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Protein damage and reactive oxygen species generation induced by the synergistic effects of ultrasound and methylene blue

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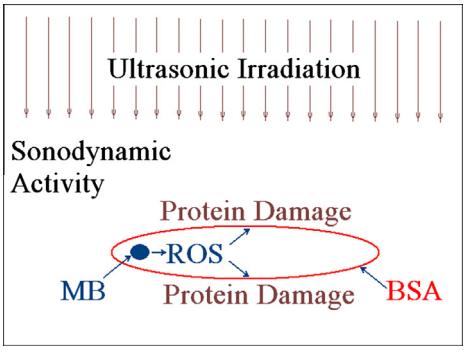
HIGHLIGHTS

- The sonodynamic damage to protein in the presence of MB were studied.
- The mechanisms of the synergistic effects of ultrasound and MB were studied.
- The protein damage induced by the synergistic effects were more serious.
- The damage of protein could be mainly due to the generation of ROS.
- Both $^1O_2$ and ·OH were the important mediators to protein damage.

GRAPHICAL ABSTRACT



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ABSTRACT

The sonodynamic damage to protein in the presence of methylene blue (MB) and the various influencing factors including ultrasonic irradiation time and MB concentration on the damage of protein were studied by fluorescence and absorption spectra. In addition, the mechanisms of the synergistic effects of ultrasound and MB were studied by oxidation–extraction photometry with several reactive oxygen species (ROS) scavengers. The results indicated that the damage of protein induced by the synergistic effects of ultrasound and MB were more serious than those that ultrasound or MB alone was applied. The damage of protein could be mainly due to the generation of ROS. The damage degree of protein increased with the increase of ultrasonic irradiation time and MB concentration because of the increased quantities of ROS generation. Both $^1O_2$ and ·OH were the important mediators of the ultrasound-inducing protein damage in the presence of MB.

Introduction

Sonodynamic therapy (SDT) is a new approach for cancer treatment on the basis of photodynamic therapy (PDT). It was firstly proposed by Japanese scholars Umemura et al. in 1989, based on the synergistic effects on tumor cells damage by the combination of the hematoporphyrin and ultrasound [1]. Ultrasound can penetrate deeply into tissues while maintaining its ability to focus energy into small volumes and locally activate the cytotoxicity of the sonosensitizer that preferentially accumulates in tumor sites [2,3]. Compared with electromagnetic modalities such as laser beams or microwaves, it is a unique advantage in the application to non-invasive treatment of non-superficial tumors [4,5], which suggests that SDT has potential value in the application for targeted therapy of tumor.

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In the latest years, SDT has been widely investigated focusing on the antitumor effects in vivo and/or in vitro and the mechanisms for the synergism between ultrasound and drugs by using different ultrasound parameters and different sonosensitizers [6]. Most of them regarded the tumor cells as assault target, and achieved the goal of treating tumors through inducing tumor cells apoptosis [7–11]. However, the intracellular targets of SDT have seldom been studied until now. The damage to intracellular substances, especially proteins that are highly abundant in cells, might be a more effective method to kill the tumor cells [12–14]. It had been reported that the changes of cytoskeletal F-actin had some correlations with Ehrlich ascites carcinoma cells apoptosis, which suggested that protein was an important subcellular target for SDT [15]. If the proteins in the tumor cells were damaged by sonosensitizers under ultrasonic irradiation, the whole cells would undergo apoptosis abnormally.

It has been reported that many compounds have sonodynamic activity. Because of their widely different structure, it is difficult to expect a universal mechanism for the synergistic effects of ultrasound and drugs [6]. Reviewing the probably mechanisms of SDT which have been studied, most experimental evidence indicated that the cell damage induced by the synergistic effects of ultrasound and drugs may contribute to the generation of reactive oxygen species (ROS) [16–21]. ROS is a class of ubiquitous molecules including both radicals and non-radicals such as superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen ($^1O_2$). These substances are constantly formed in the human body and have been implicated in a number of diseases due to their damage to cell structures, including lipids and membranes, proteins and nucleic acids, leading to cell death [22]. However, the ROS with strong oxidation activity can also exert beneficial physiologic effects for many diseases, especially, for various tumors [23].

Methylene blue (MB, Fig. 1) is a heterocyclic aromatic compound that has antifungal, antibacterial [24] and antimalarial activity [25]. It has been widely used to stain living organisms, treat methemoglobinemia [26], prevent ifosfamide-induced encephalopathy [27], and lately it has been investigated and used as photosensitizers of PDT against several types of tumors [28]. Moreover, it has been confirmed that MB possesses sonodynamic activities [29,30]. These results demonstrate that MB has potential to be used as a sonosensitizer in SDT. In this work, bovine serum albumin (BSA) was selected as a model of protein, the sonodynamic damage to protein in the presence of MB was studied by fluorescence and absorption spectra. The mechanisms of the synergistic effects of ultrasound and MB were studied by oxidation–extraction photometry with several ROS scavengers. It is wished that this report might offer some meaningful and valuable references to promoting the application of SDT at molecule level.

Experimental section

Materials

BSA (Fraction V) was obtained from Amresco (USA) and used without further purification. The BSA stock solution, 2.50×10^{-5} mol/L, was prepared in 0.05 mol/L Tris–HCl buffer solution (pH 7.40) containing 0.05 mol/L NaCl. MB, Diphenylcarbazide (DPCI), D-Mannitol (D-Man), L-Histidine (L-His) and Ascorbic acid (V_c) were all purchased from Sinopharm Chemical Reagent Co., Ltd (China). The MB stock solution (2.00×10^{-4} mol/L), the DPCI stock solution (2.50×10^{-2} mol/L) and the different ROS scavengers stock solution (5.00×10^{-2} mol/L) were all prepared in the same buffer solution. All the other materials were of analytical reagent grade and used without further purification. Doubly distilled water was used to prepare solutions.

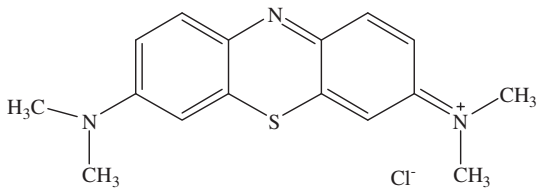

Fig. 1. Molecular structure of MB.

Apparatus

The fluorescence spectra were carried out on an F-7000 fluorescence spectrophotometer (Hitachi High-Technologies Co., Japan). Fluorescence spectra were obtained at an excitation wavelength of 280 nm, with the slit widths of both the excitation and emission set at 5.0 nm and the scanning speed of 1200 nm min^{-1}. The absorption spectra were recorded on a UV-2550 spectrophotometer (Shimadzu Co., Japan) with 1.0 cm quartz cells. The Controllable Serial-Ultrasonics apparatus (KQ5200DB, Kunshan Ultrasonic Instruments Co., Ltd. China) shown in Fig. 2 was used as irradiation source, operating at ultrasonic frequency of 40 kHz and output power of 200 W through manual adjusting. All pH measurements were made with a pHS-25 digital pH-meter (Shanghai Reaches Instrument Co., Ltd., China).

Procedures

Effect of ultrasonic irradiation time on the damage of BSA

In two conical flasks, the final concentrations of BSA were both 1.00×10^{-5} mol/L, and the final concentrations of MB were 0.00 mol/L and 1.00×10^{-5} mol/L, respectively. They were all placed in the ultrasonic irradiation apparatus and the ultrasonic irradiation time was changed from 1.0 h to 6.0 h at 1.0 h intervals. At every time interval, the solutions (10 mL) were taken out and detected by fluorescence spectrophotometer.

Effect of MB concentration on the damage of BSA

In six conical flasks, the final concentration of BSA were all 1.00×10^{-5} mol/L, and the final concentrations of MB were changed from 0.00 mol/L to 2.50×10^{-5} mol/L at 0.50×10^{-5} mol/L intervals. They were all placed in the ultrasonic irradiation apparatus for 3.0 h. Then, the solutions were taken out and detected the fluorescence spectra.

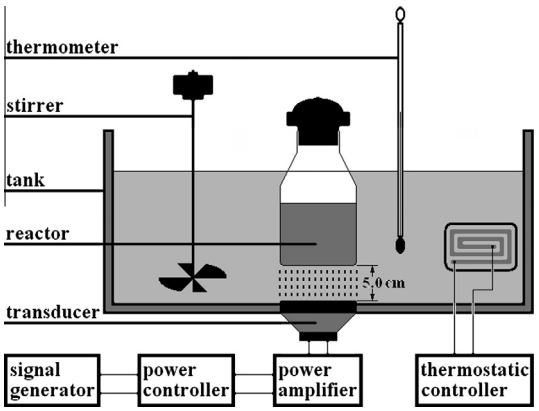

Fig. 2. The apparatus of ultrasonic irradiation.

Effect of ultrasonic irradiation time on the generation of ROS

In two conical flasks, the final concentrations of DPCI were both 5.00×10^{-3} mol/L, and the final concentrations of MB were 0.00 mol/L and 1.00×10^{-5} mol/L, respectively. They were all placed in the ultrasonic irradiation apparatus and the ultrasonic irradiation time was changed from 1.0 h to 6.0 h at 1.0 h intervals. At every time interval, the solutions (10 mL) were taken out and extracted repeatedly with Benzene–CCl_4 (1:1) mixed solution. The extraction solutions were diluted to 10 mL with the extractant and detected at 563 nm by absorption spectrophotometer.

Effect of MB concentration on the generation of ROS

In six conical flasks, the final concentrations of DPCI were all 5.00×10^{-3} mol/L, and the final concentrations of MB were changed from 0.00 mol/L to 2.50×10^{-5} mol/L at 0.50×10^{-5} mol/L intervals. They were all placed in the ultrasonic irradiation apparatus for 3.0 h. Then the solutions (10 mL) were taken out and extracted repeatedly with Benzene–CCl_4 (1:1) mixed solution. The extraction solutions were diluted to 10 mL with the extractant and detected at 563 nm by absorption spectrophotometer.

Effect of scavengers on the generation of ROS

In four conical flasks, the final concentrations of DPCI and MB were 5.00×10^{-3} mol/L and 1.00×10^{-5} mol/L, respectively. Different kinds of ROS scavengers were added into the solutions above. All of the scavengers' concentrations were 5.00×10^{-2} mol/L. They were all placed in the ultrasonic irradiation apparatus for 6.0 h. Then, the solutions (10 mL) were taken out and extracted repeatedly with Benzene–CCl_4 (1:1) mixed solution. The extraction solutions were diluted to 10 mL with the extractant and detected at 563 nm by absorption spectrophotometer.

Results and discussion

Absorption and fluorescence spectra of BSA and BSA–MB mixed solutions with and without ultrasonic irradiation

The absorption and fluorescence spectra of BSA and BSA-MB mixed solutions with and without ultrasonic irradiation are shown in Fig. 3. It can be seen from Fig. 3A that the BSA solution has a strong absorption peak at 278 nm, which is mainly caused by the transition of $\pi \rightarrow \pi^*$ of aromatic amino acid residues in BSA [31]. When MB was added into the BSA solution, the maximum absorbance intensity increases obviously, which is mainly caused by the formation of BSA–MB complex and the more exposure of the aromatic amino acid residues inside the hydrophobic cavities of BSA [32]. In Fig. 3B, it is obvious that BSA has a strong fluorescence emission peaked at 341 nm after being excited with the wavelength of 280 nm due to the existence of tryptophan (Trp) and tyrosine (Tyr) residues in BSA [33].When MB was added into the BSA solution, the fluorescence of BSA is quenched by MB due to the interaction between MB and BSA and a non-fluorescent complex is formed [32].

Under ultrasonic irradiation, the BSA solution shows hyperchromic effect and the fluorescence intensities decrease obviously compared with corresponding those without ultrasonic irradiation. These results can be explained that water can generate some ROS due to the cavitations effect of ultrasonic irradiation [34], which can lead to an unfolding of BSA due to the breaking of disulfide bonds and induce the oxidation of Trp and Tyr residues in BSA molecules [35,36]. In addition, the BSA–MB mixed solution exhibits more obvious hyperchromic effect than pure BSA solution and the fluorescence intensity of BSA–MB mixed solution decreases more strikingly. These results indicate that the synergistic effects of ultrasound and MB induce more serious damage to BSA molecules. Moreover, the absorbance of BSA–MB mixed solution at 665 nm decreases remarkably compared with corresponding that without ultrasonic irradiation. It can be concluded that MB is decomposed under ultrasonic irradiation. Therefore, after ultrasound treatment, the retention of MB in the human body would be very low. If MB could be applied to the SDT clinical treatment, the patients will no longer need a long time away from light in order to reduce side effects to the human body.

Effect of ultrasonic irradiation time on the damage of BSA and the generation of ROS

The effect of ultrasonic irradiation time on the damage of BSA was investigated by the changes of fluorescence intensities. As shown in Fig. 4, the fluorescence intensities decrease with the increase of ultrasonic irradiation time, and the fluorescence intensities of BSA–MB mixed solution are obviously lower than those of BSA solution at any ultrasonic irradiation time. These results indicate that the degree of BSA molecules damage enhances with the increasing ultrasonic irradiation time. Moreover, the relative fluorescence quenching ratios (R_{FQ}) were calculated using the equation

$$R_{FQ}(\%) = 1 - \frac{F_{BMU}}{F_{BU}} \times 100 \quad (1)$$

where F_{BU} represents the fluorescence intensity of BSA solution at different ultrasonic irradiation time, and F_{BMU} represents the fluorescence intensity of BSA–MB mixed solution at different ultrasonic irradiation time. It can be seen that the R_{FQ} increases obviously with the increase of ultrasonic irradiation time. The result indicates that the synergistic effects of ultrasound and MB induce more serious damage to BSA molecules.

In addition, the oxidation–extraction photometry method was used to determine the generated ROS in solutions under ultrasonic irradiation. The oxidation–extraction photometry method is an effective method to determine ROS [37]. In this method, DPCI can

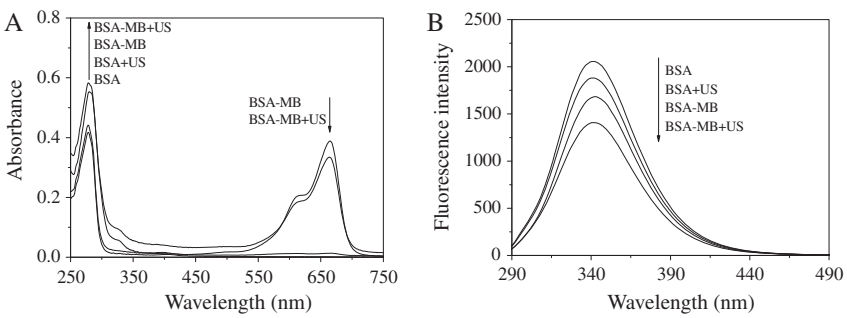

Fig. 3. Absorption spectra (A) and fluorescence spectra (B) of BSA and BSA–MB mixed solutions with and without ultrasonic irradiation, [BSA] = 1.00×10^{-5} mol/L, [MB] = 1.00×10^{-5} mol/L, t_{US} = 3.0 h, US: ultrasound.

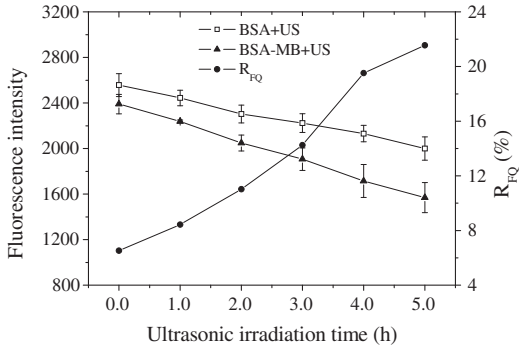

Fig. 4. Changes of fluorescence intensities of BSA and BSA–MB mixed solutions with different ultrasonic irradiation time, [BSA] = 1.00×10^{-5} mol/L, [MB] = 1.00×10^{-5} mol/L.

be oxidized by ROS into diphenylcarbonzone (DPCO), which can be extracted by organic solvents and shows the maximum absorption at 563 nm. The absorbance of DPCO at 563 nm are correlated to the quantities of ROS generation. The absorption spectra of generated DPCO in the DPCI–MB mixed solutions at different ultrasonic irradiation time are shown in Fig. 5A and the changes of absorbance of DPCO at 563 nm in the DPCI and DPCI–MB mixed solutions at different ultrasonic irradiation time are shown in Fig. 5B and the absorbance of DPCO showed in Fig. 5B are the absorbance difference values of solutions under ultrasonic irradiation and in the dark. It can be seen from Fig. 5B that the absorbance of DPCO at 563 nm increase with the increase of ultrasonic irradiation time in the presence and absence of MB. However, the absorbance of DPCO in DPCI–MB mixed solution are much higher than corresponding those in the DPCI solution at any ultrasonic irradiation time. And the difference values of their absorbance increase with the increasing ultrasonic irradiation time. These results indicate that the ability of ROS generation is very limited for simple ultrasonic irradiation. Moreover, MB can be activated by ultrasound and generate ROS more effectively, and the quantities of ROS increase with the increase of ultrasonic irradiation time. Therefore, the synergistic effects of ultrasound and MB can induce more serious damage to BSA molecules than the simple ultrasonic irradiation.

Effect of MB concentration on damage of BSA and the generation of ROS

The changes of fluorescence intensity of BSA–MB mixed solutions with different MB concentrations with and without ultrasonic irradiation are shown in Fig. 6. It can be seen that the fluorescence intensities of maximum fluorescence emission wavelength of BSA–MB mixed solutions decrease gradually with the increase of MB concentration. The reason is that the interaction exists between MB and BSA and a non-fluorescent complex is formed [32]. After being irradiated by ultrasound, the fluorescence intensity of BSA–MB mixed solutions decreases much faster compared with that without ultrasonic irradiation. These results indicate that the degree of BSA molecules damage enhances with the increase of MB concentration. Moreover, the relative fluorescence quenching ratios (R_{FQ}) were calculated using the equation

$$R_{FQ}(\%) = 1 - \frac{F_{BMU}}{F_{BM}} \times 100 \quad (2)$$

where F_{BM} represents the fluorescence intensity of BSA–MB mixed solution at different MB concentration, and F_{BMU} represents the fluorescence intensity of BSA–MB mixed solution at different MB concentration. It can be seen that the R_{FQ} increases obviously with the increase of MB concentration when MB concentration is less than 2.00×10^{-4} mol/L. The result indicates that the synergistic effects of ultrasound and MB induce more serious damage to BSA molecules than MB only.

In order to further discuss the results above, the generated ROS in solutions with and without ultrasonic irradiation were determined. The absorption spectra of generated DPCO in the DPCI–MB mixed solutions at different MB concentration are shown in Fig. 7A. and the changes of absorbance of DPCO at 563 nm with and without ultrasonic irradiation in the DPCI–MB mixed solutions at different MB concentration are shown in Fig. 7B. From Fig. 7B we can see that the absorbance of DPCO at 563 nm of DPCI–MB mixed solution without ultrasonic irradiation are very lower than those with ultrasonic irradiation. The result indicates that MB can be activated by ultrasound and generate ROS more effectively. In addition, the absorbance of DPCO at 563 nm of DPCI–MB mixed solutions increase significantly with the increasing MB concentration when MB concentration is less than 2.00×10^{-4} mol/L. The result indicates that the quantity of ROS generation increases with the increase of MB concentration under ultrasonic irradiation and the damage of BSA molecules becomes higher with the increase of MB concentration. When MB concentration is more than 2.00×10^{-4} mol/L, the absorbance of DPCO at 563 nm of DPCI–MB mixed solution decreases under ultrasonic irradiation. The reason might be that the high MB concentration would inhibit the light transmission from sonoluminescence and decrease the ROS generation. Therefore, the damage of BSA molecules decreases with the increase of MB concentration. These results are in accord with those of from Fig. 6.

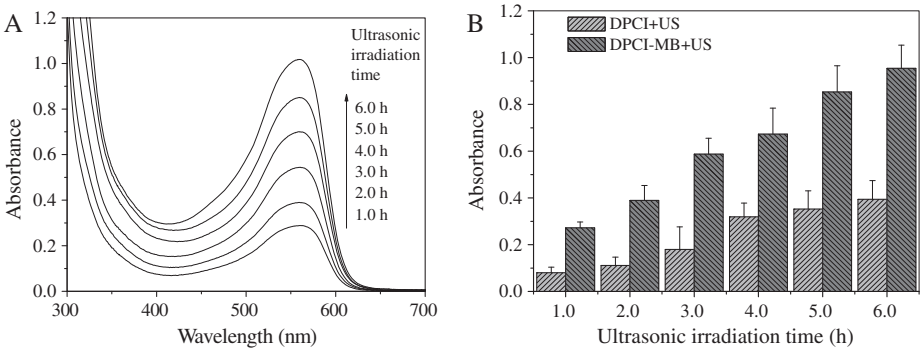

Fig. 5. Absorption spectra of generated DPCO in the DPCI–MB mixed solutions (A) and the changes of absorbance of DPCO at 563 nm in the DPCI and DPCI–MB mixed solutions (B) at different ultrasonic irradiation time, [DPCI] = 5.00×10^{-3} mol/L, [MB] = 1.00×10^{-5} mol/L.

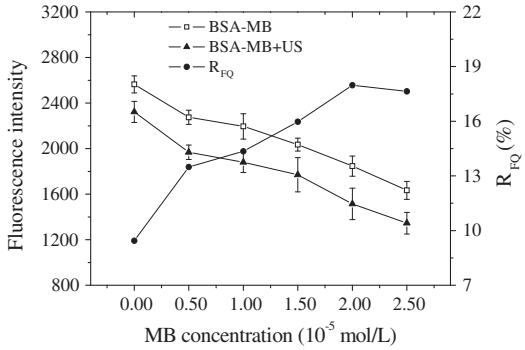

Fig. 6. Changes of fluorescence intensities of BSA–MB mixed solutions with the increase of MB concentration with and without ultrasonic irradiation, [BSA] = 1.00×10^{-5} mol/L, t_{US} = 3.0 h.

Effect of different ROS scavengers on the generation of ROS

It can be found that the synergistic effects of ultrasound and MB cause an obvious increase of ROS generation in this study, which indicates that the damage of protein is related to the generation of ROS. In order to confirm the kinds of ROS, we tested the scavenge effects of different ROS scavengers on the ROS generation in the system. D-Man is the scavenger of ·OH [38], L-His is the scavenger of $^1O_2$ and ·OH [39], and V_C can scavenge all kinds of ROS [40]. If the absorbance of DPCO at 563 nm decreases after adding some kind of scavenger, it will demonstrate that there is a kind of corresponding ROS in the system. The absorption spectra of generated DPCO in the presence of different ROS scavengers in the DPCI–MB mixed solutions under ultrasonic irradiation are shown in Fig. 8A and the effects of those scavengers on the absorbance of DPCO at 563 nm in the DPCI–MB mixed solutions under ultrasonic irradiation are shown in Fig. 8B. As shown in Fig. 8B, the absorbances of DPCO at 563 nm decrease remarkably in the presence of L-His and V_C, and the absorbance of DPCO at 563 nm decreases to some extent in the presence of D-Man. These results suggest that the synergistic effects of ultrasound and MB on the damage of BSA molecules could be mainly due to the generation of ROS. Moreover, both $^1O_2$ and ·OH are the important mediators inducing the damage of BSA molecules.

Conclusions

The damage to BSA molecules under ultrasonic irradiation in the presence of MB was studied by means of absorption and fluorescence spectra. The results indicated that the synergistic effects of ultrasound and MB could induce the damage of BSA molecules more serious. The damage degree of BSA molecules increased with the increase of ultrasonic irradiation time and MB concentration because of the increased quantities of ROS generation in the system. The mechanism of synergistic effects of ultrasound and MB was investigated by means of oxidation–extraction photometry combined with several ROS scavengers. The results indicated that the damage of BSA molecules could be mainly due to the generation of ROS, in which both $^1O_2$ and ·OH were the important mediators of the ultrasound-inducing protein damage in the presence of MB. It is wished that this paper could offer some meaningful and valuable references for studying the mechanism and promoting the application of MB in SDT tumor treatment.

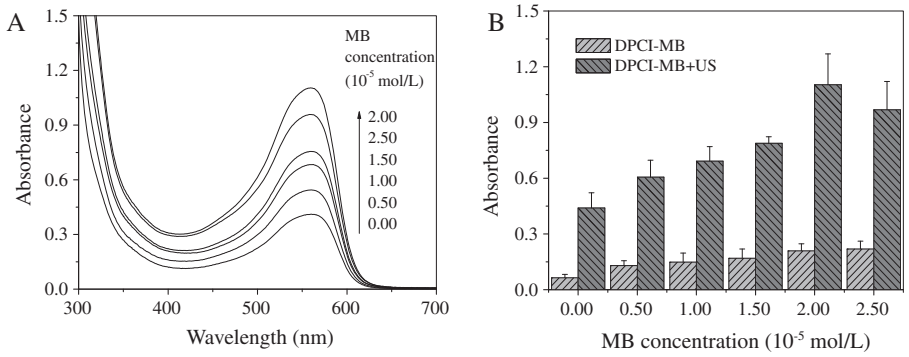

Fig. 7. Absorption spectra of generated DPCO (A) and the changes of absorbance of DPCO at 563 nm with and without ultrasonic irradiation (B) in the DPCI–MB mixed solutions at different MB concentration, [DPCI] = 5.00×10^{-3} mol/L, t_{US} = 3.0 h.

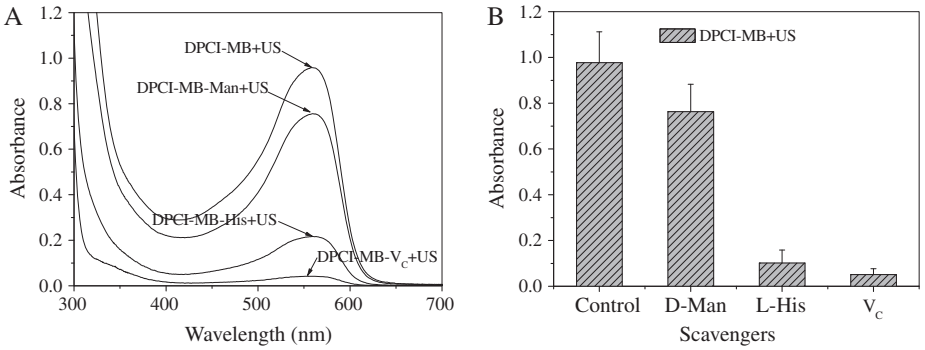

Fig. 8. Absorption spectra of generated DPCO in the presence of different ROS scavengers (A) and the effects of the different ROS scavengers on the absorbance of DPCO at 563 nm (B) in the DPCI–MB mixed solutions under ultrasonic irradiation, [DPCI] = 5.00×10^{-3} mol/L, [MB] = 1.00×10^{-5} mol/L, [D-Man] = [L-His] = [V_C] = 1.00×10^{-2} mol/L, t_{US} = 6.0 h.

Acknowledgments

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