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# Spectrometric Studies on the Sonodynamic Damage of Protein in the Presence of Levofloxacin

**Bin Liu · Jun Wang · Xin Wang · Bing-Mi Liu · Yu-Mei Kong · Dan Wang · Shu-Kun Xu**

**Abstract** Taking bovine serum albumin (BSA) as typical molecules, the sonodynamic damage of protein in the presence of Levofloxacin (LVFX) and its mechanism were studied by fluorescence and UV-vis spectra. Various influencing factors such as ultrasonic irradiation time, pH value, ionic strength and solution temperature on the damage of BSA were also discussed. The results showed that ultrasound can enhance the damage of LVFX on BSA. The damage degree of BSA was aggravated with the increase of ultrasonic irradiation time, solution temperature and ionic strength, whereas decreased with the increase of solution pH value. Furthermore, the reactive oxygen species (ROS) in reaction system were studied by oxidation and extraction photometry. Experimental results showed that the amounts of superoxide anion radical ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH$) were significantly more than that of singlet oxygen ($^1O_2$) in the presence of LVFX under ultrasonic irradiation.

**Keywords** Spectrometry · Protein · Sonodynamic damage · Levofloxacin (LVFX)

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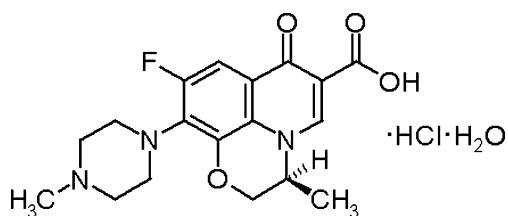Shenyang 110036, People's Republic of China

## Introduction

Sonodynamic therapy (SDT) is a promising new concept for cancer treatment by using of ultrasound. Yumita and Umemura firstly discovered the phenomenon of sonochemical activation of photosensitive materials by ultrasound [1–4]. The most widely used sonosensitizers were hematoporphyrine (Hp) and its derivatives (HpD). However, they were liable to cause severe photo-dermatitis and difficult to use in clinical practice [5]. Additionally, some non-steroidal anti-inflammatory drugs, such as piroxicam and tenoxicam, were also found to have a synergistic antitumor effect with ultrasound. But they were still far from ideal for clinical use because of high systemic doses having potential toxicity [6–8]. Therefore, searching for an efficient sonosensitizer was very significant. Recently, we focused on a new kind of fluoroquinolones, Levofloxacin (LVFX, the structure shown in Fig. 1), which had been frequently used in clinical practice with relatively mild photosensitivity reactions [9, 10]. Also, it showed few side effects, and contained clear targets, such as Topoisomerase II and Topoisomerase IV [11, 12].

Proteins are major targets for many drugs due to their important roles both in normal cells or tumor cells. The most abundant protein in the circulatory system is serum albumin. Bovine serum albumin (BSA) is highly stable and comparatively cheap, and its structure is similar to human serum albumin (HSA) in 76% [13]. So we selected BSA as a model protein and LVFX as sonosensitizer to study the BSA damage and its mechanism under ultrasonic irradiation.

In this paper, the experiment was carried out in two steps. Firstly, sonodynamic damage of BSA in the presence of LVFX was investigated. Meanwhile, the influencing factors such as ultrasonic irradiation time, solution acidity, ionic strength and solution temperature on the damage of

**Fig. 1** Molecular structure of levofloxacin (LVFX)

BSA were also studied systemically. Secondly, the mechanism of BSA damage was investigated by means of oxidation and extraction photometry [14]. Maybe, this report can offer a valuable reference to promote the application of SDT in tumor treatment at molecule level and simplify the detection of reactive oxygen species (ROS).

## Experimental section

### Materials

Levofloxacin (LVFX, ≥99.0%, Jinan Dachpharm Development Co., Ltd., China) as sonosensitizer and bovine serum albumin (BSA, ≥98.0%, Beijing Aoboxing Biotechnological Company, China) as model protein were purchased. Diphenylcarbazide (DPCI) as trapping agent and sodium azide ($NaN_3$), 2,6-Di-tert-butyl-4-methylphenol (BHT) and Vitamin C (VC) as quenchers of reactive oxygen species (ROS) were obtained from Sinopharm Chemical Reagent Co., Ltd., China. All other reagents were of analytical grade. Tris-HCl buffer solutions (0.05 M, pH 7.4, containing NaCl of 0.05 M) were used to prepare the BSA storage solutions ($2.00 \times 10^{-5}$ M, stored at 0–4 °C) and LVFX storage solutions ($5.00 \times 10^{-5}$ M). All solutions were prepared using doubly distilled water. All measurements were carried out at $25.00 \pm 0.02$ °C.

### Apparatus and instruments

UV-vis absorption spectra were recorded using UV-vis spectrophotometer (UV-2100, Beijing Purkinje General Instrument Co., Ltd., China) in the range of 200–700 nm. The fluorescence spectra were measured in a luminescence spectrometer (LS-55, PerkinElmer Inc., USA) with the excitation wavelength of 280 nm, and the excitation and emission slit width (each 12 nm), scan rate (1,200 nm/min) were constantly maintained for all the experiments. The experimentations of BSA damage under ultrasonic irradiation were carried out in a Controllable Serial-Ultrasonics apparatus (KQ5200DB, Kunshan Ultrasonic Instrument Co., Ltd. China) shown in Fig. 2. Its frequency and power were 40 kHz and 1 W/cm², respectively. The pH value of

solutions was measured with a pH meter (PHS-3C, Shanghai Leici Instrument Company, LTD, China).

### Sonodynamic damage of BSA under ultrasonic irradiation combined with LVFX

Firstly, six clean 25.00 mL volumetric flasks were marked with a–f, respectively. Four 12.50 mL BSA storage solutions ($2.00 \times 10^{-5}$ M) were taken exactly and put into volumetric flasks a–d, respectively. Then, four 5.00 mL of LVFX storage solutions ($5.00 \times 10^{-5}$ M) were added to volumetric flasks c–f, respectively. Finally, all volumetric flasks were diluted to 25.00 mL with Tris-HCl-NaCl solutions. The final concentration of BSA and LVFX were both of $1.00 \times 10^{-5}$ M. Afterwards, a, c and e were placed in a lucifuge ultrasonic irradiation apparatus. Others were only placed in the dark. After 3.0 h, the UV-vis and fluorescence spectra of each sample solution were determined to evaluate the damage of BSA molecules. In order to investigate the BSA damage process systematically, the effects of ultrasonic irradiation time, pH value, ionic strength and solution temperature were also examined.

### Identification of the reactive oxygen species (ROS)

Firstly, two aliquots of 2.00 mL DPCI and 2.00 mL LVFX were taken exactly and put into volumetric flask (a) and (b), respectively. Then, the solutions were diluted to 10.00 mL with Tris-HCl-NaCl solutions, making the final concentration of DPCI and LVFX were $5.00 \times 10^{-3}$ M and $1.00 \times 10^{-4}$ M, respectively. Afterwards, one of them was transferred into 50 mL conical flask and placed in an ultrasonic irradiation apparatus, the other was kept in the dark. After 1.0 h, the solutions were extracted repeatedly with Benzene-CCl₄ (1:1) mixed solution extractant. The extraction liquids were diluted to 10.00 mL with the extractant and detected by UV-vis spectrophotometer. In order to investigate systematically the changes of ROS level under ultrasonic irradiation,
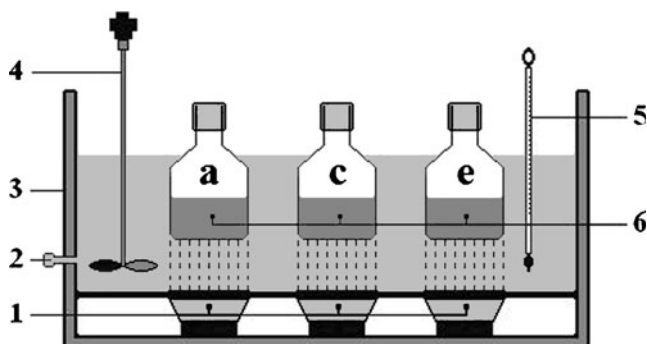


**Fig. 2** The apparatus of ultrasonic irradiation. *1*: transducer; *2*: outlet; *3*: tank; *4*: stirrer; *5*: thermometer; *6*: reaction solution. *a*: BSA; *c*: BSA + LVFX; *e*: LVFX

the effects of LVFX concentration, ultrasonic irradiation time and ultrasonic power were also studied. Furthermore, the quenching effects of NaN$_3$, BHT and VC [15–17] were investigated. All of the quencher's concentrations were 0.05 M.

## Results and discussion

### UV-vis and fluorescence spectra of BSA-LVFX solutions under ultrasonic irradiation

The damage degrees of BSA molecules with / without the ultrasonic irradiation as well as in the presence / absence of LVFX were compared by using their corresponding UV-vis and fluorescence spectra. The results were shown in Figs. 3 and 4, respectively. It can be seen from Fig. 3 that the absorption peaks of BSA solutions in the presence and absence of LVFX appear at 281 nm and 278 nm, respectively. These indicated the formation of a ground state complex due to the interaction of LVFX with BSA [18]. Furthermore, both of the BSA-LVFX solution (Fig. 3a) and BSA solution (Fig. 3c) under ultrasonic irradiation for 3.0 h showed hyperchromic effect at 281 nm and 278 nm compared with corresponding ones (Figs. 3b and d) without ultrasonic irradiation, respectively; and the BSA-LVFX solution exhibited more obvious hyperchromic effect than the BSA solution. These phenomena indicated that the synergy of ultrasonic irradiation and LVFX made the BSA molecules be damaged seriously.

The preliminary inferences can be further validated by means of the fluorescence spectra. As shown in Fig. 4, without the ultrasonic irradiation, the fluorescence intensity of BSA-LVFX solution (Fig. 4b) was quenched compared
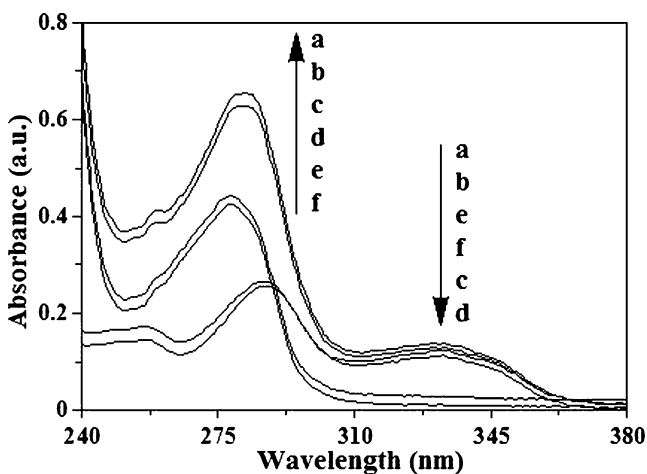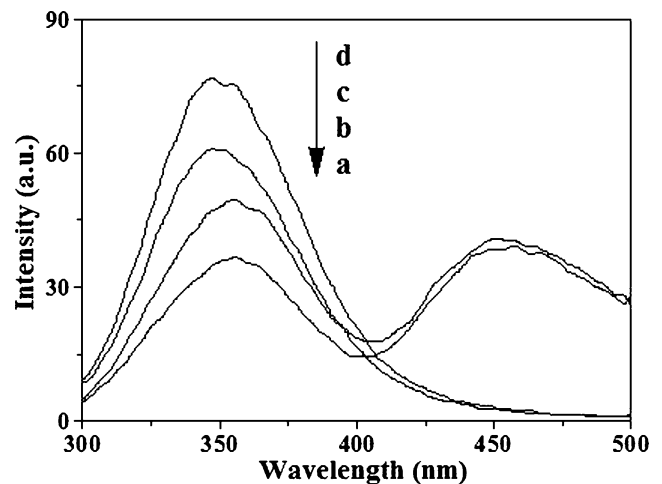


**Fig. 4** Fluorescence spectra of (*a*) BSA-LVFX with irradiation for 3.0 h, (*b*) BSA-LVFX without irradiation, (*c*) BSA with irradiation for 3.0 h and (*d*) BSA without irradiation

with the original BSA solution (Fig. 4d) because of the interaction of BSA and LVFX. Whereas with the ultrasonic irradiation, the fast decrease in fluorescence intensity of both BSA-LVFX solution (Fig. 4a) and BSA solution (Fig. 4c) was observed, and the loss of fluorescence intensity of BSA-LVFX solution was more serious than pure BSA solution. Apparently, the BSA-LVFX solution under ultrasonic irradiation emitted the weakest fluorescence in all of the samples. It is the reason that the numbers of Trp and Tyr residues, which are the intrinsic fluorescence substance of BSA, become less gradually [19, 20] due to the combination with LVFX and the generation of a large number of ROS.

### Influence of ultrasonic irradiation time on damage of BSA

The damages of BSA in the presence of LVFX were studied along with ultrasonic irradiation time altering within 5.0 h at 1.0 h interval. The concentrations of BSA and LVFX were both $1.00 \times 10^{-5}$ M.

Figure 5(a) shows that the absorbance increases along with the increase of ultrasonic irradiation time whether in the presence or absence of LVFX. However, the absorbance of BSA−LVFX solution is much higher than corresponding one of BSA solution at any ultrasonic irradiation time. That is, the hyperchromic effects of BSA-LVFX solutions are more obvious than those of the BSA solutions at any time under ultrasonic irradiation. It can be inferred that the BSA in the presence and absence of LVFX suffer from different degree damages under ultrasonic irradiation. The sonodynamic damage of BSA in BSA-LVFX solution was more serious than pure BSA solution. Moreover, the damage degrees of BSA were intensified with increasing ultrasonic irradiation time. These results can be explained as following. Activation of sonosensitive LVFX can generate a large number
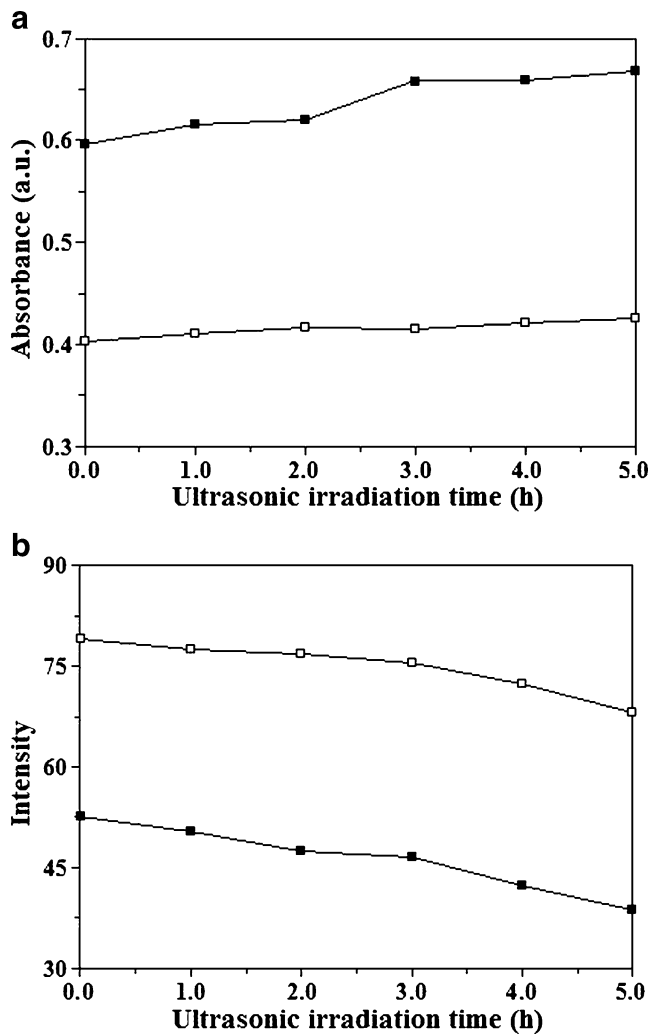


**Fig. 3** UV-vis spectra of (*a*) BSA-LVFX with irradiation for 3.0 h, (*b*) BSA-LVFX without irradiation, (*c*) BSA with irradiation for 3.0 h, (*d*) BSA without irradiation, (*e*) LVFX with irradiation for 3.0 h and (*f*) LVFX without irradiation

## a



## b



Fig. 5 Absorbance (**a**, $\lambda_{max}$=283 nm) and fluorescence intensity (**b**, $\lambda_{ex}$=280 nm and $\lambda_{em}$=352–357 nm) changes of BSA-LVFX solutions with ultrasonic irradiation time (■: with 3.0 h irradiation; □: without irradiation)

of ROS. Therefore, the damage degrees were increased in a large scale. Meanwhile, along with the prolonging of ultrasonic irradiation time, the quantity of ROS generated in the solutions also increased, and the chance of ROS attacking BSA became more and more. So, the damage of BSA became more and more serious. It was also apparently that the anticipated level of protein damage could be controlled through adjusting the time of exposure to ultrasonic irradiation [21].

Fluorescence spectra were also used to study the effect of ultrasonic irradiation time on the damage of BSA. The corresponding results were shown in Fig. 5(b). It can be seen that the fluorescence intensities decreased along with the increase of ultrasonic irradiation time. In this case, the loss of intrinsic fluorescence of BSA was mainly due to the oxidation of its Trp and Tyr residues. Nevertheless, the fluorescence intensities of BSA-LVFX solution were obviously lower than

those of BSA solution at any irradiation time. It indicated that the damage of BSA were more likely to happen in the BSA-LVFX solution because of the synergistic effects of ultrasonic irradiation and LVFX.

### Influence of solution acidity on damage of BSA

Because the pH value of microenvironment around the cancer tissue was slightly lower than that of the normal tissue, the solution acidity was studied as an influencing factor on the damage of BSA. Here, the pH values were adjusted from 5.0 to 9.0 using HCl and Tris diluent solutions. The concentrations of BSA and LVFX were both $1.00 \times 10^{-5}$ M.

As shown in Fig. 6(a), in weak acidic solution, the absorbance of BSA-LVFX solution increased along with the increase of pH value, whereas in weak alkali condition, the absorbance was lower than that at neutral pH value. Moreover, under ultrasonic irradiation the absorbance of BSA-LVFX solution expressed more obvious hyperchromic effect compared with those only kept in the dark. Correspondingly, Fig. 6(b) shows that without the ultrasonic
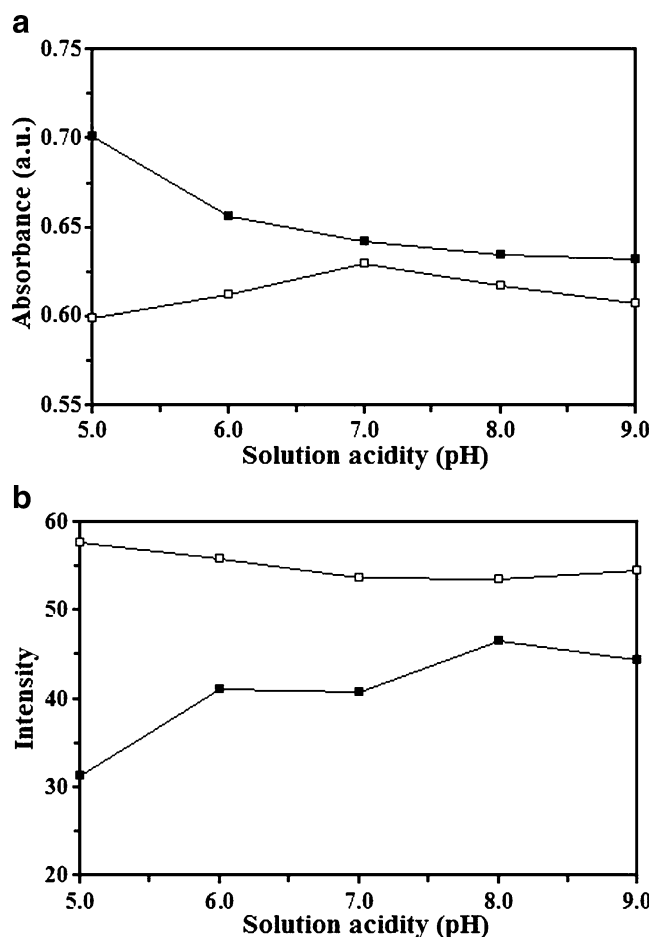
## a



## b



Fig. 6 Absorbance (**a**, $\lambda_{max}$=283 nm) and fluorescence intensity (**b**, $\lambda_{ex}$=280 nm and $\lambda_{em}$=354–358 nm) changes of BSA-LVFX solutions with solution acidity (■: with 3.0 h irradiation; □: without irradiation)
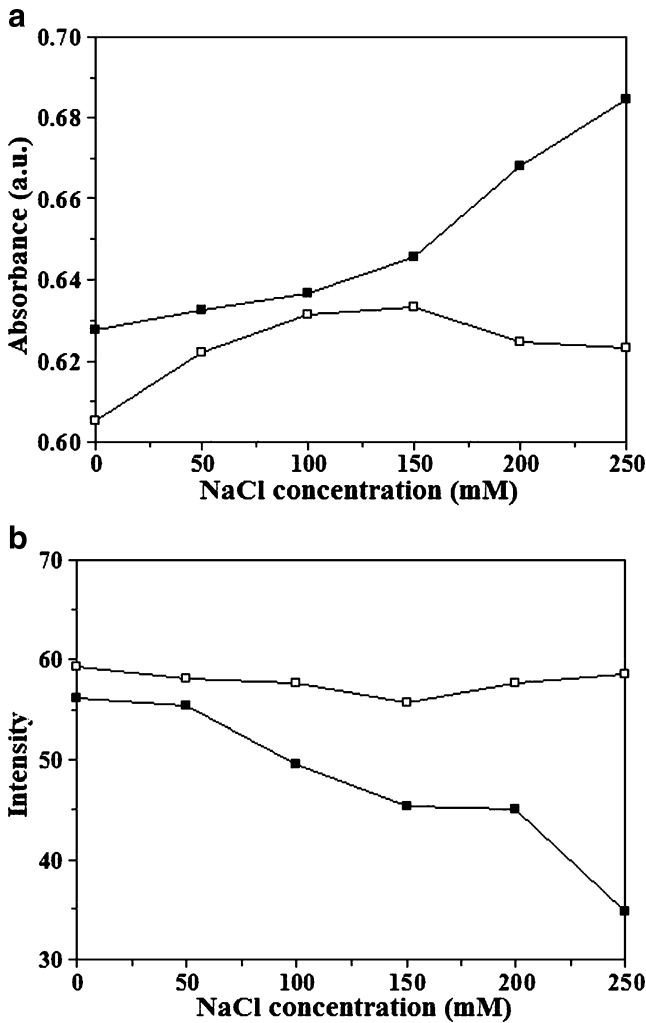
**Fig. 7** Absorbance (**a**, $\lambda_{max}$=283 nm) and fluorescence intensity (**b**, $\lambda_{ex}$=281 nm and $\lambda_{em}$=351–355 nm) changes of BSA-LVFX solutions with NaCl concentration (■: with 3.0 h irradiation; □: without irradiation)

irradiation, the fluorescence intensities of BSA-LVFX solution decreases along with the increase of pH value. Whereas the absorbance of BSA-LVFX solution increases under ultrasonic irradiation.

These results indicate that the influence of solution acidity on damage of BSA is complicated. Firstly, at the pH 5.0–7.0 range, the surface of BSA molecules bears negative charges because of pH above the isoelectric points (PI) of BSA (4.8). But, LVFX bears positive charges because of pH below 6.7, which is the PI of LVFX. Therefore, the increase of interaction force is observed with the increase of pH value. However, both BSA and LVFX bear negative charges in weak alkali condition make the interaction force decrease and the repulsion increase, so that the possibility of damage BSA decreases along with the increase of pH value. In addition, our results indicate that low pH conditions enhance sonoluminescence-induced damage to BSA structure by facilitating the donor-side mechanism of photoinhibition

[22]. At the same time, it is also a reason that the decrease in the amount and lifetime of $^1O_2$ are observed with the changing pH from 5.0 to 9.0 [23].

## Influence of ionic strength on damage of BSA

In order to determine the effect of the ionic strength on sonodynamic damage of BSA, some related experiments were carried out using different concentrations of NaCl of 0–200 mM at 50 mM interval. The concentrations of BSA and LVFX were both $1.00 \times 10^{-5}$ M.

It can be seen from Fig. 7(a) that for BSA-LVFX solution without the ultrasonic irradiation, the absorbance at 281 nm increased firstly along with the increase of solution ionic strength, and then decreased. However, under ultrasonic irradiation the absorbance increase regularly and become much higher compared to those without the ultrasonic irradiation. Figure 7(b) showed that the fluorescence intensity of BSA-LVFX solution decreased along with the increase of ionic strength under ultrasonic irradiation.
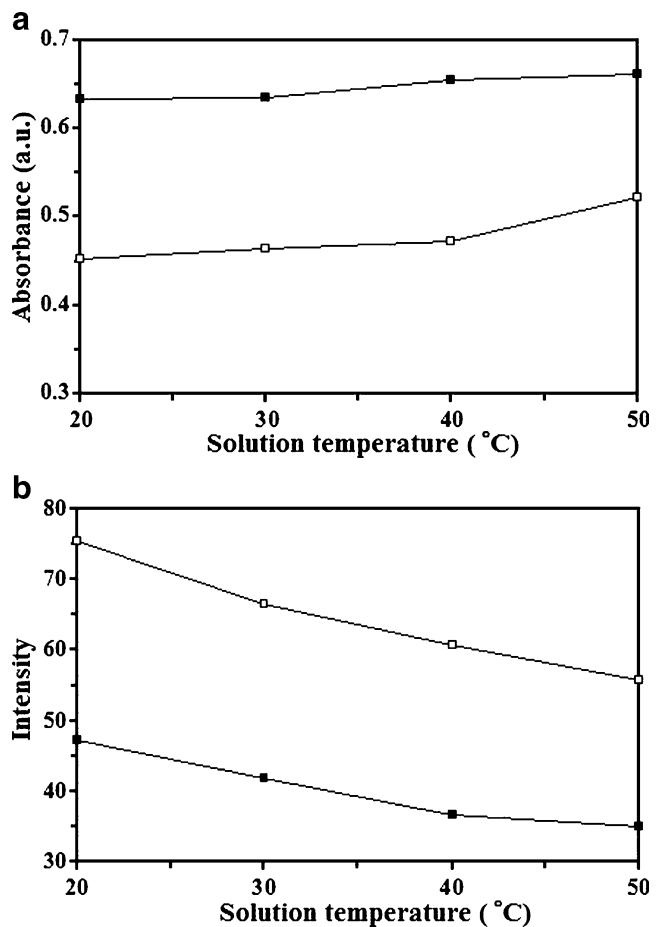


**Fig. 8** Absorbance (**a**, $\lambda_{max}$=283 nm) and fluorescence intensity (**b**, $\lambda_{ex}$=280 nm and $\lambda_{em}$=347–350 nm) changes of BSA-LVFX solutions with solution temperature (■: with 3.0 h irradiation; □: without irradiation)

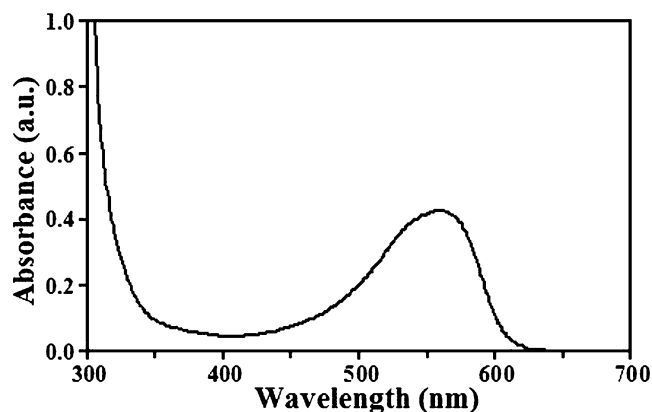**Fig. 9** Absorption spectra of DPCI solution with ultrasonic irradiation for 1.0 h



**Fig. 11** Absorbance changes of DPCO with ultrasonic irradiation time ([DPCI] = $5.0 \times 10^{-3}$ M, [LVFX] = $1.0 \times 10^{-4}$ M and $\lambda_{max}$=563 nm)

The results indicated that the BSA in BSA-LVFX solution under ultrasonic irradiation was damaged more and more serious along with the increase of ionic strength. With the increase of ionic strength, the salt bonds in BSA molecules were ruptured gradually, which resulted in the breakage of secondary structure, extension of peptide strand and exposure of Trp, Tyr and Phe residues. The extension of peptide strand caused the increase of hyperchromic effects in UV-vis spectra and quenching effects in fluorescence spectra. Nevertheless, in higher ionic strength region, the excessive ions interfere with the interaction of LVFX and BSA, which caused the decrease of hyperchromic effects in UV-vis spectra and quenching effects in fluorescence spectra. However, with the increase of the ionic strength, the cavitation effect caused by ultrasonic irradiation was strengthened. Therefore, the oxidative damage degree of BSA increases still along with the increase of the ionic strength under ultrasonic irradiation.

Influence of solution temperature on damage of BSA

To investigate the influence of the solution temperature on the damage of BSA under ultrasonic irradiation for 3.0 h in
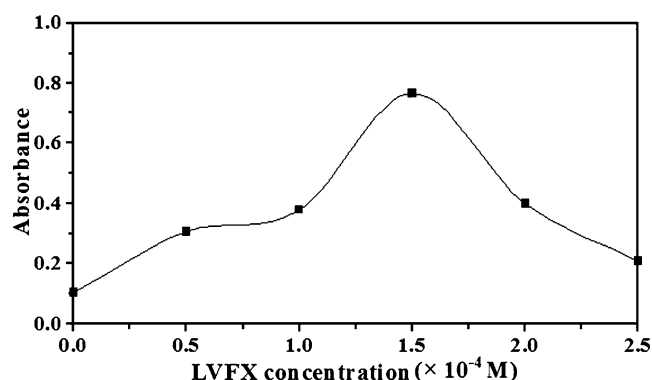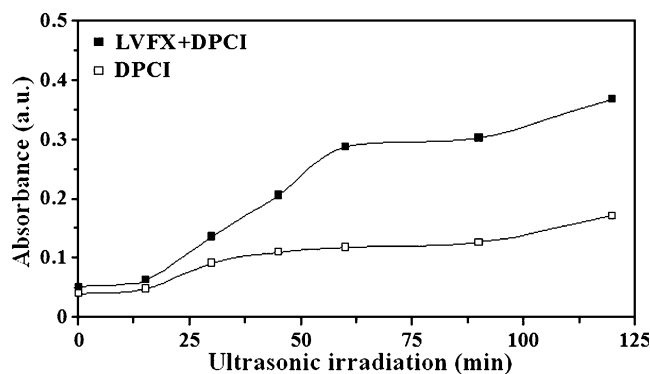
the presence of LVFX, the solution temperatures were adjusted between 20–50 °C at 10 °C intervals. The concentrations of BSA and LVFX were both $1.00 \times 10^{-5}$ M.

Figure 8(a) showed that the absorbance of BSA-LVFX solution at 283 nm either with or without ultrasonic irradiation increased along with the increase of solution temperature. Furthermore, under ultrasonic irradiation, they became much higher than those without ultrasonic irradiation. Figure 8(b) showed that the fluorescence intensity of BSA-LVFX solution decreased along with the increase of solution temperature.

The results indicated that the BSA molecules in BSA-LVFX solution were damaged more seriously along with the increase of solution temperature. In this process, the increase of the solution temperature caused conformational changes in the unfolding form of secondary structure, which resulted in the extension of peptide strand and exposure of Trp, Tyr and Phe residues. Moreover, along with the increase of the solution temperature, the accessibility of LVFX to chromophoric amino acid residues of BSA became easier and the effect of cavitations by ultrasound increased. Simultaneously, the more ROS were produced, meanwhile the decomposition rates of ROS also
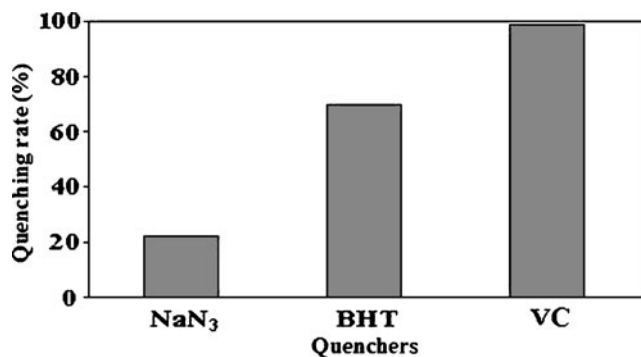


**Fig. 10** Absorbance changes of DPCO with LVFX concentration ([DPCI] = $5.0 \times 10^{-3}$ M, $\lambda_{max}$=563 nm and 1.0 h ultrasonic irradiation)



**Fig. 12** Effects of different quenchers on ROS generation under ultrasonic irradiation for 1.0 h ([NaN$_3$] = [BHT] = [VC] = $5.0 \times 10^{-2}$ M, [DPCI] = $5.0 \times 10^{-3}$ M and $\lambda_{max}$=563 nm)

increased. Therefore, the oxidative damage degree of BSA increased along with the increase of the solution temperature regardless of there was ultrasound or not.

Identification of the reactive oxygen species (ROS)

ROS are reactive form of oxygen, which can react with electron-rich bio-molecules such as lipids [24], proteins [25–28] and DNA [29, 30]. Since colorimetric techniques for testing ROS were sensitive and easy to implement in routine laboratories, they were still attractive from an analytical point of view [31]. The ROS were identified with oxidation and extraction photometry in this paper. This technique was based on the formation of a specific product upon reaction of ROS with a chemical trap [14]. The mechanism was that the diphenylcarbazide (DPCI) can capture the ROS resulted from the synergy of ultrasonic irradiation and sonosensitizer LVFX, and then it could be oxidized to diphenylthiocarbazone (DPCO), which can be extracted by organic solvent and analyzed by UV-vis spectrum. Figure 9 showed that absorption spectrum of DPCO was characterized by a strong peak at 563 nm. The changes of absorbencies of DPCO with LVFX concentration and ultrasonic irradiation time were recorded in Figs. 10 and 11, respectively. Figure 10 showed that the ROS levels increased significantly with the increasing LVFX concentration, while decreased at concentration above $1.50\times 10^{-4}$ M under ultrasonic irradiation for 1 h. The reason may be that the high LVFX concentration will have a greater barrier to light transmission from sonoluminescence. Then we investigated the changes of ROS levels with ultrasonic time and power. The results showed that ROS levels both LVFX group and the control group all increased with ultrasonic time increasing, while the former was higher obviously than the latter. It is because of the LVFX activated by US can generate a large number of ROS along with the prolonging of ultrasonic irradiation time. Additionally, the ROS levels were increased with the increasing of irradiation power, but increased slightly. This may be partly due to the increasing of power, which is conducive to the cavitations and can produce more ROS. On the other hand, excessive ROS can destroy the LVFX. In short, the irradiation power is a very complex factor. Furthermore, to identify the kind of ROS, the effects of different quenchers were investigated. Among them, the azide ion was a strong physical quencher of $^1O_2$ and was frequently employed to show involvement of $^1O_2$ in oxidation processes [15], and that, VC and BHT were quenchers of superoxide anion radical ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH$). Figure 12 showed that the quenching rate of $^1O_2$ by NaN$_3$ was obviously less than others. Therefore, it can be inferred that the amount of superoxide anion radical ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH$) was significantly more

than singlet oxygen ($^1O_2$) under ultrasonic irradiation in the presence of LVFX. Even though, for photodynamic mechanism, the $^1O_2$ played a major role [32–34].

## Conclusions

It is evident that the damage of BSA happened under low frequency (40 kHz) ultrasonic irradiation in the presence of LVFX. Also, the damage degrees of BSA molecules aggravate obviously with the increase of ultrasonic irradiation time, ionic strength and solution temperature. Nevertheless, the BSA damage decreases with the increase of solution pH value. Hence, it can be inferred that the combination action of ultrasonic irradiation and LVFX can effectively damage the BSA, so that LVFX can be used as a good sonosensitizer drug of SDT to treat cancers on the base of further investigation. Also, the mechanism on sonodynamic damage of BSA is different from photodynamic mechanism. Based on the above experimental results, both the $\cdot O_2^-$ and $\cdot OH$ played the major role in BSA damage under ultrasonic irradiation combined with LVFX.

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