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The preparation of TiO_2/hydroxylapatite (TiO_2/HA) composite and sonocatalytic damage to bovine serum albumin (BSA) under ultrasonic irradiation

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ABSTRACT

The TiO_2/hydroxylapatite (TiO_2/HA) composite with 6.0% HA molar content was prepared by precipitation method and heat-treatment at 500 °C for 40 min. The products were charactered by powder X-ray diffraction (XRD) and scanning electron microscopy (SEM). The sonocatalytic activity of the prepared TiO_2/HA composite was studied through the damage of bovine serum albumin (BSA) in aqueous solution under ultrasonic irradiation. The effects of several factors on the sonocatalytic damage of BSA molecules were evaluated by means of UV–vis and fluorescence spectra. It was found that the damage degree was aggravated with the increase of ultrasonic irradiation time, ultrasonic irradiation power, TiO_2/HA addition amount, solution acidity and ionic strength. The damage site to BSA molecules was also studied using synchronous fluorescence technology. It was observed that the damage site was mainly focused on the tyrosine (Tyr) residue of BSA molecule. These research results are of great significance for driving sonocatalytic method to treat tumor in clinic application.

1. Introduction

In recent years, the biological effects of ultrasound and its application in diseases treatment has become a rapidly expanding research area [1,2]. Especially, the ultrasonic therapy for various tumors has been developed successfully [3,4]. Some investigations showed that the responses of malignant cells to low-intensity ultrasound were not identical to those of normal cells in that the cancer cells were more prone to being killed [5]. Thus, the low-intensity ultrasound could also suppress cell proliferation and clone formation, improve the effects of anticancer chemicals and deactivate cells via indirect mechanisms [6,7]. These findings revealed that the low-intensity ultrasound has distinct potential as a technique for cancer treatment. In the meantime, many studies have also applied an ultrasonic system to wastewater treatments, and investigated the improvements to this method by using semiconductor materials (e.g. titanium dioxide (TiO_2) and zinc oxide (ZnO)) due to their high catalytic reactivity, avirulence, and low cost [8–10]. It hints that the TiO_2 or ZnO modified by using appropriate biological material can strengthen the low-intensity ultrasound to destroy the cancer cells.

Hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) is a major mineral component of human hard tissues such as bones and teeth [11,12], distributed in milk protein micelle and whey protein of cow's milk [13]. In recent years, with the development of synthesis technology the chemically synthesized HA has got closer to biological apatite in composition and structure [14,15]. Because of the excellent biocompatibility, bioactivity and non-toxicity, the specially synthesized HA has been widely used as implant materials for replacement and repair of damaged bone or teeth [16,17]. Moreover, due to the high affinity for biopolymers such as proteins, enzymes and strong adsorption capacity for organic drug molecules the HA can be acted as a drug carrier for site specific delivery and time-controlled release [18–20]. Hence, the HA has caused wide public concern in many research area.

It is well known that the protein is the most abundant macromolecule in biological cell and it is also a fundamental substance that expresses various biological functions. If the functional proteins are damaged, the cells will be destroyed from interior, resulting in the apoptosis of whole cell. In previous work, the bovine serum albumin (BSA) was selected as a model protein and the interaction with many small organic molecules (such as drug, dye, pesticide, and so on) was studied [21–23]. Meanwhile, in order to investigate the sonocatalytic method to kill cancer cells, as a pilot study the damage of BSA molecules by ultrasonic irradiation combined with TiO_2 or ZnO was also studied with satisfactory results [24,25]. As what mentioned before, the actions of the manmade TiO_2 or ZnO are generally regarded as non-recognition capability to various biomolecules. Therefore, in this paper, adopting conventional precipitation method the composite of TiO_2 and HA, TiO_2/HA, was prepared for the first time and intended to improve the recognition capability and affinity of TiO_2 particles to BSA molecules. It

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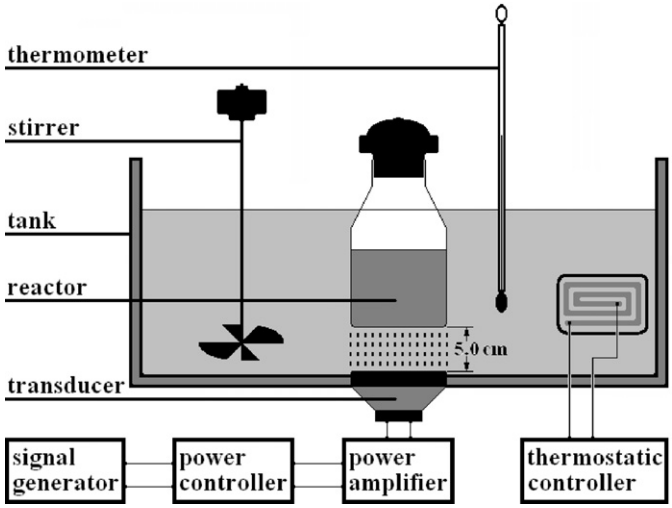

Fig. 1. Schematic illustration of experimental setup.

is hopeful to provide a valuable reference for the further research on the method of cancer treatment.

2. Experimental

2.1. Materials and chemicals

Nano-sized titanium dioxide (TiO_2) powder (anatase phase, 99.9% TiO_2 content, 20–25 nm grain diameter and 150–180 m^2/g specific surface area) was bought from Hangzhou Wanjing Xincailiao Limited Company (Hangzhou, China). Bovine serum albumin (BSA) was purchased from Abxing Biotechnological Company (Beijing, China) and was directly used without further purification. All other reagents were of analytical grade. Doubly distilled water was used as a solvent throughout the experiment.

2.2. Instruments and apparatus

The controllable serial-ultrasonics apparatus (KQ-100, Kunshan apparatus Company, China) shown in Fig. 1 was adopted as irradiation source. X-ray diffraction meter (XRD) (RINT 2500, XRD-Rigaku Corporation, Japan) was used to confirm the crystal phase. Scanning electron microscope (SEM) (JEOL JSM-5610LV, Hitachi Corporation, Japan) was used to observe the grain diameter and particle surface. UV–vis spectra were recorded with an UV–vis spectrometer (Cary 50, Varian Company, USA) and fluorescence measurements were performed on a fluorescence spectrometer (Cary 300, Varian Company, USA).

2.3. Preparation of TiO_2/HA composites

TiO_2/hydroxylapatite (TiO_2/HA) composite was prepared using a conventional precipitation method. Details on this technique were as follows: 0.075 mol/L calcium nitrate ($Ca(NO_3)_2$) solution and 0.045 mol/L ammonium phosphate ($(NH_4)_2HPO_4$) solution were separately brought to pH 11–12 with concentrated ammonia solution (25–28%). A certain amount of the nano-sized TiO_2 powder was put into the prepared $Ca(NO_3)_2$ solution in order to obtain the TiO_2/HA composite with 6.0% HA molar content. $Ca(NO_3)_2$ solution was vigorously stirred at room temperature and then added by dropwise to $(NH_4)_2HPO_4$ solution to produce gelatinous precipitates. The precipitated solution was stirred for half an hour to form TiO_2/HA composite. Then the reaction mixture was centrifuged and washed repeatedly to remove the unreacted NH_3. The sludge was filtered using filter paper to form a sticky cake and then dried at least 48 h at 60 °C. Dried cakes were crushed and ground thoroughly. Subsequently, the dried mixture with 6.0% HA molar content was divided into several parts in corundum crucibles, followed by heat-treatment for different durations at elevated temperatures in the range of 300–700 °C. At last, all the heat-treated powders were cooled to room temperature naturally for succedent sonocatalytic measurements.

2.4. Experiments of sonocatalytic damage of BSA

Four 25.00 mL BSA solutions (1.00×10^{-5} mol/L) were prepared and added into four 50 mL brown conical flask marked as a–d, respectively. One TiO_2 powder and two TiO_2/HA composites, whose addition amount were all 1.0 g/L, were added into b, c and d, respectively. Afterwards, c and d were placed in an ultrasonic irradiation apparatus, while a and b only done away from light. The temperature was dominated at 37.0 ± 0.2 °C in the whole process of experiment. After 3.0 h, samples (b, c and d) were immediately centrifuged to remove the catalysts and their transparent liquids were analyzed. The UV–vis and fluorescence spectra of each sample solution were determined, in order to evaluate the sonocatalytic damage to BSA molecules. After this, the effects of HA molar content, heat-treated temperature and heat-treated time on sonocatalytic activty of TiO_2/HA composite were reviewed by the numbers. In addition, the effects of some factors such as ultrasonic irradiation time, ultrasonic irradiation power, TiO_2/HA addition amount, solution acidity and ionic strength on the damage of BSA molecules were also studied. Lastly, the damage sites of BSA molecules were examined by synchronous fluorescence technology.

3. Results and discussion

3.1. XRD and SEM of heat-treated TiO_2/HA composite

The X-ray diffraction patterns of TiO_2/HA (with 6.0% HA molar content), TiO_2 and HA powders were shown in Fig. 2. As can be

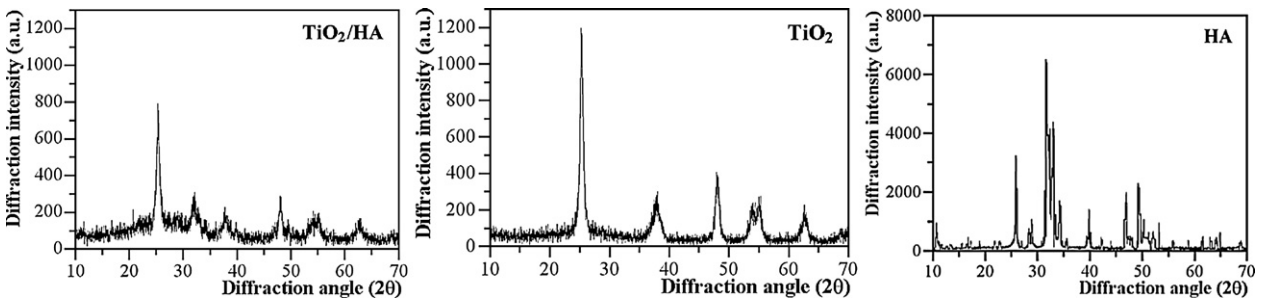

Fig. 2. XRD of TiO_2/HA composite (6.0 mol% HA, 500 °C and 50 min heat-treatment), TiO_2 (500 °C and 50 min heat-treatment) and HA (500 °C and 50 min heat-treatment).

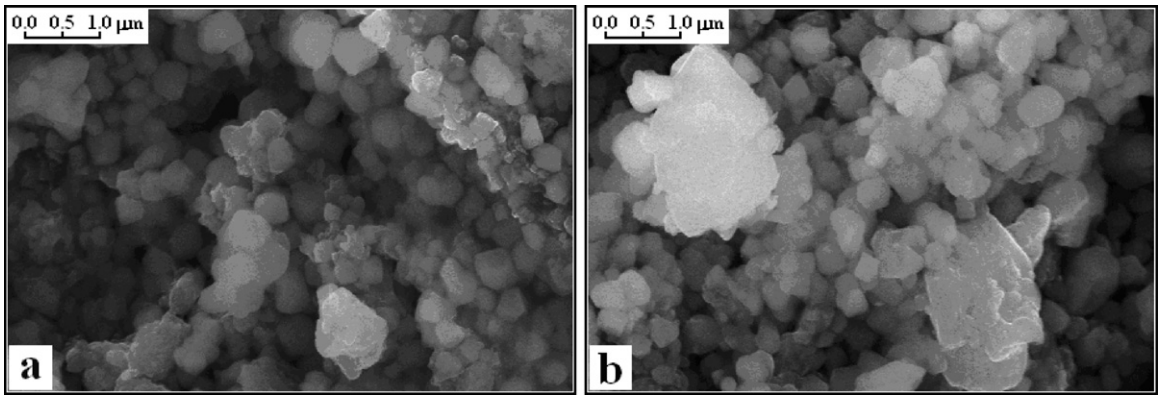

Fig. 3. SEM of TiO_2/HA composite (a) (6.0 mol% HA, 500 °C and 50 min heat-treatment) and HA (b) (500 °C and 50 min heat-treatment).

seen, the characteristic peaks of TiO_2 and HA powders are both found in the XRD pattern of TiO_2/HA composite. It indicates that the composite really contains the crystalline phase belonging to anatase TiO_2 and apatite components. However, the intensities of the diffraction peaks both become weak compared with that of the pure TiO_2 and HA powders, which is caused by the relative decrease of the molar contents of TiO_2 and HA in the TiO_2/HA composite.

The external surface of TiO_2/HA composite (with 6.0% HA molar content) and HA powder were characterized by scanning electron microscopy (SEM). It can be seen in Fig. 3a that the surface of TiO_2/HA particles becomes bright white compared with that of pure HA particles in Fig. 3b. It indicates that the surface of nano-sized TiO_2 particles are covered with the HA. In addition, it can also be proved that the average granularity of HA particles becomes large from 0.3–0.4 μm to 0.5–0.6 μm after covering nano-sized TiO_2 particles.

3.2. UV–vis and fluorescence spectra of BSA solution under ultrasonic irradiation in the presence of TiO_2/HA composite

UV–vis and fluorescence spectra are powerful tools to study the structural and compositive change of protein [26]. Herein, in order to investigate the damage of BSA molecules under different conditions, the measurements of UV–vis and fluorescence spectroscopy were carried out.

In general, the BSA in aqueous solution gives a strong absorption peak at 280 nm wavelength. It can be seen in Fig. 4a that, compared with the original BSA solution, the absorbance of BSA solution in the presence of TiO_2/HA composite without ultrasonic irradiation decreases very slightly. It illustrates that the TiO_2/HA composite has a low adsorption ability for BSA molecules. By comparison, it was found that the absorbance of BSA solution in the presence of TiO_2/HA composite or TiO_2 powder under ultrasonic irradiation obviously increased, but the former is much higher than the latter. The reason may be explained as follows. The associated action of ultrasonic irradiation and TiO_2 powder can produce added hydroxyl (•OH) radicals [8–10]. The produced •OH radicals attack the bisulfur (–S–S–) bonds between two peptide chains and hydrogen bonds among amino acid residual groups, resulting in the extension of peptide chain and the exposure of chromophoric amino acid residues. Thereby the absorbance of BSA solution increases obviously. Meanwhile, the total amount of powder stays the same. The amount of TiO_2 powder relatively decreases after adding HA. Also, as a stable and safe biological material, HA has no catalytic activity. Therefore, it can be inferred that the sonocatalytic activity of TiO_2/HA composite obviously becomes high after adding HA.

It can further be validated by means of fluorescence spectra that the addition of HA can promote the selectivity of TiO_2 powder to BSA, due to its high affinity for proteins. As a result, BSA is more prone to being damaged by TiO_2 powder effectively. As shown in Fig. 4b that the slight loss of fluorescence intensity of BSA solution in the presence of TiO_2/HA composite without ultrasonic irradiation appears compared with the BSA original solution. However, under ultrasonic irradiation the fluorescence intensities of BSA solution in the presence of TiO_2/HA composite and TiO_2 powder both decrease obviously. It can be explained that the synergistic effect of ultrasonic irradiation and TiO_2 powder destroys the tryptophan (Trp) and tyrosine (Tyr) residues which mainly contribute to

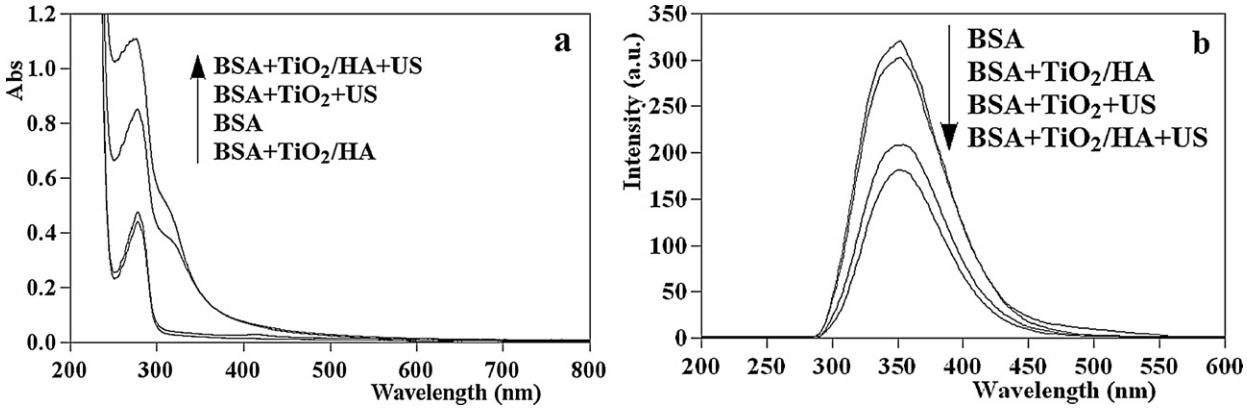

Fig. 4. UV–vis (a) and fluorescence (b) spectra of BSA, BSA + TiO_2 (500 °C and 50 min heart-treatment) and BSA + TiO_2/HA (6.0 mol% HA, 500 °C and 50 min heart-treatment) solutions under different conditions ([BSA] = 1.00×10^{-5} mol/L, [TiO_2/HA] = [TiO_2] = 1.00 g/L, pH 7.40, [NaCl] = 50 mmol/L, t_{US} = 3.0 h, P_{US} = 50 W, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL. US: ultrasonic irradiation).

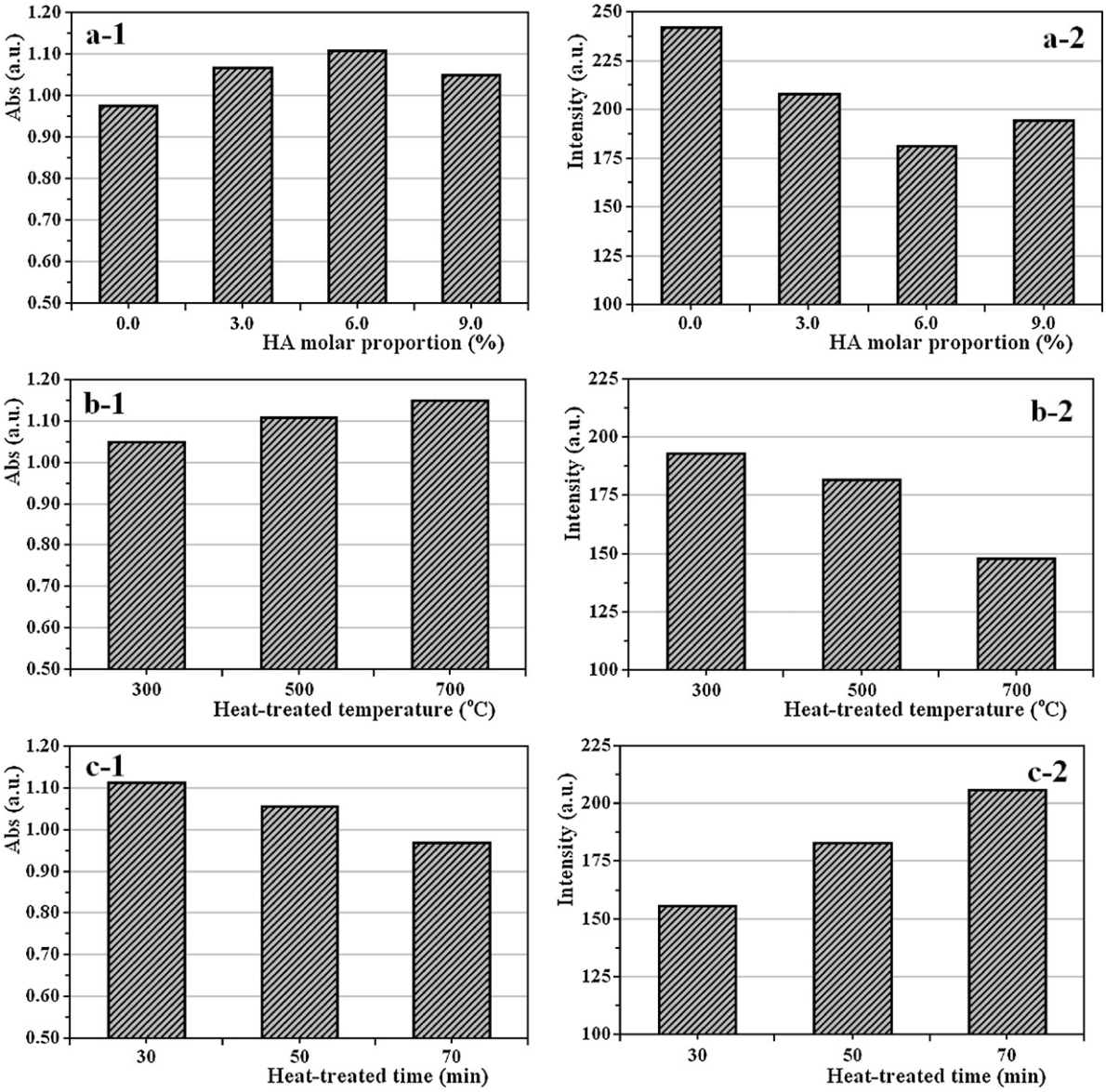

Fig. 5. Changes of UV–vis absorbance (1) and fluorescence intensity (2) of BSA + TiO_2/HA solutions with HA molar proportion (500 °C and 50 min heart-treatment) (a), heat-treated temperature (6.0 mol% HA and 50 min heart-treatment) (b) and heat-treated time (6.0 mol% HA and 500 °C heart-treatment) (c) ([BSA] = 1.00×10^{-5} mol/L, [TiO_2/HA] = 1.00 g/L, pH 7.40, [NaCl] = 50 mmol/L, t_{US} = 3.0 h, P_{US} = 50 W, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL. US: ultrasonic irradiation).

fluorescence spectra of BSA molecules. So the fluorescence intensity reduces. Furthermore, the HA helps TiO_2 powder to damage BSA effectively owning to its high affinity for proteins, even if the amount of TiO_2 powder decreases in TiO_2/HA composite. Perhaps, the TiO_2/HA composite is more suitable to be adopted for the damage of biological macromolecules in intending sonocatalytic treatment of tumors.

3.3. The effect of HA molar content, heat-treated temperature and heat-treated time on sonocatalytic activty of TiO_2/HA composite

The TiO_2/HA composite with different HA molar content from 0.0% to 9.0% at 3.0% intervals were prepared by heat-treatment at 500 °C for 50 min. And then the sonocatalytic activities of various TiO_2/HA composites were evaluated through the damage of BSA molecules in aqueous solution.

From Fig. 5a-1 and a-2 it is observed that the sonocatalytic activity of the TiO_2/HA composite for the damage of BSA increases along with the increase of the HA molar content, and then slightly decreases after 6.0% HA molar content. The reason is that the content of TiO_2 powder becomes less and less with the increase of the HA molar content, when the total amount of TiO_2/HA composite stays the same. Generally, the HA does not display sonocatalytic activity, so it is reasonable that the sonocatalytic activity of TiO_2/HA composite reduces for high HA molar content. In this study, 6.0% HA molar content was adopted, in order to obtain a high sonocatalytic activity and selectivity to BSA.

A series of TiO_2/HA composites with 6.0% HA molar content were heat-treated at elevated temperatures in the range of 300–700 °C for 50 min, and then their sonocatalytic activities were investigated through the damage of BSA molecules. In addition, the heat-treated time was changed in the range of 30–70 min and the sonocatalytic activities were also investigated.

As shown in Fig. 5b-1, the absorbance of BSA solution increases with the increase of heat-treated temperature. It indicates that the sonocatalytic activity of TiO_2/HA composite can be enhanced with continuous increase of heat-treated temperature. The same results can be obtained from the fluorescence spectra in Fig. 5b-2. Apparently, the appropriate high temperature is conducive to intensifing the combination of TiO_2 and HA and acquiring the high

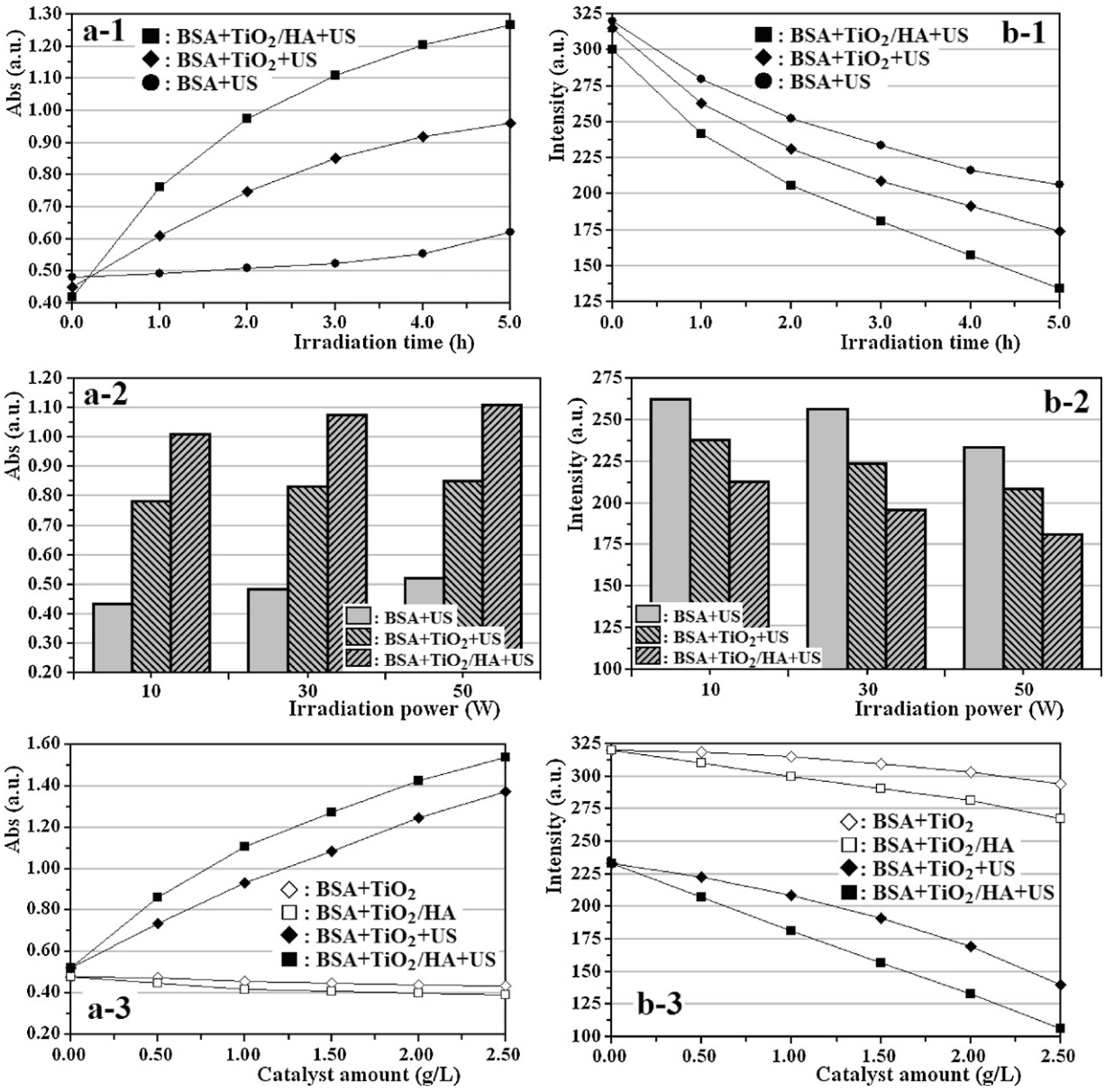

Fig. 6. Changes of UV–vis absorbance (a) and fluorescence intensity (b) of BSA, BSA + TiO_2 (500 °C and 50 min heart-treatment) and BSA + TiO_2/HA (6.0 mol% HA, 500 °C and 50 min heart-treatment) solutions with ultrasonic irradiation time (1), ultrasonic irradiation power (2) and catalyst addition amount (3) ([BSA] = 1.00×10^{-5} mol/L, [TiO_2/HA] = [TiO_2] = 1.00 g/L, pH 7.40, [NaCl] = 50 mmol/L, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL. US: ultrasonic irradiation).

active mixed crystal phase (anatase and rutile) TiO_2. In this experiment, 500 °C was chosen to heat-treat TiO_2/HA composite.

From Fig. 5c-1 it can be seen that the absorbance of BSA solution decreases along with the heat-treated time. It indicates that, due to the proportional decrease of anatase and rutile phases, the sonocatalytic activity of TiO_2/HA composite falls along with heat-treated time. The fluorescence spectra of the same BSA solution are also reviewed. As shown in Fig. 5c-2, the change of fluorescence quenching of BSA solution is weakened with the heat-treated time. In order to obtain properly high sonocatalytic activity, therefore, 50 min heat-treated time was adopted in our experiments.

3.4. The effect of ultrasonic irradiation time, ultrasonic irradiation power and TiO_2/HA addition amount on damage of BSA

From Fig. 6a-1 it can be seen that the absorbance of BSA solution under onefold ultrasonic irradiation slightly increases. Comparatively, it increases obviously with the increase of ultrasonic irradiation time in the presence of TiO_2 powder or TiO_2/HA composite. And that, the absorbance in the presence of TiO_2/HA composite is higher than the corresponding one in the presence of TiO_2 powder all through. It is because that TiO_2/HA composite not only produces more and more •OH radicals along with the increase of ultrasonic irradiation time and the chance that •OH radicals attack BSA molecules becomes greater, but also displays a high recognition capability to BSA molecules. Therefore, the damage of BSA caused by TiO_2/HA composite becomes more serious by TiO_2 powder.

The similar results can be further proved via fluorescence spectra as depicted in Fig. 6b-1. With the gradual increase of ultrasonic irradiation time, the fluorescence intensity of BSA solution decreases slightly under onefold ultrasonic irradiation. But it decreases fleetly in the presence of TiO_2 powder or TiO_2/HA composite. Moreover, the fluorescence intensity of BSA solutions in the presence of TiO_2/HA composite is lower than the corresponding examples in the presence of TiO_2 powder. It can be inferred that

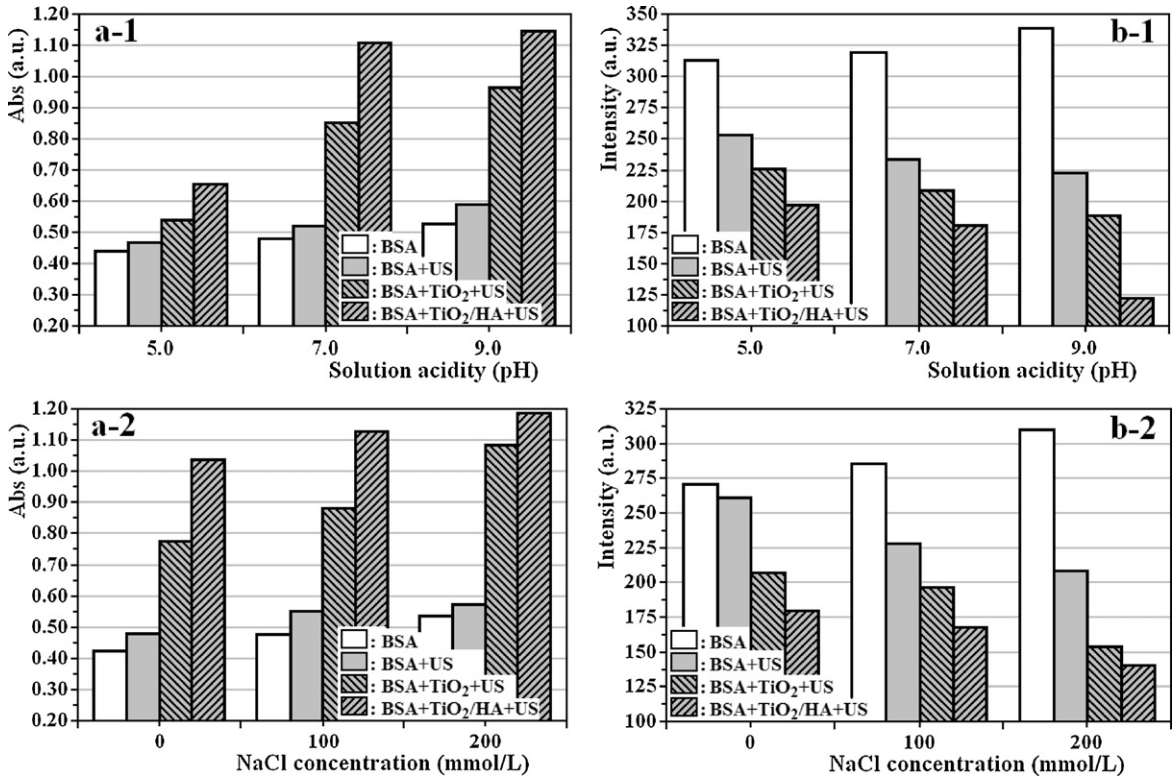

Fig. 7. Changes of UV–vis absorbance (a) and Fluorescence intensity (b) of BSA, BSA + TiO_2 (500 °C and 50 min heart-treatment) and BSA + TiO_2/HA (6.0 mol% HA, 500 °C and 50 min heart-treatment) solutions with solution acidity ([NaCl] = 50 mmol/L) (1) and NaCl concentration (pH 7.40) (2) ([BSA] = 1.00×10^{-5} mol/L, [TiO_2/HA] = [TiO_2] = 1.00 g/L, t_{US} = 3.0 h, P_{US} = 50 W, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL. US: ultrasonic irradiation).

the recognition capability of TiO_2/HA to BSA molecules is crucial originally.

Next, the ultrasonic irradiation power was varied from 10 W to 50 W and the effect on the sonocatalytic damage of BSA was studied. Fig. 6a-2 and 6b-2 reveal that the ultrasonic irradiation power has a considerable effect on the damage of BSA. In the case of onefold ultrasonic irradiation, the damage degree slightly enhances with increasing output power. But it aggravates obviously after adding the catalysts. Being similar to photocatalytic reaction, the high output power can provide the more chances that the •OH radicals form [27]. Furthermore, when the output power is 50 W, the damage degree in the presence of TiO_2/HA composite is more obvious than the corresponding one in the presence of TiO_2 powder.

The effects of different TiO_2/HA composite addition amounts (0.0–2.5 g/L) on the sonocatalytic damage of BSA molecules were investigated. It can be seen in Fig. 6a-3 that, no matter which kind of catalysts, without ultrasonic irradiation the absorbance of BSA solution at 278 nm displays a slight decrease. It attributes to the adsorption of increasing catalyst particles to BSA molecules. By comparison, under ultrasonic irradiation, the spectral hyperchromic effects of BSA solutions become more and more obvious with the increase of catalyst addition amount. Moreover, the absorbance of BSA solution in the presence of TiO_2/HA composite is higher than the corresponding one in the presence of TiO_2 powder. It can be inferred that, with the increase of TiO_2/HA addition amount, the advantage of selectivity of HA becomes apparent.

The corresponding results can be further validated by means of fluorescence spectra. As depicted in Fig. 6b-3, with the increase of the catalyst amount, under ultrasonic irradiation the fluorescence intensities of BSA solutions are reduced for both TiO_2/HA composite or TiO_2 powder. And the former is more obvious than the latter. These results suggest that, as expected, HA can promote the selectivity of TiO_2 powder for the damage to BSA.

3.5. The effect of solution acidity and NaCl concentration on damage to BSA

It is well known that the solution acidity can influence the property and structure of biomolecules [28,29]. Moreover, it has been reported that a slightly lower pH value is an important characteristic differentiating tumor tissues from the normal ones [8,30]. Therefore, the determinations of UV–vis and fluorescence spectra with various pH values (5.0, 7.0 and 9.0) were carried out, to obtain the effect of solution acidity on the sonocatalytic damage of BSA molecules.

As shown in Fig. 7a-1, without ultrasonic irradiation, the absorbance of pure BSA solution slightly increases with altering pH values up, which indicates that the increasing pH value detroys the inner hydrogen bonds and causes the extension of peptide chain and exposure of chromophoric amino acid residues. And that the absorbance more increases under ultrasonic irradiation. It demonstrates that the structure and composition of BSA molecule is further destroyed. After adding the catalysts, the hyperchromic effects of BSA solutions become more obvious in base-adjusted solutions than in acid-adjusted solution. Furthermore, the absorbance of BSA solution in the presence of TiO_2/HA composite is higher than the corresponding ones in the presence of TiO_2 powder. Several reasons can be used to explain these phenomena. The reviewed pH values are between the isoelectric points of BSA (pH 4.8) and TiO_2 powder (pH 9.5). With the increase of the pH value, the negative charges on the surface of BSA molecules become more and more. Hence, the BSA molecules are become easy to approach the surface of TiO_2 particles with the increase of pH value, and then are damaged easily. So the absorbance of BSA solutions increases.

Fluorescence spectra were also used to study the effect of solution acidity on the damage of BSA. As shown in Fig. 7b-1, at any pH

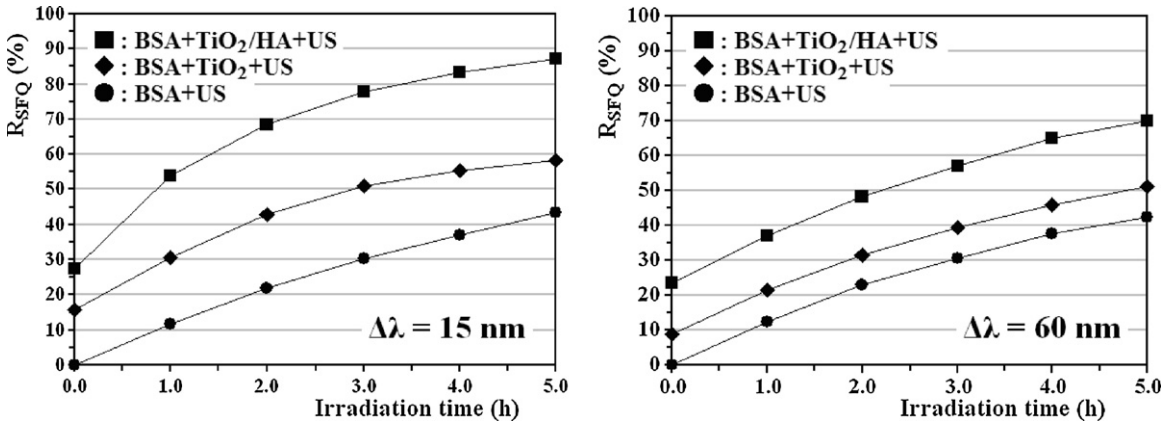

Fig. 8. Synchronous fluorescence quenching ratio (R_{SFQ}) of BSA, BSA + TiO_2 (500 °C and 50 min heart-treatment) and BSA+TiO_2/HA (6.0 mol% HA, 500 °C and 50 min heart-treatment) solutions with ultrasonic irradiation time ([BSA] = 1.00×10^{-5} mol/L, [TiO_2/HA] = [TiO_2] = 1.00 g/L, pH 7.40, [NaCl] = 50 mmol/L, P_{US} = 50 W, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL. US: ultrasonic irradiation).

value, the fluorescence intensities of BSA solutions under ultrasonic irradiation decrease compared to pure BSA solution, especially those in the presence of catalyst (TiO_2 powder or TiO_2/HA composite). Moreover, along with the increase of pH value, the fluorescence quenching is strengthened. Likewise, the fluorescence intensity in the case of TiO_2/HA composite is lower than that in the case of pure TiO_2 powder.

It has been well known that, due to unceasing changes of Na^+ concentration, all kinds of cells can survive and continue. Hence, the NaCl was adopted to adjust the ionic strength and the effect on the sonocatalytic damage of BSA molecules was studied in the NaCl concentration range of 0–200 mmol/L at interval of 100 mmol/L.

As can be seen from Fig. 7a-2, whether with or without ultrasonic irradiation, the absorbance of BSA solutions slightly increases with the increase of the NaCl concentration. It illuminates that the inner salt bonds are gradually weakened with the increase of NaCl concentration. After adding TiO_2/HA composite or TiO_2 powder, the UV–vis spectra of the BSA solutions show obvious hyperchromic effects. And the absorbance increases gradually with the increase of NaCl concentration. It indicates that the damage degree to BSA molecules can be aggravated in high NaCl concentration. It is because the increase of the ionic strength causes the extension of peptide chain, which helps to the damage of BSA. Likewise, the absorbance of BSA solution in the presence of TiO_2/HA composite is higher than the corresponding examples in the presence of TiO_2 powder.

Correspondingly, Fig. 7b-2 shows the fluorescence quenching in the presence of TiO_2/HA composite or TiO_2 powder is more obvious at any NaCl concentration, compared with that of pure BSA solution with or without ultrasonic irradiation. With the increase of NaCl concentration, the fluorescence quenching is strengthened. Moreover, the fluorescence intensity in the presence of TiO_2/HA composite is lower than that in the presence of TiO_2 powder. The phenomena can also prove that the HA facilitates the fixed damage of BSA by TiO_2 powder.

3.6. Synchronous fluorescence spectra of BSA solution under ultrasonic irradiation combined with TiO_2/HA composite

Synchronous fluorescence spectroscopy technique was firstly introduced by Lloyd in 1971 [31]. It involves the simultaneous scanning of the excitation and emission monochromators while maintaining a fixed wavelength interval ($\Delta\lambda$) between both. The synchronous fluorescence spectra can give some important information about the molecular environment in the vicinity of the fluorescent molecules. In this study, the synchronous fluorescence spectroscopy was mainly used to consider the damage site of BSA molecules. When the difference value ($\Delta\lambda$) between excitation wavelength and emission wavelength are stabilized at 15 nm or 60 nm, respectively, the synchronous fluorescence gives the characteristic information of Tyr residues or Trp residues [32].

The synchronous fluorescence spectra of BSA solutions in the presence of TiO_2 powder and TiO_2/HA composite with the increase of ultrasonic irradiation time were determined, respectively. The ratios of synchronous fluorescence quenching (R_{SFQ}) for the two cases were calculated by using the equation $R_{SFQ} = 1 - F/F_0$, in which F_0 was the fluorescence intensity of original BSA solution without ultrasonic irradiation, F was the fluorescence intensity at different ultrasonic irradiation time. From Fig. 8, it can be seen that in the presence of TiO_2 powder or TiO_2/HA composite, the R_{SFQ} of BSA for both $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm increase gradually with the increase of ultrasonic irradiation time. Moreover, the R_{SFQ} in the presence of TiO_2/HA composite is more obvious than that in the presence of TiO_2 powder. These phenomena indicate that the TiO_2/HA composite in association with ultrasonic irradiation can aggravate the damage of BSA effectively. That is, the addition of HA can help the TiO_2 powder damage BSA molecules. In addition, it is apparent that in the presence of TiO_2/HA composite or TiO_2 powder the R_{SFQ} at $\Delta\lambda$ = 15 nm is higher than that at $\Delta\lambda$ = 60 nm. It demonstrates that the damage site to BSA molecules are both mainly at Tyr residue under ultrasonic irradiation in the presence of TiO_2/HA composite or TiO_2 powder.

3.7. Possible damage mechanism to BSA under ultrasonic irradiation combined with TiO_2/HA composite

There have been some reports on adopting sonocatalytic method to damage biomolecules [24,25,33]. However, the corresponding mechanism, even less on the sonocatalytic damage of BSA in the presence of TiO_2/HA composite, has not been established. Here, we tentatively proposed the following explanation to the damage of BSA molecules under ultrasonic irradiation combined with TiO_2/HA composite.

It is well known that the partial temperature of "hot spot" resulted from ultrasonic cavitations in water medium can reach 5000 °C [34]. Such high temperature sufficiently brings a lot of holes on the surface of TiO_2 to produce •OH radicals [10]. Also, it can enable water molecules split to directly produce •OH radicals, but the efficiency is not high [35]. These generated •OH radicals would destroy the weak disulfide linkages which maintain the secondary structure of BSA molecules [24]. Synchronously, it is considered that the formation of •OH radicals results in the change of the

partial pH value around the microenvironment of BSA molecules. It badly weakens the inner hydrogen bonds. The destruction of bisulfur bonds and the decrease of hydrogen bonds cause the extension of peptide chain. The UV–vis spectrum shows the hyperchromic effect due to the exposure of chromophore groups of hydrophobic amino acid residues, but the fluorescence spectrum displays the fluorescence quenching due to the destruction of microenvironment around fluorescent Trp and Tyr residues. Regrettably, the artificially synthesized TiO_2 particles are regarded as non-identifiability to any protein molecule. As a biomimetic material, the HA has good biocompatibility and high affinity for proteins. So it can help TiO_2 particles approach BSA molecules and damage them effectively. However, the detailed mechanism on sonocatalytic damage of biological macromolecules needs further research.

4. Conclusions

TiO_2/hydroxylapatite (TiO_2/HA) composite was prepared by a conventional precipitation method. The sonacatalytic damage of BSA molecules under ultrasonic irradiation in the presence of prepared TiO_2/HA composite was detected by means of UV–vis and fluorescence spectra. Meanwhile, the effects of HA molar content, heat-treated temperature and heat-treated time on the sonocatalytic activity of TiO_2/HA composite were studied. In addition, some influence factors such as ultrasonic irradiation time, ultrasonic irradiation power, catalyst addition amount, solution acidity and ionic strength on the sonacatalytic damage of BSA were also reviewed. The results showed that, the sonocatalytic activity of TiO_2/HA composite increased with the increase of HA molar content until to 6.0 mol% HA, and then slightly decreases. While it increased and decreased, respectively, all through along with the increase of heat-treated temperature and heat-treated time. The damage degree to BSA molecules was aggravated with the increase of ultrasonic irradiation time, ultrasonic irradiation power, TiO_2/HA composite addition amount, solution acidity and ionic strength. Furthermore, the damage site to BSA molecules was also estimated by synchronous fluorescence spectroscopy. It was found to be mainly at Tyr residue in the presence of TiO_2/HA composite. However, the onefold ultrasonic irradiation or ultrasonic irradiation combined with pure TiO_2 powder damage the Trp and Tyr residues with the same probability. Of course, the fluorescence quenching in the presence of TiO_2/HA composite was more obvious than that in the presence of TiO_2 powder. Hence, it can be inferred from the experimental results that the combination action of ultrasonic irradiation and TiO_2/HA composite can damage the protein molecules effectively.

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