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ESR and UV–Vis Studies of Semiquinone Radical Anion and Hydroquinone Generated by Irradiation of 15-Deacetyl-13-glycine Substituted Hypocrellin B

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15-Deacetyl-13-glycine substituted hypocrellin B (GDHB) is a new type of hypocrellin derivative with enhanced red absorption longer than 600 nm and water solubility. When an anaerobic DMSO or DMSO–buffer (pH 7.4) solution of GDHB was illuminated with >470 nm light, a strong electron spin resonance (ESR) signal was formed. The ESR signal was assigned to the semiquinone anion radical of GDHB ($GDHB^{\bullet -}$) based on a series of experiments. $GDHB^{\bullet -}$ was predominantly photoproducted via the self-electron transfer between the excited- and ground-state species. Decay of this species, both in the presence and absence of electron donor, was consistent with second-order kinetics. In aqueous solution, the TEMPO counter-spin experiment indicated the formation of $GDHB^{\bullet -}$ that could not be detected by ESR method directly. The formation of $GDHB^{\bullet -}$ and hydroquinone of GDHB ($GDHBH^{\bullet -}$) was also confirmed by spectrometric method. These findings suggested that GDHB was at least a favorable type I phototherapeutic agent.

Keywords: 15-Deacetyl-13-glycine substituted HB, semiquinone anion radical, hydroquinone, ESR spectra, spin counteraction, spectrophotometric measurements

INTRODUCTION

Photodynamic therapy (PDT), which involves the use of photosensitizing chemicals combined with light, is an alternative trend to traditional chemotherapy for cancer treatment. A light-activated sensitizer can transfer energy from its triplet state by two processes, directly to molecular oxygen with generation of singlet oxygen ($^1O_2$) (type II reaction) or by interaction with solvent or substrate by electron or proton transfer with generation of radicals (type I reaction).

Hypocrellin A and B (HA and HB) (Figure 1) are new types of photosensitive pigments and medicines,[1,2] which derive their names from *Hypocrella bambusae* (B. et Br) sacs, growing abundantly in the northwestern region of Yunnan Province in China. These lipid-soluble 4,9-dihydroxy-3,10-perylenequinone derivatives exhibit several advantages over the presently used haematoporphyrin derivatives (HPD), such as

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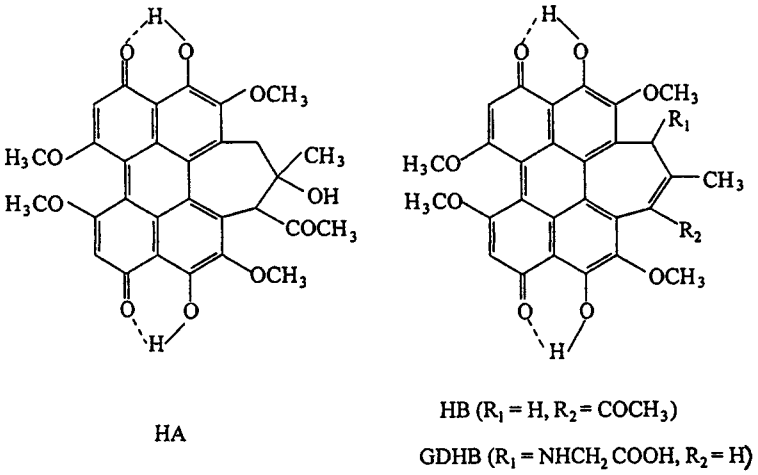


FIGURE 1 Chemical structures of HA, HB, GDHB.

ease of preparation and purification relative to HPD, small aggregation tendency which decreases the efficiency of HPD, strong red light absorptivity and significantly reduced normal tissue photosensitivity because of the fast metabolism *in vivo*.[3] So, hypocrellins have been successfully employed as antitumor agent in treatment of various skin diseases.[4–6] Recently, substantial advance has been made in various aspects of hypocrellins. For example, a number of hypocrellins and their analogs have been observed to be potent inhibitors of protein kinase C, a key enzyme in cellular differentiation and proliferation via a type I and/or type II photosensitization.[7] And HA has been used to probe artherosclerotic deposits via a laser induced fluorence-guide angioplasty system.[8] Of all most attractive finding is their activities against cancers and the acquired immunodeficiency syndrome (AIDS). Hudson *et al.* had demonstrated that hypocrellins inactivated HIV-I almost as efficiently as hypericin done.[9] A series of works have been done by Miller *et al.* to investigate the pharmacokinetics, intracellular uptake kinetics and distribution tumoricidal activity, cutaneous photosensitivity and genotoxicity, the optimal properties of cyto- and phototoxicity *in vitro*, acute and chronic toxicity *in vivo* and so on of hypocrellins and their derivatives. They have found that ethanolaminated HB and butylaminated HB elicit phototoxity *in vitro* primarily via the type II mechanism, with some type I activity under stringently hypoxic conditions. Transcutaneous phototherapy with ethanolaminated HB permanently ablates EMT6/Ed tumors growing in the flanks of Balb/c mice, with minimal cutaneous effects. It was found that some hypocrellins exhibit even more preferential lysosomal localization than their parent hypocrellins, which can be used to enhance the drug selectivity. The findings, a rapid clearance rate of HB from plasma and full incorporation into murine tumor within 2 h of intravenous administration, might explain that hypocrellins generally exhibit much less delayed skin photosensitization in both animals and humans than porphyrins such as photofrin II. And HB exhibits a qualitatively similar tissue distribution pattern to that of photofrin II but faster.[10–14]

However, natural hypocrellins do not display sufficiently strong absorptivity at wavelengths longer than 600 nm and they are insoluble in water. These disadvantages limit their application in PDT. For these reasons, we have recently synthesized a new type of long-wavelength perylenequinone with enhanced water solubility – 15-deacetyl-13-glycine substituted hypocrellin B (GDHB) (Figures 1 and 2) in high yield, and

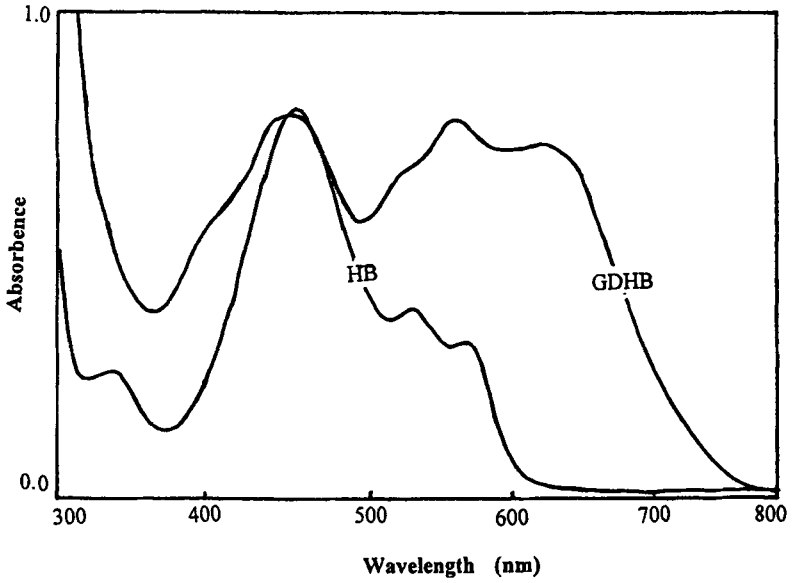


FIGURE 2 Absorption spectrum of HB and GDHB in DMSO.

investigated the photodynamic properties of the new compound. Evidence is accumulating that the generation of reactive oxygen species is intimately associated with the photodynamic effect of many sensitizers. The semiquinone anion radical of hypocrellin was considered to be a key intermediate in the cytotoxic reactions through the ability of this radical to generate toxic species such as H_2O_2 and $HO^{\bullet}$ in the presence of oxygen and its direct participation in the reaction with the substrates in the absence of oxygen.[15–18] And it has been shown that not only the active oxygen species but also the quinone radical (anion radical and cation radical) participated in the photodynamic damage caused by HA.[17,19] The hydroquinone, which can be oxidized to form semiquinone radical anion and active oxygen species via one-electron oxidation, might also play an important role in the cytotoxic reactions.

GDHB is a new type of hypocrellin derivative and possesses enhanced red absorptivity in the domain of phototherapeutic window longer than 600 nm and enhanced water solubility. So investigating in detail the formation mechanisms of semiquinone radical anion and hydroquinone is of critical importance. In this paper, we report the identification and the properties of the $GDHB^{\bullet-}$ and $GDHBH_2$. The results show that at least type I mechanism is involved in the photosensitization of 15-deacetyl-13-glycine substituted hypocrellin B.

MATERIALS AND METHODS

HA and HB were extracted from fungus sacs of *Hypocrella bambusae* and were purified by recrystallization from acetone. GDHB (Figure 1) was synthesized in connection with other work (Yu-zhu Song *et al.*, unpublished data). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) and 2,2,6,6-tetramethyl-4-piperidone-N-oxyl radical (TEMPO) were purchased from Aldrich Chemical Company. Superoxide dismutase (SOD), cysteine and reduced glutathione were purchased from Biotech Technology Corporation, Chinese Academy of Sciences. 1-Benzyl-1,4-dihydronicotinamide (BNAH) was prepared by reduction of the nicotinamide chloride with dithionite. Reduced nicotinamide adenine dinucleotide (NADH), dimethyl sulphoxide (DMSO) and other

solvents, all of analytical grades, were purchased from Beijing Chemical Plant. Water was freshly distilled before use. The solutions were purged with oxygen, air and argon according to experimental requirements. The required high-purity solvents were prepared by further purification of the commercial products, and no impurities were detected by absorption and/or fluorescence spectroscopy. The pH values of phosphate buffer solutions (PBS) were adjusted with 0.2 M KH_2PO_4 and K_2HPO_4 solutions. Aqueous solutions of GDHB were prepared as follows: GDHB was dissolved in DMSO first, then diluted with PBS of different pH values, the final concentration was 0.5% DMSO by volume.

Measurements of the ESR spectra were carried out on a Bruker ESP 300E spectrometer operating at room temperature (x band; microwave frequency, 9.5 GHz). Samples were purged with oxygen or argon for 30 min in the dark according to experimental requirements, then they (30 μl) were injected quantitatively into specially made quartz capillaries for ESR analysis. A 450 W medium-pressure sodium lamp was used as light source, and a long pass filter was employed to eliminate light of wavelength shorter than 470 nm. In all of these experiments, solutions were irradiated outside the cavity in capillaries and the ESR spectra were recorded within 3 mm after exposure.

Absorption spectra were recorded with Shimadzu PC1601 Spectrophotometer using 1 cm glass cuvettes with a long neck. Argon was bubbled through to remove oxygen and then the cuvettes were sealed with a rubber stopper.

RESULTS

ESR Measurements of Free Radicals Produced during Photosensitization of GDHB

Illumination of GDHB (1 mM) in an anaerobic DMSO or DMSO–buffer (pH 7.4, 1 : 1 by volume) solution for 1 min generated the strong ESR signal

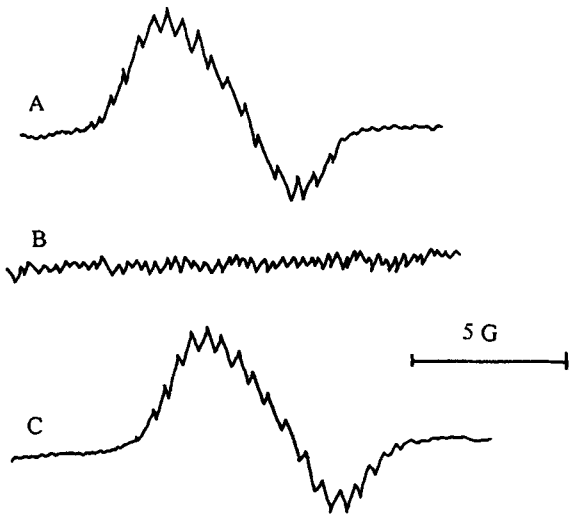


FIGURE 3 Spectrum A: ESR spectrum from a deaerated DMSO solution of GDHB (1 mM) on irradiation above 470 nm for 1 min. Spectrum B: same as spectrum A, but oxygen was bubbled through the solution after illumination. Spectrum C: same as spectrum A, but in the presence of BNAH (1 mM) and irradiation for 30 s. Instrument settings: microwave power, 8.00 mW; modulation amplitude, 0.102 G; scan range, 50 G; receiver gain, 1×10^5 (spectrum A, B); 1×10^4 (spectrum C).

as shown in Figure 3, spectrum A, with $g = 2.0038$. The hyperfine structure of this ESR spectrum was similar to but simpler than that of the semiquinone radical anion of HB.[22] At the same time the color of the illuminated mixture changed from deep blue to green. The intensity of ESR signal increased rapidly with the irradiation time and intensity, and decreased very slowly in the dark. When the sample was exposed to oxygen, the ESR signal was quenched rapidly (Figure 3, spectrum B). The ESR signal intensity of the GDHB radical also increased with increasing GDHB concentration. The strong concentration effect indicated that the GDHB radical might be formed via electron transfer between the excited- and ground-state species:

$$GDHB^* + GDHB \rightarrow GDHB^{\bullet -} + GDHB^{\bullet +} \quad (1)$$

In order to identify the ESR signal shown in Figure 3, the following experiments were carried out.

(1) Irradiation of an anaerobic DMSO solution containing GDHB (1 mM) and BNAH (5 mM), a typical electron donor, for 30 s leads to the generation of an ESR signal. The ESR spectrum obtained (Figure 3, spectrum C) was similar to that obtained in the absence of BNAH. It can also be seen that the addition of BNAH intensifies the ESR signal significantly. The color of the sample changed from deep blue to green ascribed to the GDHB radical thus generated. In the presence of electron donor (D), $GDHB^{\bullet -}$ may be generated by Equation (2):

$$GDHB^{*} + D \rightarrow GDHB^{\bullet -} + D^{\bullet +} \qquad (2)$$

(2) A series of other electron donor with different redox potentials were added to the solutions of GDHB. In all cases the ESR signals of the GDHB were intensified in comparison with that obtained without electron donor after 2 min illumination (Table I). Table I also showed that the intensity increased sharply with the decrease in the redox potentials of the donors added. This further confirmed the anionic nature of the GDHB radical.

(3) The ESR signal of the GDHB radial shown in Figure 3A was quenched completely by the purge of oxygen. Illumination of an aerobic DMSO solution containing GDHB and DMPO with > 470 nm light lead to the formation of a large amount of $DMPO-O_2^{\bullet -}$ adducts, accompanied by the disappearance of the ESR signal of GDHB anion radical. The ESR spectrum (Figure 4, spectrum A) is characterized by

TABLE I Effect of electron donor with different redox potentials on the intensity of the ESR signal of GDHB

Electron donor	Relative intensity	$E_{(D^{\bullet +}/D)}$ (V) (vs. normal hydrogen electrode)
GDHB	1	—
Glutathione	53	0.87
Cysteine	121	0.63
NADH	268	0.28

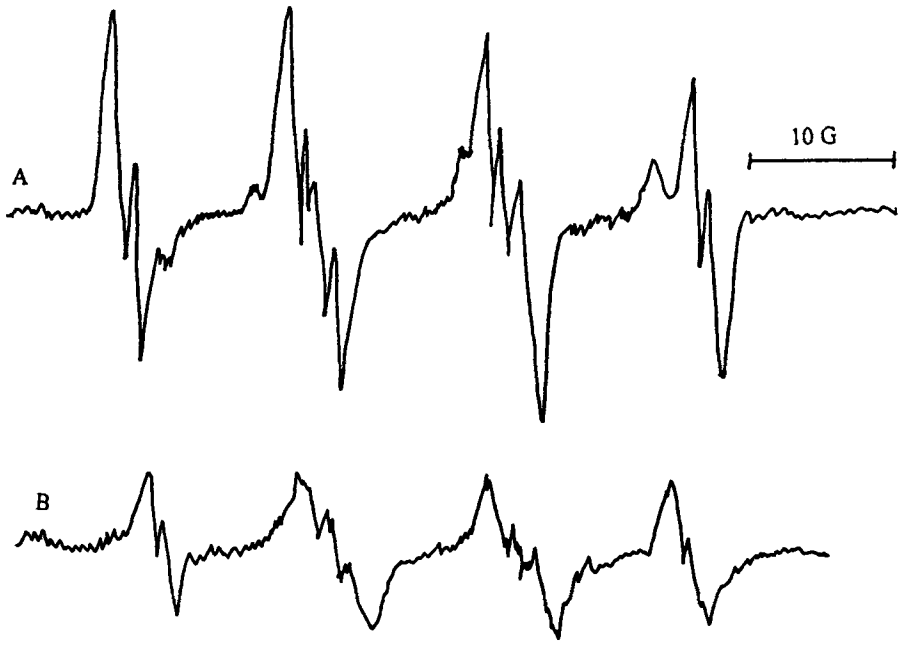


FIGURE 4 Spectrum A: ESR spectrum of DMPO–superoxide radical adducts produced on irradiation of an oxygenated DMSO solution of GDHB (1 mM) and DMPO (45 mM). Spectrum B: same as spectrum A, but in the presence of SOD (25 $\mu g\,ml^{-1}$). Instrument settings: microwave power, 5.05 mW; modulation amplitude, 1.835 G; scan range, 100 G; receiver gain, 2×10^5.

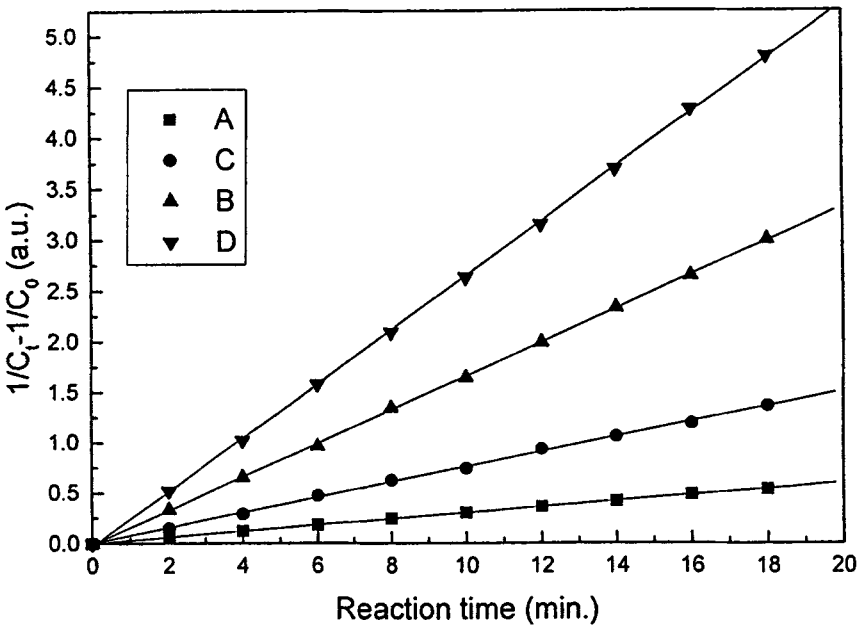


FIGURE 5 Dependence of the reciprocal GDHB semiquinone radical anion concentration on the time of dark reaction. Line A: in DMSO; line B: in DMSO–Buffer (1 : 1 by volume, pH 7.4); line C: same as line A, but in the presence of NADH (5 mM); line D: same as line B, but in the presence of NADH (5 mM).

hyperfine coupling constant of $a^N = 12.9$ G, $a^H_\beta = 10.5$ G, $a^H_\gamma = 1.5$ G are in good agreement with the literature. [15] The addition of SOD (25 μg ml^{-1}) prior to illumination reduces the formation of the DMPO–$O_2^{\bullet-}$ adduct (Figure 4, spectrum B), which further confirmed the formation of superoxide anion radical detected by DMPO spin trapping. We suggested that the generation of $O_2^{\bullet-}$ from GDHB on photoexcitation process resembles that from the parent hypocrellins, [16] Equation (3):

$$GDHB^{\bullet-} + O_2 \rightarrow GDHB + O_2^{\bullet-} \qquad (3)$$

This suggested that the ESR signal shown in Figure 3A should be ascribed to $GDHB^{\bullet-}$ rather than other species such as GDHB cation radical.

(4) The kinetics of the decay of the radicals was measured by recording the decrease of the amplitude of the ESR signal from its steady state level after illumination was stopped. Figure 5 shows such a record in DMSO (line A) and DMSO–buffer (pH 7.4) (line B). Both of the lines are consistent with second-order kinetics indicating a radical–radical annihilation reaction taken place [Equation (4)]:

$$GDHB^{\bullet-} + GDHB^{\bullet-} \xrightarrow{H^+} GDHBH^- + GDHB \qquad (4)$$

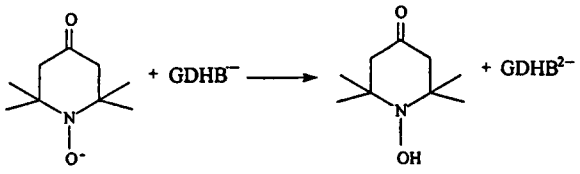
In the presence of water, the decay rate of $GDHB^{\bullet-}$ generated via Equation (2) is much faster than that in DMSO. In the presence of electron donor, $GDHB^{\bullet-}$ might also decay via Equation (5) corresponding to pseudo-first kinetic besides Equation (4):

$$GDHB^{\bullet-} + NADH \rightarrow GDHBH^- + NAD^{\bullet} \qquad (5)$$

The decay of $GDHB^{\bullet-}$ in the presence of electron donor was also consistent with second-order of kinetics (Figure 5C and D) suggested that the decay via Equation (4) was predominant under our experimental conditions.

Spin Elimination of TEMPO by $GDHB^{\bullet-}$ in Anaerobic Aqueous Solution

GDHB anion radical in anaerobic aqueous solution could not be detected by ESR method directly in the absence or presence of electron donor. But the color of the solution changed from blue (the color of GDHB) to green then immediately to pink-orange (the color of $GDHBH^-$) during the irradiation. When exposed to oxygen, the pink-orange changed back to blue indicating intermediates that can be oxidized by oxygen must be generated. In order to determine whether $GDHB^{\bullet-}$ generated during this process, the following experiment was adapted since $GDHB^{\bullet-}$ can eliminate the spin of TEMPO in aqueous solution via Equation (6):



(6)

When the anaerobic aqueous solutions of GDHB (1 mM) and TEMPO (50 μM) were irradiated, the ESR signal intensity of TEMPO radical decreased exponentially with the irradiation time. No significant degradation was observed in solutions exposed to air, no degradation was observed when aerated and anaerobic solutions of TEMPO were exposed to GDHB in the darkness or to light without GDHB. Thus, the spin elimination was not mediated by active oxygen species because it is suppressed in aerated solution.

The result indicated that the spin elimination of TEMPO was caused by the reaction of TEMPO with $GDHB^{\bullet-}$ as shown in Equation (6). This result was supported by the fact that an electron donor, such as BNAH, NADH, strongly enhanced the GDHB sensitized photodegradation of TEMPO in anaerobic solution (Figure 6). And the decrease in GDHB concentration slowed down the spin elimination of TEMPO.

The further identification of the intermediates in terms of color was also made by spectrophotometric method below.

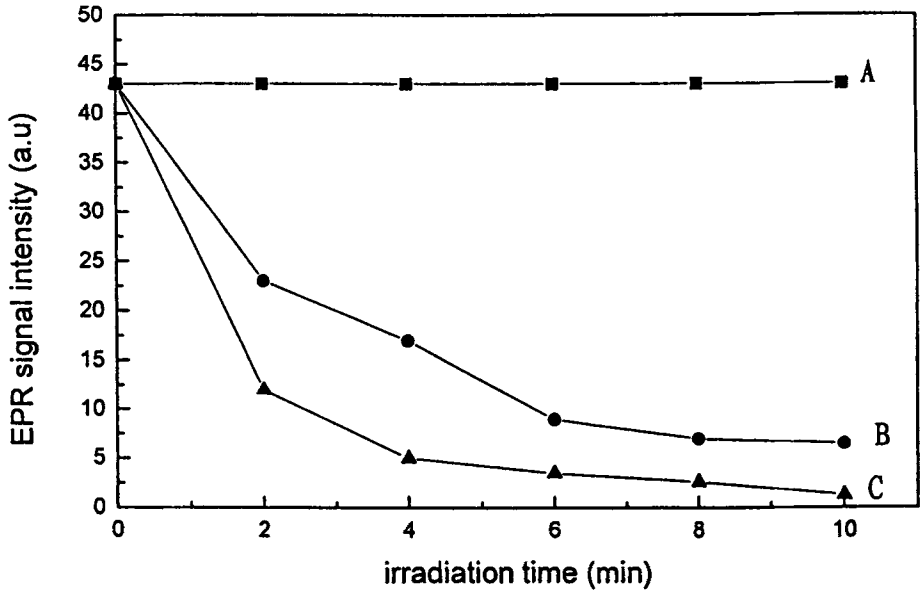


FIGURE 6 Spin elimination of TEMPO (50 μM) by GDHB (1 mM) in an aqueous solution at pH 8.0 (5% DMSO) as a function of illumination time using > 470 nm light. Line A: under aerobic conditions; line B: under anaerobic conditions; line C: same as line B, but NADH (5 mM) was added. Instrument settings: modulation amplitude, 1.032 G, receiver gain, 1×10^4.

Spectrophotometric Measurements of Deoxygenated Solutions of GDHB and Electron Donor

Deaerated aqueous solutions containing GDHB (50 μM) were irradiated with >470 nm light, absorbed only by the sensitizer, in the presence of NADH (1 mM). Examples of the photoinduced optical changes for samples incubated at pH 5.8 and 8.0 were shown in Figure 7A and B respectively. It could be seen that illumination induced decay of the maxima at 620 nm, characteristic of GDHB, and is concomitant with the formation of new absorption maxima at shorter wavelength.

At pH 5.8, the reaction goes to completion, with total elimination of the absorption from GDHB, and formation of new absorption bands at 504 nm. During the exposure the color of the sample changes from blue to pink-orange. There is only one isobestic point at 544 nm indicating that only two light-absorbing species, i.e. the starting quinone GDHB and, most likely, hydroquinone, $GDHBH^-$, exist (in terms of HB, the maximum of HBH_2 was at about 502–520 nm[20–22]). The latter species could be formed in two one-electron transfer reaction [Equation (8)] or during recombination of the radicals [Equation (4)], these two processes may be catalyzed by protonation of the radical anion $GDHB^{\bullet-}$ at low pH. When the sample exposed to oxygen, pink-orange changed back to deep blue, indicating that the intermediate was oxidized by oxygen to return to GDHB:

$$GDHB^{\bullet-} \xrightarrow{H^+} GDHBH^{\bullet} \quad (7)$$

$$GDHBH^{\bullet} \xrightarrow{D,H^+} GDHBH_2 \quad (8)$$

At pH 8.0, a similar result was obtained. But the decay of the absorption maxima at 620 nm was much slower than at pH 5.8, and the newly formed absorption at 508 nm with the isobestic point at 548 nm. Both of them had a red shift. During the exposure, the color of sample changed from deep blue to green then immediately to pink-orange, but the green transient could not be recorded by spectrophotometry due to its rapid decay.

DISCUSSION

Illumination of GDHB in DMSO or DMSO–buffer (pH 7.4, 1 : 1 by volume) generated a strong signal. This ESR signal was intensified in the presence of electron donors and was quenched by oxygen. Other identified experiments also indicated that the signal should be ascribed to the semiquinone anion radical of GDHB instead of other species.

In aqueous solution, the generation of $GDHB^{\bullet-}$ could not be detected by ESR and UV–Vis measurements. So the spin elimination of TEMPO was adapted to confirm the photogeneration of $GDHB^{\bullet-}$. The results show that the $GDHB^{\bullet-}$ was also formed in the aqueous solution in terms of type I mechanism.

The spectrophotometric measurements indicate that on illumination GDHB is directly reduced to its two-electron reduction product, i.e. hydroquinone ($GDHBH^-$) in aqueous solution. The absorption maximum of $GDHBH^-$, which is located at 504 nm at pH 5.8, shifts little bathochromically with increase in pH value of the solution, such as 508 nm at pH 8.0.

No transient species were detected in aqueous solution using spectrophotometric method, may be explained by a rapid transfer of a second electron to $GDHB^{\bullet-}$ [Equation (8)], and/or fast disproportionation of radicals, according to Equation (4). And the lifetime of the radical at pH 5.8 is shorter than that at pH 8.0, which is due to the protonation of the anion radical Equation (7).

In summary, the identification of the semiquinone radical anion and hydroquinone of GDHB by ESR and absorption spectroscopies show that GDHB was at least favorable type I photother-apeutic agent with enhanced red absorptivity and water solubility.

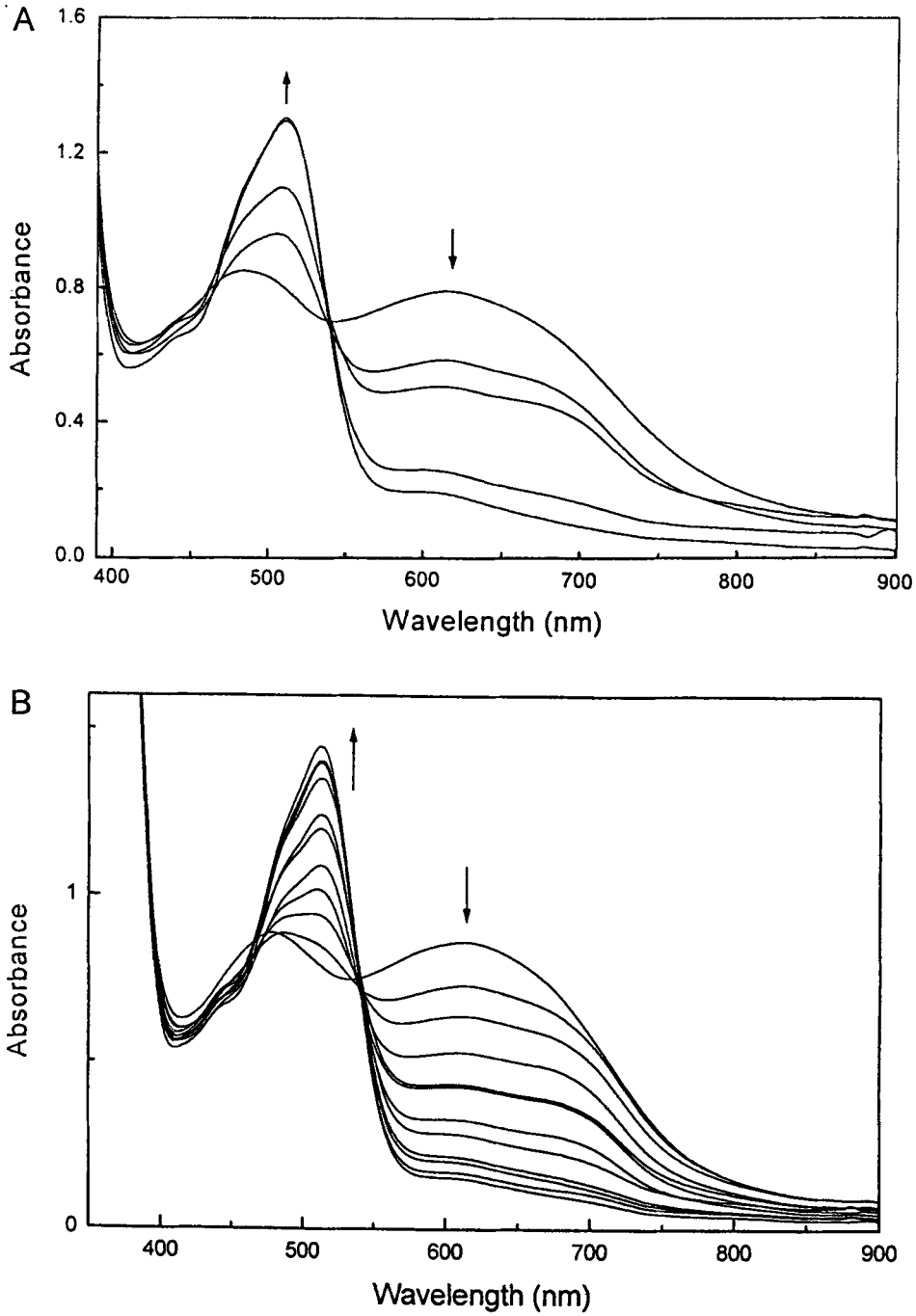


FIGURE 7 Absorption spectra from deoxygenated aqueous solution (5% DMSO) contain GDHB (50 μM) and NADH (1 mM) upon irradiation for (A) 0, 5, 10, 25, 40, 60 s, at pH 5.8. (B) 0, 5, 15, 30, 60, 90, 120, 180, 300, 480, 720, 900 s, at pH 8.0.

Acknowle dgeme nt

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