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International
Journal
Of Modern Engineering Research (IJMER)

Investigation on photo-oxidation Reaction of Human Oxyhemoglobin Induced by Tryptophan under Ultraviolet Light

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ABSTRACT:*Redox reaction is one of the most important biochemical processes in living organisms. In this research, the photo-oxidized mechanism of oxyhemoglobin (HbO_2) has been investigated in vitro by Ultraviolet-Visible (UV-Vis) absorption, synchronous fluorescence, Circular Dichroism (CD) spectroscopy, laser flash photolysis spectrometer. The results of UV-Vis absorption spectra showed that the maximum absorption peak at 414 nm in Soret band of HbO_2 was blue-shifted irradiated by UV light and the maximum absorption peak intensity of Q band was decreased at 540 and 575 nm. Three new bands appeared near 537, 577 and 630 nm, and consequently oxyhemoglobin (HbO_2Fe (II)) was oxidized to hemiglobin (metHbFe(III)). The effect sequence of wavelength on the strength of the photo-oxidation was 280 nm> 254 nm> 430 nm> 406 nm. Tryptophan (Trp) has the highest molar extinction coefficient in UV region, Trp absorbed 280 nm light to the excited state, and then energy was transmitted to the iron porphyrin, which absorbed the light energy and was excited, then caused photooxidation process to occur. After the addition of free tryptophan, the △OD value of HbO_2 of the transient absorption increased, indicating that the energy transfer between tryptophan and HbO_2 ferroporphyrin, which increased the excitation energy of HbO_2 to facilitate the photooxidation reaction. Hydrogen ions (H^+) played a catalytic role in promoting nucleophilic attack of water molecules. Preliminary study results showed that the initial photoionization occurs on the Trp residues to form excited states or free radicals, and then the energy was quickly transferred to the heme, which promoted the internal electron transfer process of the HbO_2. Fluorescence and CD spectra revealed that there was not significant changes in the structure of the protein during photo-reaction period. For the study of the reaction of HbO_2 under light-induced reaction process, it is helpful to study the light regulation of oxyhemoglobin's oxygen-binding and other functions, which will provide a certain basis for the research in the fields of medicine, photobiology, and life sciences.*

KEY WARDS:Hemoglobin, Photo-induced, Redox Reaction, Tryptophan, Spectroscopy

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Date of Submission: 09-04-2026 Date of acceptance: 22-04-2026

I. INTRODUCTION

With the rapid development of photodynamic therapy (PDT) in the medical field[1,2], light, as a necessary physical factor in people's life, is also increasingly favored by multi-disciplinary scholars[3]. Proteins are a large group of functional macromolecules, and their specific structure is the material basis for their function. Due to its unique structure, hemoglobin can absorb certain wavelengths of light and undergo structural changes, thus affecting the physiological functions of hemoglobin in transporting oxygen and carbon dioxide and maintaining the acid-base balance of the blood. In recent years, the effect of photo-excitation on the structure and function of hemoglobin has aroused great interest for scholars[4]. Light causes chemical reactions of chemicals to be seen everywhere in living organisms. The 193 nm pulsed laser induced hydrogen peroxide to generate hydroxyl radicals, which caused chemical reactions of substrate polypeptides and proteins[5]. Some

biochemicals are sensitive to light due to their structure and can produce different biochemical reactions. The results of Liang Ma et al demonstrated that photoactivated phyA and phyB promoted plant salt tolerance by increasing SOS2-mediated phosphorylation and degradation of PIF1 and PIF3, thus broadening our understanding of how plants adapt to salt stress according to their dynamic light environment[6]. The iron porphyrin structure in hemeproteins is similar to chlorophyll, its active center is a cyclic large conjugated structure. The light at different wavelengths can induce structural changes in the active centers of iron porphyrins, so that the expression of the biological function of the heme protein is affected similar to chlorophyll's excellent optical and electrical properties. The oxidation and reduction trends of cytochrome c (Cyt c) after ultraviolet irradiation varied with wavelength, which was closely related to its sixth coordination methionine (Met), that is, the whole reaction was the energy and electron transfer process between heme center iron porphyrin, amino acid, water and other molecules[7]. The photolysis kinetics of Mb axial ligands also have attracted considerable attention, M. Nollmann[8] and P. Etchegouin[9] have studied photoinduced deoxygenation of hemoglobin by resonance Raman spectroscopy and surface enhanced Raman spectroscopy, respectively. Zhou, H.W., et al have found that irradiation of specific wavelength induced ferric reduction in metMb, the degree of photo reduction was depending on the wavelength of incident light, and low temperature and alkaline conditions was favorable for the photo reduction of metMb[10-12]. The reaction mechanism was as follows: after the porphyrins were photoexcited, the electrons were transferred to the D - orbitals of the trivalent iron, promoting the reduction reaction. H.Y.Cao, etal found that methemoglobin (metHb), deoxyhemoglobin (deoxyHb), oxygenated hemoglobin (HbO_2) and carboxyhemoglobin (HbCO) were emitted to their corresponding excited states and the decay processes to the ground state indicated they had almost the same time irradiated by 266 nm laser, and the photo-induced redox reaction of hemoprotein with ultraviolet lights had more obvious effects than the reaction irradiated by the iron porphyrin characteristic absorption wavelength (406 nm) [13]. Light-induced oxidation of the reaction center dimer and periplasmic cytochromes for wild type and mutants of cytochrome c (cycA, cytC4 and pufC) of Rubrivivaxgelatinosus and Rhodobactersphaeroides was detected by fast kinetic difference absorption changes in intact cells exposed to excitation by a laser with of 802 nm wavelength and 2 W power on a 1×1 mm^2 spot of a culture of whole cells of Rvx. Under continuous excitation, concentrations of oxidized cytochromes increased in three phases where light intensity, electron transfer rate and the number of reduced cytochromes were the rate liming steps, respectively[14]. The researches of Sang-Hyum Ahn et al showed that the red light could increased cytochrome c oxidase activity in the electron transport system, reduced inflammation and increase antioxidant reactions to promote cell regeneration. So, they concluded that minimization of blue light exposure and the general application of red light treatment strategies are anticipated to show synergistic effects with existing treatments for retinal disease and glaucoma and should be considered a necessary prospect for the future[15].

The absorption of visible light by protein is mainly due to their heme auxiliary group, while that ultraviolet absorption in the region between 250 nm and 330 nm is mainly due to the contribution of three aromatic amino acids, such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). Trp has the highest molar extinction coefficient, and the strongest absorption capacity in the ultraviolet region.

Hemoglobin (Hb) is a well-known respiratory hemoprotein, which has the physiological function of carrying oxygen molecules to body tissues[16]. Hb is a tetramer composed of two alpha chains and two beta chains. The effects of 810 nm light-emitting diode (LED) photobiomodulation (PBM) on cerebral metabolism and cerebral hemodynamic were investigated by using a broadband near-infrared spectroscopy (bb-NIRS) under aesthesia conditions with isoflurane. PBM was applied to seven Sprague-Dawley rats at 50 mW/cm2 power density. PBM evoked the gradual increases of $\Delta(HbO_2)$ and Δ(HHb)during light stimulation. Meanwhile, Δ(oxCCO)was significantly decreased right after the onset of stimulation, which improve oxygen to the brain[17]. Photoacoustic imaging (PAI) that merges optical illumination and ultrasound detection has been rapidly gaining popularity and explored for biomedical imaging application in the last two decades. Endogenous photosensitive substances, such as Hb and melanin, can absorb certain light energy and provide structural, functional, molecular and kinetic information for clinical applications in breast, skin, blood vessels, carotid arteries, musculoskeletal, gastrointestinal and adipose tissue imaging, etc[18]. If the body's blood oxygen content is low, it will cause symptoms such as fatigue, sleepiness, lack of energy, and decreased thinking ability. The blood oxygen content is insufficient for a long time, which can cause acid-base balance disorder, also can cause damage to the brain, heart and other important organs. Therefore, understanding the changes of Hb structure caused by light will provide some basis for the diagnosis and treatment of brain, blood, heart and other diseases.

Trp can affect electron transfer rate in the process of photooxidation and reduction of many proteins[4, 19]. However, as an endogenous aromatic amino acid molecule of Hb, whether tryptophan also absorb light energy of a certain wavelength and transmit it to Hb, resulting in a certain change in the latter, and what is the mechanism of the change in the energy transfer between the two? There is no systematic study. Therefore, the effect of Trp on HbO_2 photooxidation was systematically studied by UV-Vis absorption, fluorescence, laser

flash photolysis and circular dichroism. For the study of the reaction process of HbO_2 that has the ability to bind and transport oxygen and the exploration of the mechanism of the oxidized process under light-induced reaction process, it is helpful to study the light regulation of HbO2's oxygen-binding and other functions, which will provide a certain basis for the research in the fields of medicine, photobiology, and life sciences, as well as providing useful information and data for the purposeful regulation of the important physiological functions in the body in the clinic.

II. Materials and Methods

Instruments and Reagent

Instruments：V-560 UV-Vis Spectrometer (Jasco company, Japan), Julabo F-12 Refrigeration and heating circulator (Julabo company, Germany), F-6000 Fluorescence spectrometer (Shimadzu Corporation, Japan), J-810 Circular dichroism spectrometer (Jasco company, Japan), LP-980 Laser flash photolysis (Edinburgh Instruments, UK)

Preparation of HbO_2：1×10^{-4} mol • L^{-1} met Hb was reduced with an excess of sodium dithionite with a concentration of 0.25 mol • L^{-1}. Excess sodium dithionite was removed by ultrafiltration, then high purity O_2 was passed through the DeoxyHb for 15 min(2 ~ 3 bubbles per second)The concentration of HbO_2 solution was quantified by measuring the absorption value at 415(ε 415=129 $mM^{-1}cm^{-1}$)，541(ε 541=13.8 $mM^{-1}cm^{-1}$)，577 nm(ε 577=14.6 $mM^{-1}cm^{-1}$) [20].

Experimental Methods

UV-Vis spectra: The absorption spectra of HbO_2 were recorded at room temperature (25 ℃) on a V-560 spectrophotometer (Jasco, Japan) equipped with 1.0 cm quartz cells and were performed by keeping the HbO_2 in pH7.4 Na_2HPO_4-NaH_2PO_4 buffer with a concentration of 0.05mol·L^{-1}. The spectra of oxidation state were determined by adding 0.1 mol·L^{-1} potassium ferricyanide (K3Fe (CN)6) solution to HbO_2 solution, and incubating for 2 min each time. The spectra of the reduced state was determined by adding 0.2 mol·L^{-1} sodium dithionite (Na_2SO_4) solution to HbO_2 solution and incubating for 2 min each time. The absorbance spectra were recorded in the wavelength of 220 –700 nm contrasted to 50 mM pH7.4 NaH_2PO_4-Na_2HPO_4, the slit width was 2 nm, scanning rate was 200 nm·min-1, response time was medium.

Fluorescence spectra: Fluorescence measurements were performed on an FP-6500 fluorescence spectrophotometer (Jasco, Japan) equipped with a 150 W xenon lamp source and 1.0 cm quartz cell at a scanning speed of 500 nm/min. The fluorescence emission spectra were recorded in the wavelength of 290–450 nm upon excitation wavelength at 280 nm and both excitation and emission bandwidths were 5 nm. The temperature was controlled by refrigerating and heating circulator (Julabo F-12 ME, Germany). The sample was excited by 266 nm laser provided by the third and fourth harmonics generated by the Nd:YAG Q-switched laser. Detection light was provided by a 150 W xenon lamp, and two beams of light were vertically concentrated on a 1 cm quartz cuvette.

The transient absorption signal was detected by Hamamastu R928 photomultiplier tube, split by array Czerny-Turner monochromator, and finally transmitted to computer terminal. Circular dichroism (CD) spectra were run on a J-810 Circular dichroism chromatograph (Jasco, Japan) using a 0.1 cm path length cell at 0.1 nm intervals, with 3 time scans averaged for each CD spectrum in the range of 190 -250 nm at 298 k. HbO_2 was prepared with 0.05 M phosphate buffer of pH.

III. Result and Discussion

Characterization of Hemoglobin with Different Valence

There are two forms of Fe (II) and Fe (III) in the heme active center Fe atom of hemoglobin (Hb), while Hb in Fe (II) has two forms of deoxyhemoglobin (DeoxyHb) and oxyhemoglobin (HbO_2). Oxygen molecule of deoxyHb dissociated from hemoglobin molecules detected by Raman spectroscopy, at this time Fe (II) was far away from porphyrin plane, and its electrons were in a high spin state. The Fe (II) of HbO_2 combined with O_2 on the basis of five coordination (deoxyHb) to form six ligands, the Fe (II) plane sinks and was pulled into the porphyrin ring plane, and the electrons were in a low spin state[21]. The nuclear resonance vibrational spectroscopy combined with DFT analyses gave new insights into the nature of the Fe-O2 bond of oxy heme by revealing the effect of heme peripheral substitutions on the vibrational dynamics of heme Fe atom, where the main Fe-O_2 stretching band of the native protein was characterized at ~420 cm-1[22]. Fe (II) in the heme active center of reduced hemoglobin could be oxidized to Fe (III), hemoglobin in the form of Fe (III) was called metHb, and its heme auxiliary group was called heme. UV - Vis absorption spectra showed that metHb had characteristic absorption peaks at 406, 503, 537, 577 and 630 nm, while deoxyHb had characteristic absorption peaks at 430 and 555 nm, HbO_2 had characteristic absorption peaks at 414, 540 and 575, showed in Fig. 1.

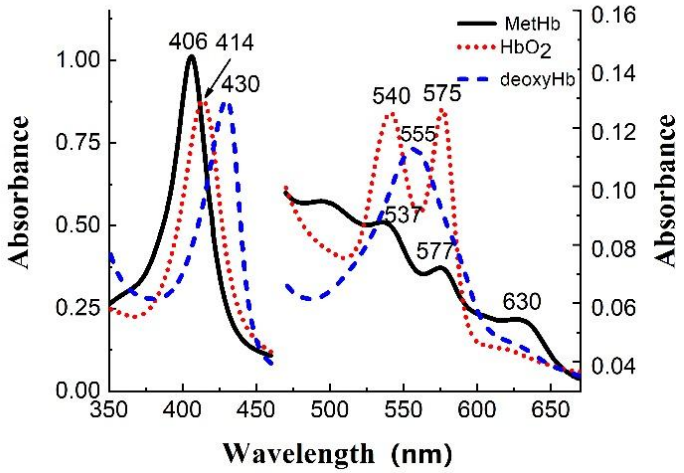

Fig. 1. The UV-Vis spectra of Hbs

Chemical Reaction and Light Reaction of HbO_2

The prepared HbO_2 remained stable in the dark for half an hour, indicating that its autoxidation rate was relatively slow. On this basis, we carried out chemical redox reaction and light reaction. Fig. 2a was the UV - Vis absorption spectra of HbO2 after adding chemical oxidant k3[Fe(CN)6]. For the curve from a to f, the characteristic peak of 414 nm in Soret band moved blue to 406 nm, and the characteristic peaks of 540 and 575 nm in Q band decreased, eventually resulted in four new absorption peaks appearing at 503, 537, 577 and 630 nm, while forming three equal absorption points at 411, 524 and 591 nm, i.e HbO2 was completely oxidized to metHb. Fig. 2b was the UV - Vis absorption spectra of HbO_2 after adding the chemical reducing agent $Na_2S_2O_4$. For the curve from a to f, the characteristic peak of 414 nm shifted to 430 nm with the increase of $Na_2S_2O_4$ concentration, while the absorption peak intensity at 540 and 575 nm decreased to form a large absorption peak at 555 nm, at the same time, five equal absorption points were formed at 421, 505, 532, 556 and 575 nm, i.e. HbO_2 was completely reduced to deoxy-Hb. At room temperature, HbO_2 solution was irradiated with a single wavelength of 280 nm light separated by xenon lamp. The UV - Vis absorption spectra of HbO_2 were shown in Fig. 2c, the results could be seen from the Fig. 2c that the absorption peak at 414 nm of Soret band decreased and shifted blue with the increase of illumination time, and the absorption peaks at 540 nm and 575 nm of Soret band disappeared, and four new peaks appeared near 503 nm, 537 nm, 577 nm and 630 nm, while three equal absorption points were formed at 411 nm, 524 nm and 591 nm. By comparing with the spectral curves Fig. 2a and Fig. 2b, we could see that this trend of Fig. 2c is consistent with the trend of adding chemical oxidant K3[Fe(CN)6], so illumination could make HbO2 be oxidized to form metHb. In Fig. 2c, the Soret band of HbO_2 finally shifted blue to 407 nm, but didn't reach the state of complete oxidation, which were attributed to the denaturation of a small amount of protein[19].

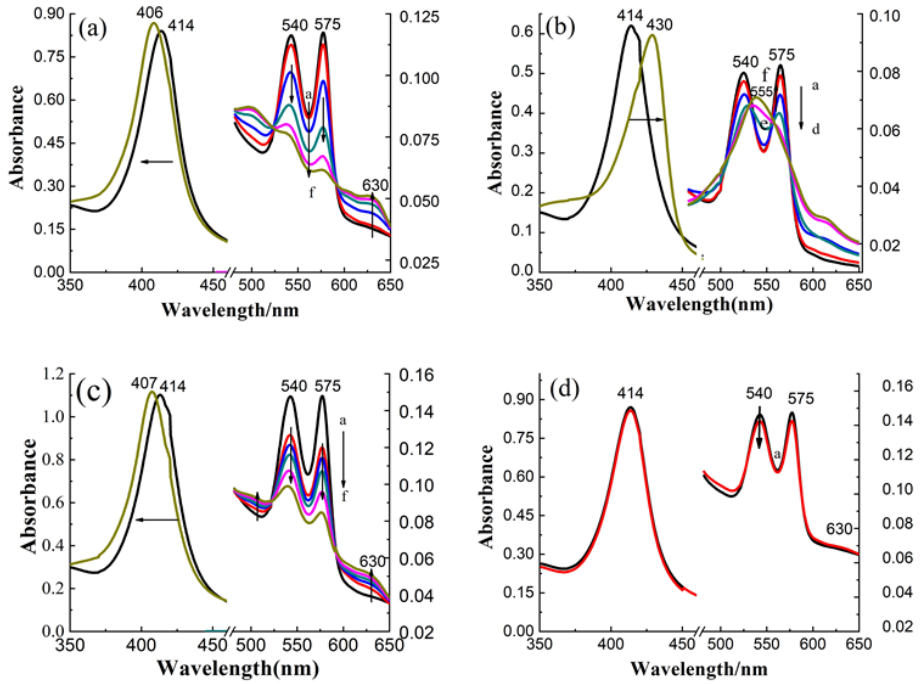

Fig. 2.UV-Vis absorption spectra of redox reaction of HbO_2 under different conditions

(**a**) Chemical oxidation with potassium ferricyanide agent, (**b**) Chemical reduction with sodium dithionite agent, (**c**) Irradiation of xenon lamp at 280 nm

（a）a → f: HbO_2 added potassium ferricyanide (0, 0.5, 1, 1.5, 2, 2.5) × 10^{-5} mol·L^{-1}, respectively.

（b）a → f: HbO_2 added sodium dithionite (0, 2, 4, 6, 8, 10) × 10^{-4} mol·L^{-1}, respectively.

（c）a→ f: Irradiation time is 0, 5, 10, 15, 20 and 25 min, respectively.

Wavelength Selection of Photo Oxidation

In order to study the oxidation of HbO_2 irradiated with different wavelengths of light, different fixed wavelengths (254, 280, 406, and 430 nm) separated from xenon lamps on RF - 6000 fluorescence spectrometer were used to irradiate HbO_2 solution for 30 min. After irradiation with different wavelengths, the intensity of the characteristic absorption peak at 414 nm in Soret band of HbO_2 decreased and shifted blue, while the intensity of the characteristic absorption peak at 540 nm and 575 nm in Q band decreased significantly as shown in Fig.**3a**. Under different wavelengths of irradiation, the peak area at 540 nm and 575 nm increased with the increase of light exposure time, but the change trend was different. The peak area of 540 nm and 575 nm decreased most when irradiated with 280 nm light, i.e. the oxidation product Hb - Fe (III) was the most. The degree of HbO_2 oxidation caused by light irradiation of four wavelengths shown in Fig.**3b**and **3c** was 280 nm > 254 nm > 430 nm > 406 nm, which showed that HbO_2 could been oxidized by UV and visible light, and the oxidation degree was not directly proportional to the irradiation energy.

The most obvious reason for oxidation reaction of HbO_2 caused by 280 nm light might be that Trp easily absorbed 280 nm light. Trp transitioned to an excited sates by absorbing a large number of photons, and transferred excitation energy to ground states of iron porphyrin groups, thus initiated photooxidation process[**Error! Reference source not found.**].280 nm light energy was absorbed in a large amount, and the energy was relatively high, so it was more favorable for photo oxidation than 254 nm. Absorption peaks of 406 and 430 nm are not characteristic absorption peaks of amino acids, and their energy was relatively low, making it difficult to excite iron porphyrin groups to excited states. Therefore, bivalent iron in HbO_2 was not easily oxidized to trivalent iron.

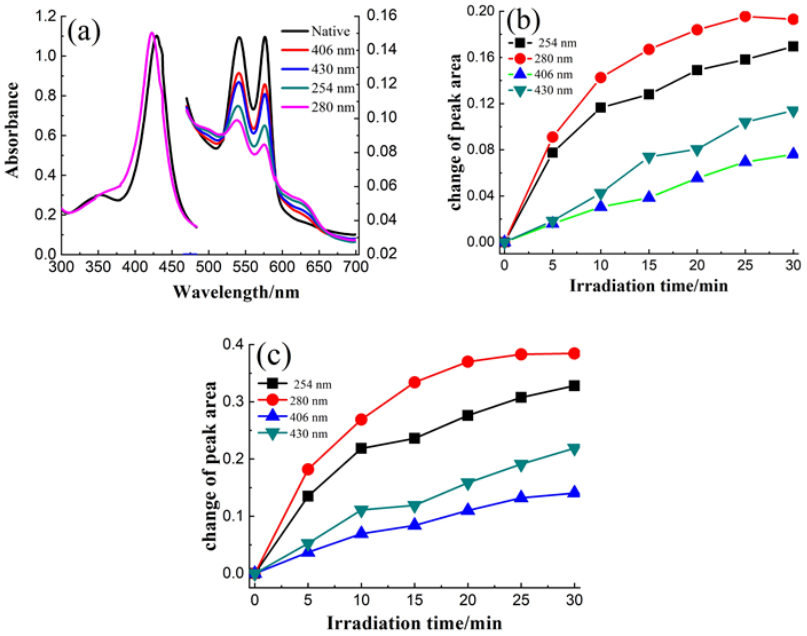

Fig. 3.UV-Vis absorbance spectra of HbO_2 (pH = 7.4) irradiated by xenon lamp for 30 min (**a**)and the peak area changes at different wavelengths， Peak area changes at 540 nm(**b**); Peak area changes at 575 nm(**c**).

Effect of Free Trp on Photooxidation of HbO_2

According to the above experiment, the oxidation degree of HbO_2 irradiated with 280 nm wavelength was the strongest. It is known that 280 nm is the maximum absorption wavelength of Trp. In order to explore the contribution of Trp residues to HbO_2 photooxidation, free Trp was added to protein solution in this experiment. In the absence of light irradiation, the UV absorption spectra of HbO_2 were almost identical in the presence and absence of free Trp, which indicated that the chemical reaction between free Trp and HbO_2 basically didn't happen in the absence of irradiation. Figure 4a and 4b showed the graphs of the peak area of HbO_2 added to Trp irradiated with 280 nm wavelength light over time, respectively. The absorption peaks at 414 nm in Soret band shifted blue and the intensity of absorption peaks at 540 nm and 575 nm in Q band were increased after the HbO_2 solution added with free Trp was irradiated with 280 nm light for 25 min. After free Trp was added, the change of peak area at 540 and 575 nm was most obvious, that is, the oxidized product of HbFe (Ⅲ) was the most. We considered that when Trp was stimulated by 280 nm light, and intermolecular energy transfer occurred between Trp and iron porphyrin, which made porphyrin more likely to reach excited state. The energy of HbO_2 without added Trp was low and it was difficult to excite the electron to excited state of porphyrin iron, so the addition of free Trp will promote the process of photooxidation reaction.

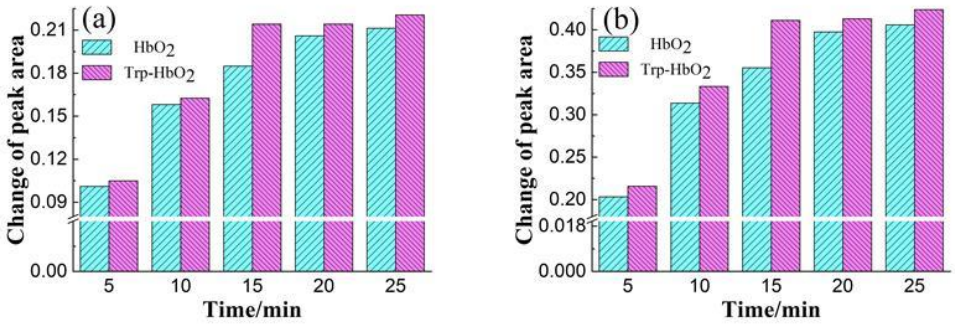

Fig. 4. Peak area change of HbO_2 added Trp by irradiation at 280 nm（**a**） Area changes at 540 nm; (**b**) Area changes at 575 nm.

Transient Absorption Spectra of HbO_2

In order to further explore the contribution of Trp residues in HbO_2 photooxidation process, 266 nm pulsed laser was used to excite HbO_2 solution containing free trp. The transient absorption spectra at different times were obtained. The Figure 5 showed that the transient absorption peak intensity first increased and then decreased with time after 266 nm laser excitation, and the transient absorption peak intensity was maximum when the time is 596 nm. Compared with HbO_2 transient absorption spectra without free Trp, HbO_2 absorption peak intensity around 415 nm were obviously enhanced after free Trp was added, and a new broad absorption peak was generated at about 340 nm.

The absorption peak at 340 nm belonged to Trp•+ absorption peak[**Error! Reference source not found.**], which showed that Trp•+ was generated after 266 nm excitation, energy transfer occurred among Trp, protein molecules and porphyrin ring, which increases the intensity of porphyrin ring absorption peak. The UV - Vis spectra of Trp also showed that the characteristic absorption peak at 280 nm disappeared after laser excitation, which indicated that conjugated double bonds of trp broke and amino acid ion free radicals were formed. The above results showed that Trp, as an oxidant in the non-chemical oxidation process, was similar to the chlorophyll photoreaction process and was a "middleman" in energy transfer. The energy released by the Trp was absorbed by porphyrin when it returned to the electron ground state through the radiation-free relaxation channel, resulting in intermolecular energy transfer. Porphyrins were more likely to reach excited state, which promoted the electron transfer of iron porphyrin of HbO_2. The whole reaction was a process of energy and electron transfer among porphyrin, tryptophan and iron, which took place after photoexcitation of amino acids in solution.

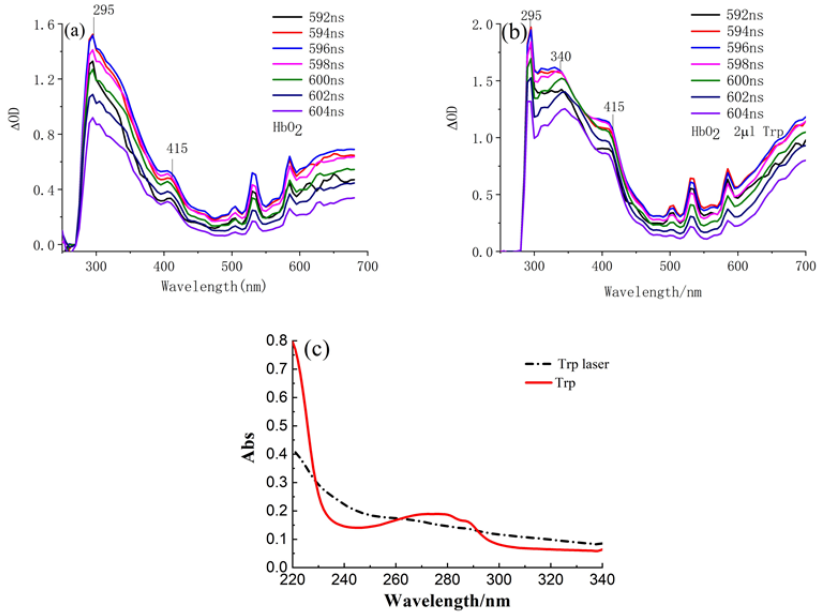

Fig. 5. Transient absorption spectra-without Trp (a) and with 2μL Trp(b) recorded at different time after 266 nm laser photolysis of HbO_2, and the UV absorbance spectra before and after 266 nm laser photolysis

Effect of pH on Photooxidation

The biological activity of enzymes and other biological macromolecules can be influenced and regulated by acid-base balance of groups close to the active site. Under the acidic condition, the histidine residue's structure of Hb was protonated and hydrogen bond transferred detected by Raman spectroscopy**[Error! Reference source not found.]**.At pH 9.0, HbO_2 was decomposed from tetramer into dimer - $(abcd)_2$, trimer - (abc) and monomer d, **Error! Reference source not found.** while the form of metHb was decomposed into trimer and monomer[**Error! Reference source not found.**]. Which indicated that a part of the subunits of hemoglobin will broke off from the subunits under the condition of given acid or alkali, that is, the quaternary structure changed. For this reason, we investigated the photooxidation of HbO_2 in buffer solutions with pH 5.0, 6.0, 7.4 and 8.0. After HbO_2 was incubated in the dark for 25 min, the characteristic absorption peak intensity did not change at pH 6.0, 7.4 and 8.0, while at pH 5.0, the characteristic absorption peak intensity of HbO_2 only slightly decreased, but the characteristic peak did not disappear and new peak did not be form, which indicated that pH had little influence on the UV-visible absorption spectra of HbO_2 in the dark. Fig.**6a** and **6b** showed the absorption peak areas of HbO_2 protein at 540 and 575 nm with light

exposure time under irradiation of 280 nm xenon lamp at different pH values. The absorption peak area both 540 and 575 nm decreased after irradiation for 25 min , and the decrease was greater under the slightly acidic condition, that is, the higher the H^+ concentration in the solution, the greater the change of absorption peak area at 540 and 575 nm, the easier the oxidation reaction would occur. The Soret band shifted blue after irradiation, and the degree of blue shift was greater under acidic conditions. At pH 5.0 and 6.0, the Soret band finally moved blue to 406 nm, and at pH 7.4 and 8.0, the final state of complete oxidation did not reach. The final stage of HbO_2 oxidation required the addition of oxidant $K_3[Fe(CN)_6]$ before it was completely converted to metHb. Which showed that HbO_2 was easy to be oxidized by irradiation under acidic conditions, and H^+ played a role as a catalyst in promoting nucleophilic attack of water molecules, which was consistent with Liu Peipei's conclusion that higher concentration of hydrogen ions was beneficial to hemoglobin oxygen release by Raman spectroscopy[**Error! Reference source not found.**].

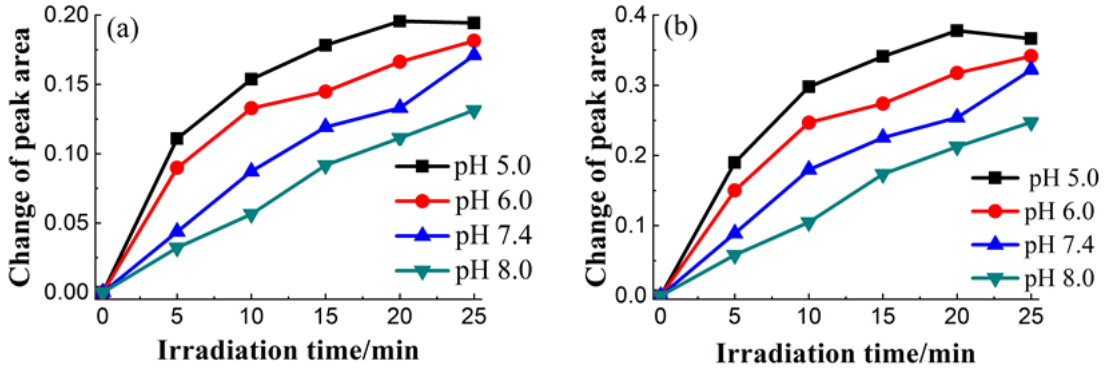

Fig. 6. The peak area changes curves of HbO_2 irradiated by xenon lamp for 30 min at different pH(a) Area changes at 540 nm; (b) Area changes at 575 nm

Fluorescence Spectra of HbO_2

The fluorescence of protein was contributed by tryptophan residue (Trp) and tyrosine residue (Tyr). There were 6 Trp and 12 Tyr residues in hemoglobin molecules, so Hb had endogenous fluorescence[**Error! Reference source not found.**].Therefore, measuring the change of fluorescence intensity of hemoglobin before and after illumination could reflect the change of Hb tertiary structure. Fig.**7** showed that HbO_2 had maximum fluorescence emission peaks at 330 nm excited by 296 nm. The light with 296 nm wavelength mainly excited Trp residues, and the intensity and position of the generated fluorescence was related to the changes of Trp residues and their surrounding environment. The Trp residues embedded in proteins had the maximum emission peak between 326 and 332 nm. The position of the 330 nm characteristic absorption peak didn't change during the irradiation, indicating that the microenvironment and hydrophobicity of HbO_2 didn't change significantly after 30 min of illumination. After irradiation, the fluorescence intensity increased from 1924 to 2068, possibly due to the change in the relative position or distance between heme and Trp, resulting in an increase in energy transfer efficiency[**Error! Reference source not found.**].

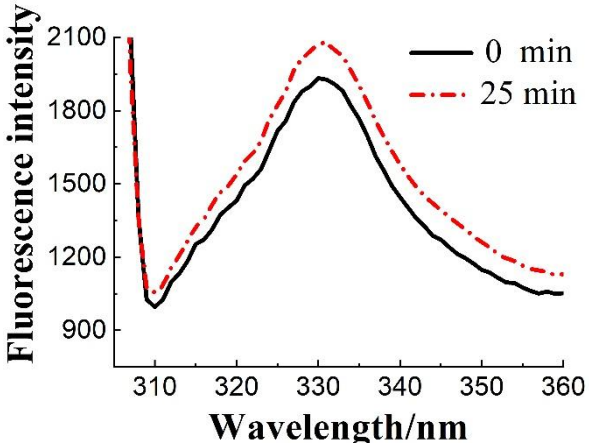

Fig. 7. The emission spectrum of HbO_2 at 296 nm

The CD spectra of HbO_2 oxidizad by irradiation

Circular dichroism spectroscopy (CD) plays an important role in the determination of protein structure[**Error! Reference source not found.Error! Reference source not found.**].It has been widely used in the study of protein conformational changes in solution, and can quickly and effectively determine the structural information of protein molecules.

The secondary structure change of the protein was studied by the CD spectra of HbO_2 before and after UV irradiation in pH 7.4, 0.05 mol·L^{-1} phosphate buffer (Fig.**8**). The CD signal recorded two negative values: 208 nm and 222 nm. In the far ultraviolet region (190-250 nm), the position and peak intensity of the characteristic peak were basically unchanged. It was known that an increase in α -helix content indicated a more stable protein structure, while an increase in β - folding content indicated an unstable protein structure in the secondary structural units of proteins. According to the secondary structure analysis software of CD spectrometer, secondary structure of the protein was still dominated by α -helix after light oxidation, and the protein was not denatured.

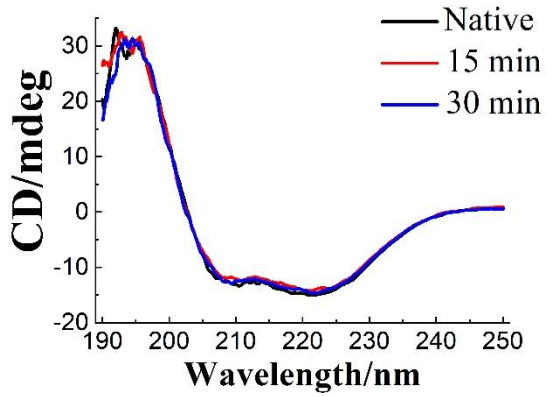

Fig. 8. The CD spectra of HbO_2 after irradiation with 280 nm

Photoinduced oxidation mechanism of HbO_2

The outer electronic structures of Fe (III) and Fe (II) are d5 and d6, respectively. No light, Fe (II) electron configuration was in a strong field and low spin, and the sixth coordination of HbO_2 was occupied by O_2. Light made iron porphyrin to excited state, the energy of coordination bond between Fe and O increased, coordination ability weakened, and oxygen was in a state of leaving. After the addition of free Trp, at first, the laser excited tryptophan, and the photoreaction starts from Trp, the excited state or free radical form of Trp returned to the electron ground state through a radiation-free relaxation channel, and the excited energy was transferred to iron porphyrin, resulting in intermolecular energy transfer. The high energy transfer efficiency between Trp donor and heme receptor contributed to the internal electron transfer process of HbO_2 iron porphyrin. When pH was acidic, the electrons of hexacoordinated Fe (II) transferred to porphyrin to form porphyrin free radicals, and Fe (II) was oxidized to Fe (III) (Fig. 9). When pH was neutral or alkaline, the oxidation rate was slower than acid. It showed that H+ played a role as a catalyst in promoting the nucleophilic attack of water molecules. In the course of the oxidation of heme iron of protein excited by light, 406 nm and 430 nm were characteristic absorption peaks of porphyrin ring in Soret band, but the oxidation degree of HbO_2 irradiated by light wasn't as good as 254 nm and 280 nm, indicating that the lower absorption energy of porphyrin was difficult to oxidize Fe (II). Trp has the highest molar extinction coefficient in the ultraviolet region, so irradiation of 254 nm and 280 nm wavelength was easily absorbed by colored amino acids such as tryptophan and then energy was transferred to iron porphyrin, thus initiating photooxidation process. The fluorescence spectra showed that irradiation caused the fluorescence intensity of amino acids to increase, that is, the tertiary structure changed, and the reason for the change of the tertiary structure was that the relative position or distance between heme and Trp, resulting in an increase in energy transfer efficiency between them. The CD spectra showed that the secondary structure of HbO_2 was still dominated by α -helix with the increase of irradiation time, so protein didn't denature for short-term illumination.

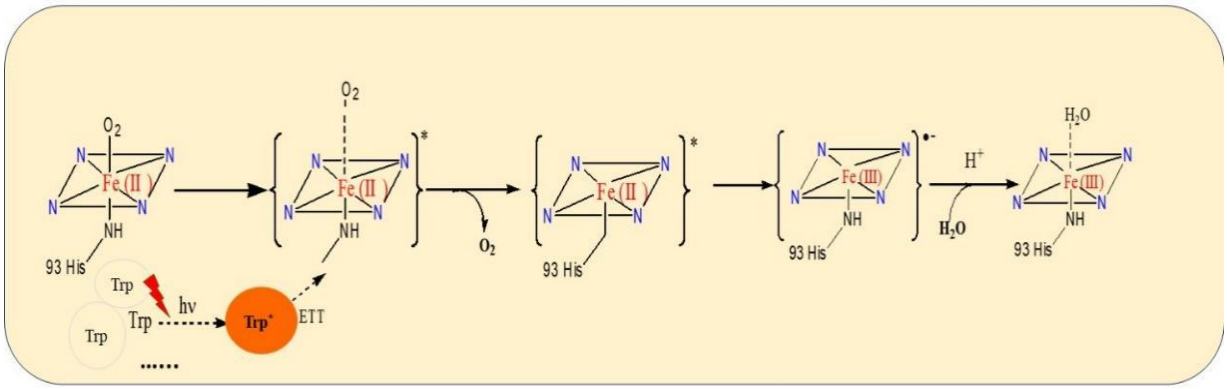

Fig. 9. Photo-induced reaction mechanism of HbO_2

From the above spectra and data results, it can be seen that HbO_2 iron porphyrin spectra has changed significantly before and after irradiation. The photooxidation mechanism of HbO_2 was photoinduced intermolecular electron and energy transfer.

IV. Conclusion

The photooxidation reaction process of HbO_2 has been studied by various spectral techniques. We found that Fe(Ⅱ) in HbO_2 could be oxidized to Fe(Ⅲ). Under the irradiation of 280 nm light, Trp absorbed energy to excite state and the energy was transmitted to porphyrin, which in turn excited iron porphyrin to carry out photooxidation process. 406 and 430 nm weren't characteristic absorption peaks of Trp, so the effect of photooxidation wasn't obvious. After the addition of free Trp, the ΔOD value of HbO_2 increased, and the energy transfer between Trp and HbO_2 increased the energy of excited HbO_2, making it easy to carry out photooxidation reaction. Light energy was absorbed by tryptophan, and the energy transfer process between iron porphyrin of heme and amino acid molecules was helpful for photooxidation reaction. This reaction belongs to Ligand-to-Metal Charge Transfer (LMCT) Photochemistry. The pH was favorable for the photooxidation of HbO_2 under certain acidic conditions. The secondary and tertiary structures of HbO_2 didn't significantly were changed under the irradiation of ultraviolet light by fluorescence and CD spectra. The above results will provide a basis for the study of the structure and function of biomolecules under the induction of light.

Acknowledgements

This work is financially supported by the National Natural Science Foundation of China (NO. 21571025，No.21601024， No.21601025), the subject construction project-the interdisciplinary project of Dalian University (DLUXK-2023-YB-007), the project of Liaoning Province Education Department (JYTMS20230380).

VI.Declaration of Conflicting Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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