the DMPO-CH_3 adduct.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (6)$$

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2 \quad (7)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad (8)$$

$$^{\cdot}OH + CH_3SCH_3 \rightarrow H_3C{-}\underset{\underset{O^{\cdot}}{|}}{\overset{\overset{OH}{|}}{S}}{-}CH_3 \rightarrow CH_3SO_2H + {}^{\cdot}CH_3 \quad (9)$$

SOD Inhibitable Cytochrome C Reduction

The inset of Fig. 7 showed the visible absorption spectrum of cytochrome c produced during irradiation of an oxygen-saturated solution containing Al^{3+}-HB (0.05 mg ml^{-1}) and cytochrome c (85 μM) with 578 nm light. The increasing absorbance at 550 nm reflects the reduction process of cytochrome c (Cyt Fe^{3+}).[25,26] Superoxide anion radical may be quantified by determining the amount of reduced cytochrome c (Cyt Fe^{2+}) produced by $O_2^{\cdot-}$ that is inhibited by SOD.[25–27] Line 1 in Fig. 7 showed that the amount of SOD-inhibitable reduction of cytochrome c (Cyt Fe^{2+}), corresponding to the $O_2^{\cdot-}$ concentration photogenerated by Al^{3+}-HB, was expressed as a function of illumination time.

Control experiments indicated that Al^{3+}-HB, oxygen and light were all essential for the reduction cytochrome c (line 2, Fig. 7). The addition of SOD (60 $\mu g\,ml^{-1}$) prior to illumination prevented the reduction of cytochrome c (line 3, Fig. 7). The addition of electron donor NADH, significantly enhanced the production of superoxide anion radical (line 4, Fig. 7), confirming that $[Al^{3+}\text{-HB}]^{\cdot-}$ could be the precursor for the formation of $O_2^{\cdot-}$. The rate of reduction of cytochrome c was unchanged after the addition of catalase (50 $\mu g\,ml^{-1}$), ruling out H_2O_2 as a significant factor.

To test the possible contribution of $^1O_2$ in the formation of $O_2^{\cdot-}$ by Al^{3+}-HB, we performed the reduction of cytochrome c in the presence of the $^1O_2$ quencher DABCO (10 mM).[20] The formation of reduced cytochrome c was not decreased by the addition of DABCO; furthermore, the formation of $O_2^{\cdot-}$ was not obviously enhanced in the deuterated water. These findings suggested that the reduction of $^1O_2$ to $O_2^{\cdot-}$ could be ignored. These results were consistent with those of DMPO spin trapping experiments.

Photogeneration of Singlet Oxygen $^1O_2$

EPR Spin Trapping

When an air-saturated DMSO solution of Al^{3+}-HB (0.5 $mg\,ml^{-1}$) and TEMP (20 mM) was irradiated at room temperature, an EPR spectrum of triplet peaks with equal intensity, characteristic of a nitroxide radical, was observed (inset of Fig. 8). The hyperfine splitting constant and g factor of the photosensitized oxidation product of TEMP by Al^{3+}-HB were identical to those of commercial TEMPO (α^N = 16.3 G, g = 2.0056). In the absence of Al^{3+}-HB, oxygen or irradiation, no EPR signal could be detected (line 2, Fig. 8). These data demonstrated that the formation of the nitroxide radical was a photodynamic process.

It has previously reported that TEMPO might be generated from the reaction of TEMP and singlet oxygen.[28] To provide further evidence to support the involvement of $^1O_2$ in Al^{3+}-HB photosensitizing process, the following experiments were carried out. In the presence of $^1O_2$ scavenger (DABCO or NaN_3), the EPR signal was suppressed (line 3, Fig. 8). The effect of deuterium solution on the yield of TEMPO was also studied. It was found that the intensity of the EPR signal increased by approximately three times when DMSO was replaced by fully deuterated DMSO as solvent (data not shown). These two powerful tools, both of which are diagnostics for $^1O_2$, suggest that TEMPO is derived from the reaction of TEMP with $^1O_2$. Since oxygen does not quench the fluorescence of Al^{3+}-HB under our experimental conditions, the generation of $^1O_2$ by Al^{3+}-HB photosensitization is considered to proceed via energy transfer from triplet Al^{3+}-HB to ground state oxygen (Eq. (10)).

$$^3(Al^{3+}\text{-HB}) + O_2 \rightarrow {}^1O_2 + Al^{3+}\text{-HB} \qquad (10)$$

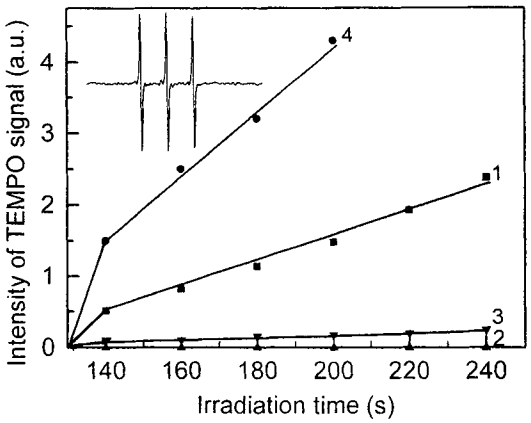

FIGURE 8 The EPR signal intensity of TEMPO formed in an air-saturated DMSO solution of Al^{3+}-HB (0.5 $mg\,ml^{-1}$) and TEMP (20 mM) as a function of illumination time using 532 nm light (line 1). Line A same as line 1 except that Al^{3+}-HB, oxygen or illumination was omitted. Line 3: same line 1 except that NaN_3 (2 mM) was added. Line 4: same as line 1 except that Al^{3+}-HB was replaced by HB (0.3 mM). The inset is a typical EPR spectrum of TEMPO formed during irradiation of solution under the same conditions as line 1.

Competition between Singlet Oxygen and the Semiquinone Anion Radical of Al^{3+}-HB

TEMPO could also be used to detect $[Al^{3+}\text{-HB}]^{\cdot -}$ via Eq. (11). This unique and dual role of TEMPO could be conveniently used to detect the competition between the $^1O_2$ and $[Al^{3+}\text{-HB}]^{\cdot -}$ as the oxygen concentration changed.[29,30]

$$[Al^{3+}\text{-HB}]^{-} + \text{TEMPO} \rightarrow \text{TEMPOH} + [Al^{3+}\text{-HB}]^{2-} \quad (11)$$

When the sealed air-saturated DMSO solution of Al^{3+}-HB (0.5 mg/ml) and TEMP (20 mM) was irradiated, the ESR signal intensity of TEMPO increased to the maximum within about 4 min and the decreased with further irradiation accompanied by appearance of a new spectrum (Fig. 9A). The new spectrum is similar to the Al^{3+}-HB semiquinone anion radical signal in spectral shape and splitting parameter. With further irradiation, the TEMPO signal intensity decreased slowly while the $[Al^{3+}\text{-HB}]^{\cdot -}$ signal intensity increased subsequently and then reached a plateau (Fig. 9B).

The increasing TEMPO signal intensity reflects the reaction process of TEMP with $^1O_2$. The excited state of Al^{3+}-HB could react with its ground state via electron transfer to generate the semiquinone anion radical and the semiquinone cation radical. As mentioned above, the cation radical of quinone is difficult to detect under our experimental conditions. Moreover, the addition of electron donor, such as NADH, significantly increased the intensity of this EPR signal (not shown). Therefore, the detected EPR spectrum might be ascribed to the semiquinone anion radical of Al^{3+}-HB. The decrease of oxygen concentration in the sealed solution suppressed the formation of $^1O_2$ via Eq. (10), but relatively promoted the production of $[Al^{3+}\text{-HB}]^{\cdot -}$ via Eq. (2). The $[Al^{3+}\text{-HB}]^{\cdot -}$ signal intensity increases with irradiation time because the rate of spin counteraction of TEMPO by $[Al^{3+}\text{-HB}]^{\cdot -}$ is much lower than that of the production of $[Al^{3+}\text{-HB}]^{\cdot -}$.

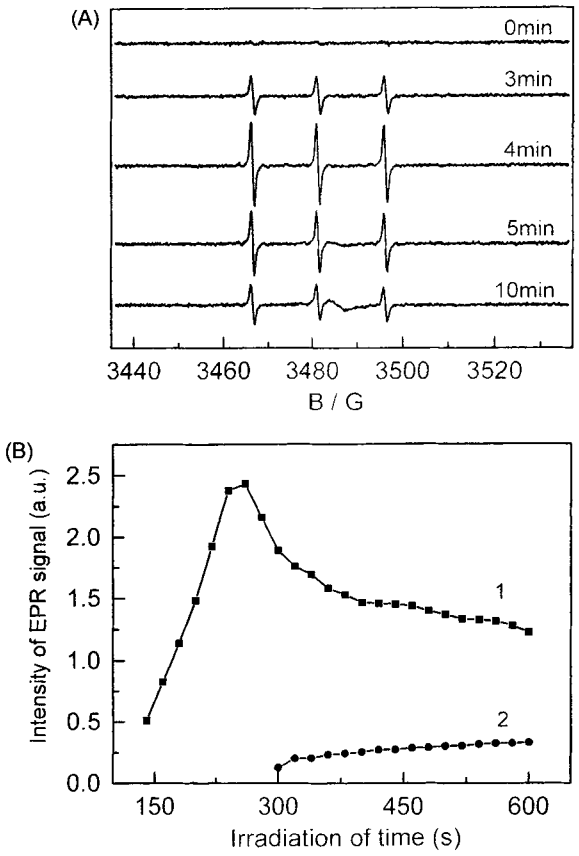

FIGURE 9 The EPR spectra produced from the sealed air-saturated DMSO solution containing Al^{3+}-HB (0.5 mg ml^{-1}) and TEMP (20 mM) with the different irradiation time. (B) The dependence of the EPR signal intensity or irradiation time in the above sealed system. Line 1: TEMPO radical; Line 2: $[Al^{3+}\text{-HB}]^{\cdot -}$.

When the production rate of $[Al^{3+}\text{-HB}]^{\cdot -}$ and its exhaustion rate became comparable, the $[Al^{3+}\text{-HB}]^{\cdot -}$ signal intensity would reach to a plateau.

Oxygen plays a key role in the competition between singlet oxygen and semiquinone anion radical in Al^{3+}-HB photosensitization process. The accumulation of $[Al^{3+}\text{-HB}]^{\cdot -}$ may replace that of singlet oxygen ($^1O_2$) as oxygen was depleted.

CONCLUSION

Al^{3+}-HB is a novel potential phototherapeutic agent and readily soluble in water. The EPR and

spectrophotometric investigations demonstrate that the semiquinone anion radical ($[Al^{3+}\text{-}HB]^{\cdot -}$), superoxide, anion radical ($O_2^{\cdot -}$) and singlet oxygen ($^1O_2$) can be produced by Al^{3+}-HB photosensitization. The semiquinone anion radical of Al^{3+}-HB is photogenerated via the self-electron transfer between the excited and ground state species in the absence of electron donor. In the presence of electron donors, the electron transfer from electron donor to triplet Al^{3+}-HB also generates the $[Al^{3+}\text{-}HB]^{\cdot -}$. $O_2^{\cdot -}$ is produced via the electron transfer from $[Al^{3+}\text{-}HB]^{\cdot -}$ to O_2. However, the possibility that triplet Al^{3+}-HB might transfer one electron directly to the oxygen molecule to form $O_2^{\cdot -}$ could not be ruled out. $^1O_2$ can be produced via the energy transfer from triplet Al^{3+}-HB to ground state molecular oxygen. The competition and transformation between active oxygen species and semiquinone anion radical in the Al^{3+}-HB photosensitization process depends on the content of oxygen. Nevertheless, the *in vitro* and *in vivo* investigations on this potential photosensitizer yet need to be done to survey its phototoxicity and pharmacology.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (No. 39870090).

References

[1] Jiang, L.J. (1990) "The structure properties, photochemical reactions and reaction mechanisms of hypocrellin (I)", *Chinese Science Bulletin* **21**, 1608–1616.

[2] Jiang, L.J. (1990) "The structure properties, photochemical reactions and reaction mechanisms of hypocrellin (II)", *Chinese Science Bulletin* **21**, 1681–1690.

[3] Hudson, J.B., Zhou, J., Chen, J., Harris, L., Yip, L. and Towers, G.H.N. (1994) "Hypocrellin, from *Hypocrella bambuase*, is phototoxic to human immunodeficiency virus", *Photochemistry and Photobiology* **60**, 253–255.

[4] Fehr, M.J., Carpenter, S.L., Wannemuehler, Y. and Petrich, J.W. (1995) "Roles of oxygen and photoinduced acidification in the light-dependent antiviral activity of hypocrellin A", *Biochemistry* **34**, 15845–15848.

[5] Hirayama, J., Ikebuchi, K., Abe, H., Kwon, K.W., Ohnishi, Y., Horiuchi, M., Shinagawa, M., Ikuta, K., Kamo, N. and Sekiguchi, S. (1997) "Photoinactivation of virus infectivity by hypocrellin A", *Photochemistry and Photobiology* **66**, 697–700.

[6] Miller, G.G., Brown, K., Ballangrud, A.M., Barajas, O., Xiao, Z., Tulip, J., Lown, J.W., Leithoff, J.M., Allalunis-Turner, M.J., Mehta, R.D. and Moore, R.B. (1997) "Preclinical assessment of hypocrellin B and hypocrellin B derivative as sensitizers for photodynamic therapy of caner: progress update", *Photochemistry and Photobiology* **65**, 714–722.

[7] Zhang, J., Cao, E.H., Li, J.F., Zhang, T.C. and Ma, W.J. (1998) "Photodynamic effects of hypocrellin A on three human malignant cell lines by inducing apoptotic cell death", *Journal of Photochemistry and Photobiology B: Biology* **43**, 106–111.

[8] Xu, Y.M., Zhao, H.X. and Zhang, Z.Y. (1998) "Raman spectroscopic study if microcosmic and photosensitive damage on the liposomes of the mixed phospholipids sensitized by hypocrellin and its derivatives", *Journal of Photochemistry and Photobiology B: Biology* **43**, 41–46.

[9] Zou, W., An, J.Y. and Jian, L.J. (1996) "Damage to pBR322 DNA photosensitized by hypocrellin A in liposomes and its derivative in solution", *Journal of Photochemistry and Photobiology B: Biology* **33**, 73–78.

[10] Diwu, Z.J. and Lown, J.W. (1992) "Photosensitization with anticancer agents 12. Perylenequinonoid pigments, a novel type of singlet oxygen sensitizer", *Journal of Photochemistry and Photobiology A: Chemistry* **64**, 273–287.

[11] Divu, Z.J. and Lown, J.W. (1992) "Photosensitization therapeutic agents: formation of tautomeric semiquinone radicals", *Journal of Photochemistry and Photobiology A: Chemistry* **69**, 191–199.

[12] Moan, J., Peng, Q., Evensen, J.F., Berg, K., Western, A. and Rimington, C. (1987) "Photosensitizing efficiencies, tumor and cellular uptake of different photosensitizing drugs relevant for photodynamic therapy of cancer", *Photochemistry and Photobiology* **46**, 713–721.

[13] Oenbrink, G., Jurgenlimke, P. and Gabel, D. (1988) "Accumulation of porphyrins in cells: influence of hydrophobicity aggregation and protein binding", *Photochemistry and Photobiology* **48**, 451–456.

[14] Ma, J.H., Zhao, J.Q. and Jiang, L.J. (2000) "Synthesis, characterization and photogeneration of active oxygen species of a new water-soluble photosensitizer", submitted for publication.

[15] Hu, Y.Z. and Jiang, L.J. (1996) "Characteristics of the reaction between semiquinone radical anion of hypocrellin A and oxygen in aprotic media", *Journal of Photochemistry and Photobiology A: Chemistry* **94**, 37–41.

[16] An, J.Y., Hu, Y.Z. and Jiang, L.J. (1996) "Reactivity of semiquinone radical anions of hydroxyl perylenequinone with oxygen", *Journal of Photochemistry and Photobiology B: Biology* **33**, 261–266.

[17] He, Y.Y., An, J.Y. and Jiang, L.J. (1998) "Photodynamic action of a water-soluble hypocrellin derivative with enhanced absorptivity in the phototherapeutic window I: ESR and UV–Vis. studies on the photogeneration of the semiquinone anion radical and hydroquinone", *Journal of Photochemistry and Photobiology A: Chemistry* **115**, 213–221.

[18] Mayer, J. and Krasluklanis, R. (1991) "Absorption spectra of the radical ions of quinines, a pulse radiolysis study",

Journal of Chemical Society Faraday Translations **87**, 2943–2947.

[19] Lang, K., Wagnerova, D.M., Stopka, P. and Damerau, W. (1992) "Reduction of dioxygen to superoxide photosensitized by anthraquinone-2-sulphonate", *Journal of Photochemistry and Photobiology A: Chemistry* **67**, 187–195.

[20] Lissi, E.A., Encinas, M.V., Lemp, E. and Rubio, M.A. (1993) "Singlet oxygen O_2 ($^1\Delta_g$) bimolecular processes. Solvent and compartmentalization effects", *Chemical Review* **93**, 699–723.

[21] Hadjur, C., Wagnieres, G., Monnier, P. and van de Bergn, H. (1997) "EPR and spectroscopic studies of free radicals ($O_2^{-\cdot}$ $^{\cdot}OH$, BPD-$MA^{\cdot-}$) and singlet oxygen $^1O_2$ generated by irradiation of benzoporphyrin derivative monoacid ring A", *Photochemistry and Photobiology* **65**, 818–827.

[22] Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1982) "Production of hydroxyl radical by decomposition of superoxide spin-trapped adducts", *Molecular Pharmacology* **21**, 262–265.

[23] Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1980) "Spin trapping of superoxide and hydroxyl radical: practical aspects", *Archives of Biochemistry and Biophysics* **200**, 1–16.

[24] Klein, S.M., Cohen, G. and Cederbaum, A.I. (1981) "Production of formaldehyde during metabolism of dimethylsulfoxide by hydroxyl radical generating systems", *Biochemistry* **20**, 6006–6012.

[25] Fridovich, I. (1985) In: Greenwald, R.A., ed, Cytochrome c CRC Handbook of methods for Oxygen Radical Research, (CRC Press, Boca Raton, FL), pp. 121–122.

[26] Fridovich, I. (1970) "Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase", *Journal of Biological Chemistry* **245**, 4053–4057.

[27] Hadjur, C. and Jardon, P. (1995) "Quantitative analysis of superoxide anion radicals photosensitized by hypericin in a model membrane using the cytochorme c reduction method", *Journal of Photochemistry and Photobiology B: Biology* **29**, 147–156.

[28] Lion, Y., Delmelle, M. and van de Vorst, A. (1976) "New method of detecting singlet oxygen production", *Nature* **263**, 442–443.

[29] Moan, J. (1980) "A new method to detect porphyrin radicals in aqueous solutions", *Acta Chemica Scandinavica B* **34**, 519–521.

[30] He, Y.Y., An, J.Y. and Jiang, L.J. (1999) "Glycoconjugated hypocrellin: Photosensitized generation of free radicals ($O_2^{-\cdot}$ $^{\cdot}OH$, and $GHB^{\cdot-}$ and singlet oxygen $^1O_2$", *Free Radical Biology and Medicine* **27**, 203–212.