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**Abstract.** Hemoglobin, one of the most important proteins in the human body, is composed of “heme” groups (iron-containing rings) and “globins” (proteins). We investigate the two-photon excited fluorescence of hemoglobin and its subunit components (heme and globin). We measure the hemoglobin fluorescence lifetime by using a streak camera of ps resolution and confirm that its lifetime is in femtosecond scale. In the study of the fluorescence properties of heme and globin, the experimental results reveal that heme is the sole fluorophore of hemoglobin. Hemoglobin fluorescence can be effectively excited only via two-photon process, because heme has a centrosymmetric molecular structure and two-photon allowed transition is forbidden for single-photon process and vice versa due to the Laporte parity selection rule. © 2015 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.20.10.105014]

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## 1 Introduction

Hemoglobin primarily serves to carry oxygen in red blood cells. In addition, it transports other important gases, such as respiratory carbon dioxide and the regulatory molecule nitric oxide in red blood cells, and functions as an antioxidant and/or a regulator of iron metabolism in other cells, including dopaminergic neurons, macrophages, alveolar cells, and mesangial cells.<sup>1,2</sup> A hemoglobin molecule has a quaternary structure characteristic of four protein subunits. Each of them is composed of a globular protein, called globin, tightly associated with a heme B [iron(II) protoporphyrin IX] group as a coenzyme. Here, heme B is the most common type of heme.

For a long time, hemoglobin was considered a nonfluorescent chromophore because it was believed that nonradiative decay dominated excited-state relaxation.<sup>3</sup> Recently, we discovered strong fluorescence emission from hemoglobin via short-wavelength two-photon excitation.<sup>4</sup> In our previous study, we found that two-photon excited fluorescence (TPEF) of hemoglobin peaked at 438 nm, and the fluorescence lifetime was extremely short so that the measured fluorescence decay curve was identical to the system response of our time-correlated single-photon counting (TCSPC) detection system.<sup>4</sup> We also measured the two-photon action cross sections of hemoglobin under excitation wavelengths from 550 to 800 nm and found that the shorter wavelength provides significantly higher excitation efficiency.<sup>5</sup> *In vivo* high-resolution imaging of red blood cells and microvasculature was achieved by using two-photon excited hemoglobin fluorescence signals.<sup>4–6</sup> The results demonstrated the potential applications of hemoglobin

fluorescence in the study of microvascular diseases, organ complications (retinopathy, neuropathy, and nephropathy), early cancer progression (neoangiogenesis in connective tissue under the epithelium), and neuroscience research (brain function and diseases). However, the mechanism of two-photon excited hemoglobin fluorescence is still not clear. A better understanding of the mechanism will give significant insight into the molecular structure and electronic energy state of hemoglobin. The mechanism of hemoglobin fluorescence may also inspire the design of a new fluorescence probe with an extremely short fluorescence lifetime, so that the new fluorescence probe can be readily distinguished from other fluorophores. This kind of probe may lead to further applications in life science research. In this work, we first study hemoglobin fluorescence decay with high temporal resolution using a streak camera. Then we characterize the fluorescence properties of heme and globin to identify the fluorophore of the hemoglobin molecule. Finally, we explore the mechanism of two-photon excited hemoglobin fluorescence and explain why its fluorescence was not observed under single-photon excitation.

## 2 Materials and Methods

### 2.1 Sample Preparation

Hemoglobin samples were extracted from the fresh blood of the New Zealand white rabbit, as described in the previous study.<sup>4</sup> To prepare the heme sample, hemin [iron(III) protoporphyrin IX] was purchased from Sigma-Aldrich (51280) and heme was reduced from the hemin by sodium dithionite.<sup>7</sup> The

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preparation of the heme and hemin solutions is critically important for this study because both heme and hemin can aggregate into dimer or even polymer in the aqueous solution, whereas they remain as monomer in the organic solvent. We chose ethylene glycol as the organic solvent because it resists sodium dithionite, and 70% (v/v) ethylene glycol can keep heme or hemin in the form of monomer.<sup>7,8</sup> In detail, the aqueous and organic solutions of heme and hemin were made with double-distilled water and 70% (v/v) ethylene glycol as solvent, respectively. Because heme belongs to the group of porphyrin complexes, the absorption spectrum of a typical porphyrin complex consists of a strong transition to the second singlet-excited state ( $S_0$  to  $S_2$ ) at about 400 nm (the Soret band) and a weak transition to the first singlet-excited state ( $S_0$  to  $S_1$ ) at about 550 nm (the Q-band).<sup>9,10</sup> The Soret- and Q-band absorptions both arise from  $\pi - \pi^*$  transitions.<sup>9,10</sup> The experimentally measured absorption spectra of the heme and hemin monomer samples are shown in Fig. 1(a). The red shift of the Soret band (from 400 to 418 nm) and the appearance of the Q band in the absorption of the heme sample indicated that heme had been successfully reduced from hemin. The globin was isolated from methemoglobin (H2500, Sigma-Aldrich) following the standard methyl ethyl ketone extraction method.<sup>11,12</sup> The ratio of Soret-band absorption of hemin at 405 nm over the protein absorption of globin at 280 nm was used to determine the purity of the extracted globin sample.<sup>12</sup> The ratio was kept below 0.5, meaning that the residual hemin concentration in the extracted globin sample was not over 5%.<sup>12</sup> In this study, the absorbance of all sample solutions was measured by a commercial absorption spectrometer (Ultrospec 4300pro UV/Visible Spectrophotometer, GE Healthcare) to determine the concentration of the substances in the solutions. In the measurements of two-photon excited fluorescence from the samples, we set the focal point of the excitation light a few microns below the sample surface to guarantee the measured samples were optically thin and to avoid the possible reabsorption of the fluorescence signal.

## 2.2 Experimental Setup

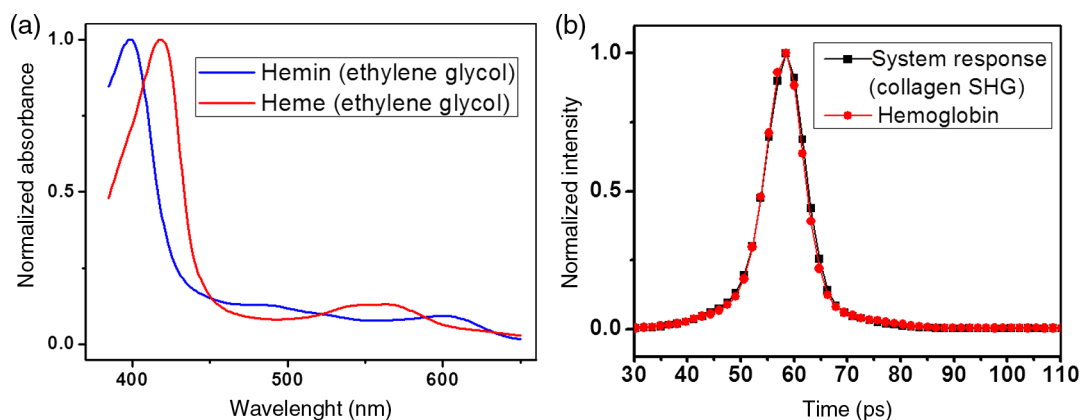
The two-photon system for the measurement of hemoglobin fluorescence lifetime was identical to that used in our previous study, except the TCSPC system with  $\sim 230$  ps system response

was replaced with an advanced streak camera of ps temporal resolution (C5680, Hamamatsu).<sup>4</sup> The 5-mW and 720-nm excitation was from a Ti:sapphire femtosecond laser (Mira, Coherent), and the fluorescence signal was coupled to the spectroscopy of the streak camera. The spectral range of the system was set from 422 to 470 nm, covering the fluorescence emission from the hemoglobin. The slit width of the spectroscopy was set to 100  $\mu\text{m}$ , and the microchannel plate gain of the streak camera was set to 32. The system response of the streak camera detection system was calibrated by the second-harmonic generation (SHG) of collagen (C9879, Sigma-Aldrich), and the integration time was 3 min to ensure good signal-to-noise ratio.

The details of the wavelength- and time-resolved TPEF imaging system for the study of TPEF signals of heme and globin in the excitation wavelengths from 550 to 750 nm were described in Ref. 5. Briefly, excitation ranging from 700 to 750 nm was provided directly by a Ti:sapphire femtosecond laser (Chameleon Ultra II, Coherent), and excitation from 550 to 650 nm was produced by the SHG of a Ti:sapphire-pumped optical parametric oscillator (Chameleon OPO, Coherent). A pair of galvo mirrors (6210H, Cambridge Technology) was used for  $x$ - $y$  lateral scanning, and a water immersion objective (UAPO40XW 340, 1.15NA, Olympus) focused the light into the sample. The laser beam was scanned over an area in the sample, typically  $90 \times 90 \mu\text{m}$ . This reduced the dwelling time over each spot on the sample and avoided possible photodamage. The excitation and emission light were separated by a 510-nm dichroic mirror (FF510-Di01, Semrock). The emission light was further filtered by two short-pass filters (SP01-532RU and SP01-633RS, Semrock) and coupled in a spectrograph (ranging from 312 to 507 nm) equipped with a multichannel TCSPC module (PML-16-3-C and SPC-150, Becker and Hickl). At each excitation wavelength, the pulse duration was measured by an autocorrelator (Pulsecheck-50, APE GmbH), and the average power was measured by a power meter (Model 2832-C, Newport).

## 3 Results and Discussions

We first study the hemoglobin fluorescence decay with high temporal resolution using a streak camera. The measured system response and fluorescence decay curve from the hemoglobin



**Fig. 1** Absorption spectra and two-photon excited fluorescence (TPEF) decay curve. (a) Normalized absorption spectra of heme and hemin in organic solvent. (b) TPEF decay of hemoglobin measured by streak camera and the system response.

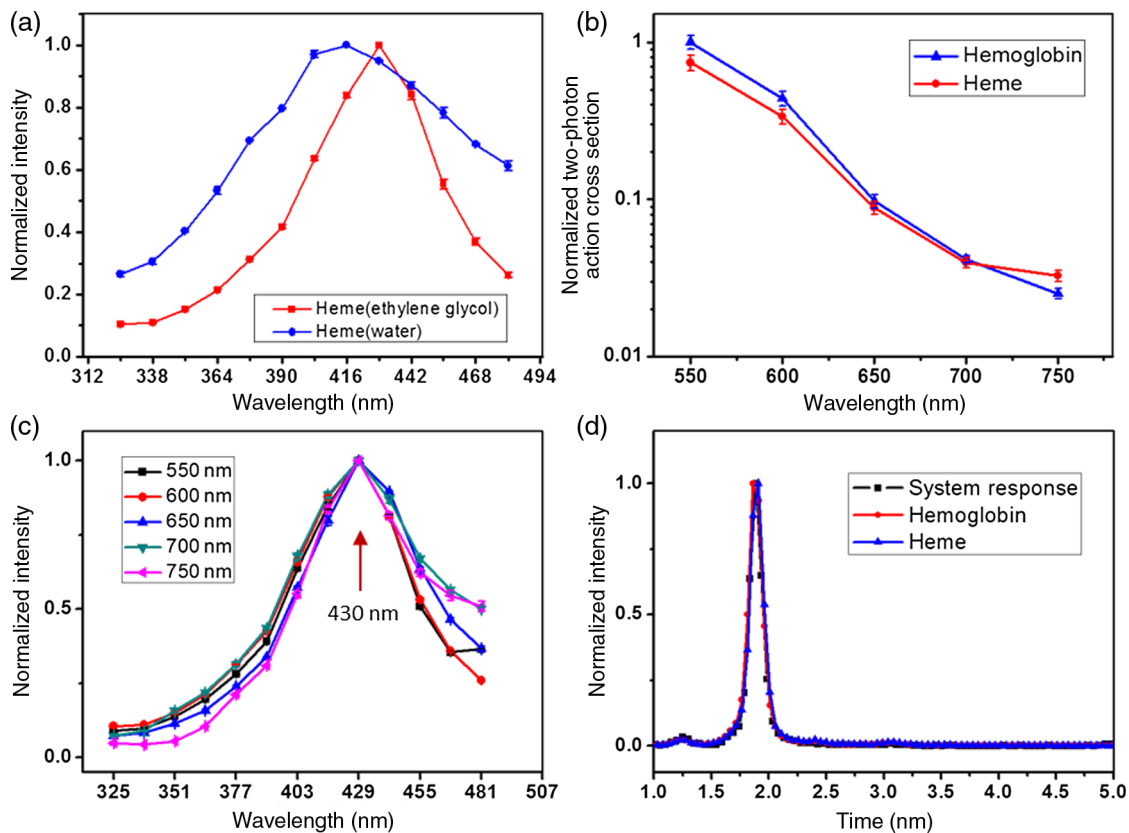
solution of concentration 2.5 mM on a heme basis are shown in Fig. 1(b). It was found that although the temporal resolution of the streak camera was significantly higher than the previously used TCSPC system, the measured hemoglobin fluorescence decay curve was still identical to the system response, indicating that the hemoglobin fluorescence lifetime must be in femtosecond scale. The result is consistent with previous work reporting that cytochrome c with heme C, similar to hemoglobin with heme B, produced a lifetime in femtosecond scale.<sup>13–15</sup> Because the decay of the hemoglobin fluorescence was far beyond the resolutions of both the streak camera and the TCSPC system, we conducted the rest of the experiments with the TCSPC system, which had much higher sensitivity to the fluorescence signals.

To identify the fluorophore of the hemoglobin molecule, we characterized the fluorescence properties of heme using a wavelength- and time-resolved TPEF imaging system. We first studied the TPEF signals of heme in the water and ethylene glycol solutions, corresponding to heme in dimer and monomer forms, respectively. The TPEF spectra excited at 600 nm are shown in Fig. 2(a). It was found that the fluorescence band of the heme monomer is significantly sharper than the heme dimer, and the fluorescence excitation efficiency of the monomer is over six times higher than the dimer. The results demonstrate that monomer is an essential form of heme to emit fluorescence efficiently. In hemoglobin, heme is enclosed by globin, meaning that heme is in the monomer form. Next, we measured the TPEF properties of the heme monomer in organic solvent, and the results are

summarized in Figs. 2(b)–2(d). It was found that the TPEF intensity of the heme monomer is quadratically dependent on the excitation power, confirming that the fluorescence is generated via the two-photon excitation process. The two-photon action cross sections of the heme monomer at different excitation wavelengths are shown in Fig. 2(b). The expression for calculating the two-photon action cross section of a fluorophore is<sup>4,5,16</sup>

$$\eta(\lambda_{\text{ex}}) = \frac{\epsilon I_f \tau_p \lambda_{\text{ex}}^2}{P_{\text{ave}}^2}, \quad (1)$$

where  $\lambda_{\text{ex}}$ ,  $I_f$ ,  $\tau_p$ ,  $P_{\text{ave}}$ , and  $\epsilon$  are the excitation wavelength, measured fluorescence intensity, excitation pulse duration, average excitation power, and proportional constant, respectively. As can be seen, the two-photon action cross sections of heme and hemoglobin at equivalent concentrations of 1.5 mM on a heme basis exhibit a similar dependence on the excitation wavelength. In particular, the short wavelength provides significantly higher excitation efficiency. As shown in Fig. 2(c), the TPEF spectra of the heme monomer under different excitation wavelengths all peaked at  $\sim 430$  nm regardless of the excitation wavelengths, indicating that the 430-nm-peaked TPEF emission could correspond to the strong Soret-band absorption of heme monomer at  $\sim 418$  nm. Similarly to the well-studied metalloporphyrins of Soret fluorescence emissions, though the Soret emission from second-excited singlet state  $S_2$  seems to violate Kasha's rule, it becomes possible in a porphyrin system



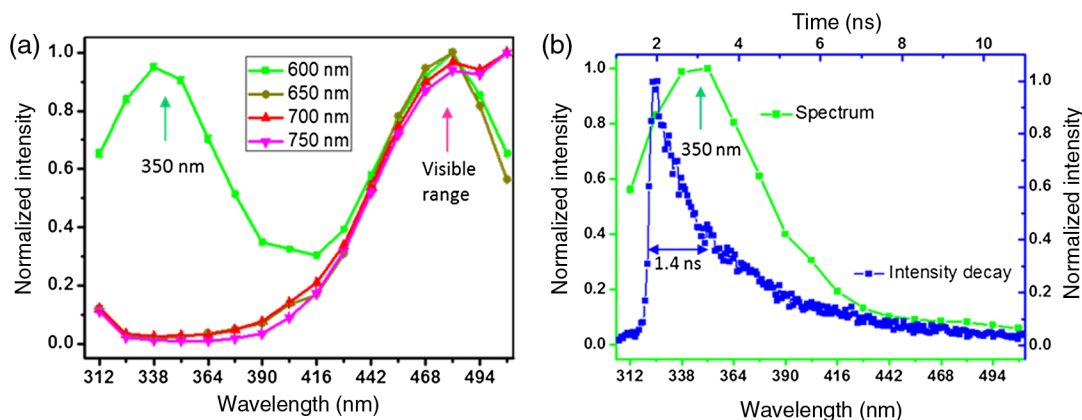
**Fig. 2** TPEF properties of heme. (a) TPEF spectra of heme monomer and dimer under 600-nm excitation; (b) normalized two-photon action cross sections of heme and hemoglobin at the same concentration; (c) TPEF emission spectra of heme under excitation from 550 to 750 nm; (d) TPEF decay curves of heme and hemoglobin under 600-nm excitation.

due to the small Franck–Condon factor for  $S_2 - S_1$  internal conversion.<sup>17,18</sup> The TPEF decays of heme and hemoglobin at a representative excitation wavelength (600 nm) are displayed in Fig. 2(d). It was not a surprise that the fluorescence decay curve of the heme monomer is the same as the hemoglobin fluorescence decay and identical to the system response ( $\sim 150$  ps FWHM).

In the study of the fluorescence properties of globin, we measured the fluorescence spectra of globin extracted from Sigma methemoglobin. The results for the excitation from 600 to 750 nm are shown in Fig. 3(a). Since globin contains tryptophan, the TPEF signal of globin exhibits strong tryptophan fluorescence peaked at  $\sim 350$  nm, similar to other proteins under 600-nm excitation.<sup>19</sup> In addition, strong fluorescence peaked in the visible wavelength range (longer than 400 nm) was observed under all the excitation wavelengths. However, this visible wavelength fluorescence signal has never been reported in any study of natural globin fluorescence. To confirm whether the visible wavelength fluorescence was truly from natural globin, we followed the standard protocol to produce methemoglobin by adding potassium ferricyanide into a freshly prepared hemoglobin solution.<sup>20</sup> The globin was then extracted from our homemade methemoglobin. The TPEF signals of the globin extracted from the homemade methemoglobin are shown in Fig. 3(b). It was found that the globin emitted strong tryptophan fluorescence only at 600-nm excitation, whereas no visible wavelength fluorescence was observed under the excitation from 600 to 750 nm. The discrepancy in TPEF spectral characteristics between the globins extracted from Sigma and homemade methemoglobin samples may result from the oxidation of proteins (here, Sigma methemoglobin) after long-term exposure to oxidative stress.<sup>21</sup> Therefore, the globin extracted from the homemade methemoglobin was used in the following study of globin TPEF properties. We confirmed that the tryptophan fluorescence intensity of globin is quadratically dependent on the power of 600-nm excitation, showing that fluorescence is from a two-photon excitation process. The TPEF decay of globin from the homemade methemoglobin is shown in Fig. 3(b). The lifetime of tryptophan fluorescence is  $\sim 1.4$  ns, much longer than the TPEF lifetimes of heme and hemoglobin. Based on the experimental evidence, we conclude that globin emits tryptophan fluorescence that is not observed in hemoglobin fluorescence only because of quenching from the heme in

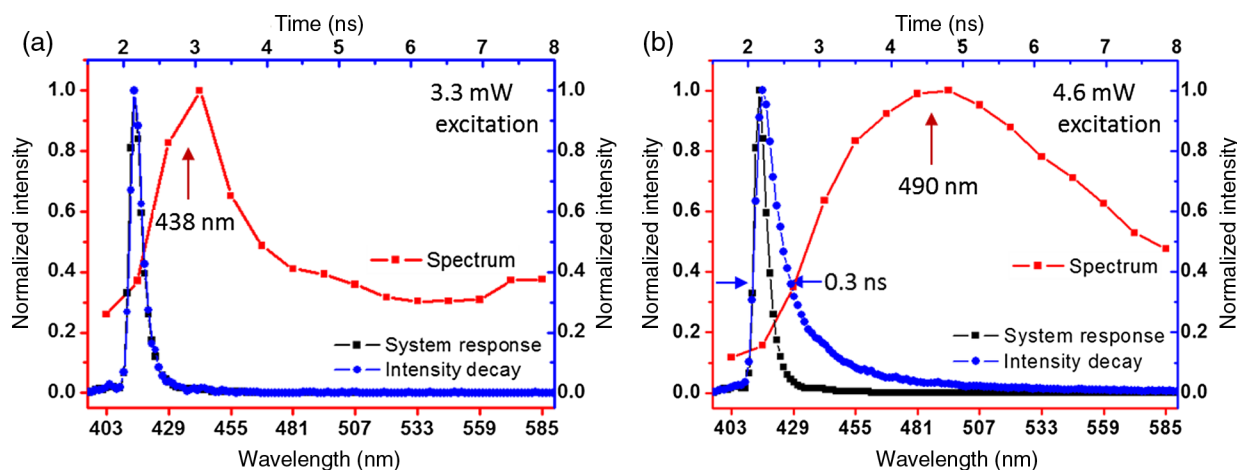
hemoglobin.<sup>22</sup> Therefore, heme is identified as the sole fluorophore of hemoglobin, and it emits fluorescence efficiently only as monomer.

It has been reported that new fluorophores could be generated from nonfluorescent molecules via photodamage caused by tightly focused excitation light.<sup>23,24</sup> To address whether photodamage plays a role in TPEF emission from hemoglobin, we examined its photostability experimentally under a variety of excitation conditions. In detail, hemoglobin solution was sandwiched between microscope cover glasses. A 740-nm femtosecond laser was focused at a fixed point in the solution, and TPEF signals were recorded continuously. It was found that when the excitation power at the sample was not over 3.3 mW, the spectral and temporal characteristics of TPEF signals remained unchanged over 5 min of continuous exposure to excitation laser, as shown in Fig. 4(a). This is identical to our previous findings.<sup>4</sup> When the excitation power at the sample increased to 4.6 mW, the TPEF signals were the same as 3.3-mW excitation when exposure time was within a time period of  $13 \pm 8$  s ( $n = 9$ ). However, after this period of time, the TPEF signal intensity increased by over a factor of 10, and the fluorescence spectrum and decay curve become completely different, as shown in Fig. 4(b), indicating a change of molecular structure or photodamage. Specifically, the TPEF spectrum become broader and the peak red-shifted to  $\sim 490$  nm from 438 nm. The TPEF lifetime was  $\sim 0.3$  ns. We found that the change of the TPEF signal from the photodamaged hemoglobin is permanent, but the mechanism of its TPEF emission is unknown. Though it has been reported that heme could be degraded into fluorescent yellow pigment in unstable hemoglobin,<sup>25</sup> further investigations are required to verify whether the dramatic changes of fluorescence properties in photodamaged hemoglobin originate from the heme degradation product. The detailed dynamics of photodamage can be seen in the movie made with continuous recording of TPEF spectra and decay curves over 30 s (see Video 1). It was found that photodamage occurs in a shorter period of time with an increase in excitation power. When the excitation power at the sample was over 6.5 mW, the TPEF characteristics changed from the Soret-like signals shown in Fig. 4(a) to the photodamaged ones shown in Fig. 4(b) within 2 s. Overall, the results demonstrate that the Soret-like TPEF signal is intrinsic to hemoglobin and not caused by any form of photodamage.



**Fig. 3** TPEF properties of globin. (a) TPEF spectra of globin extracted from Sigma methemoglobin under excitation from 600 to 750 nm; (b) TPEF spectra and decay curve of the globin extracted from homemade methemoglobin under 600-nm excitation.





**Fig. 4** TPEF characteristics of hemoglobin. (a) TPEF spectra and decay curve of hemoglobin under 3.3-mW excitation without photodamage. (b) TPEF spectra and decay curve of hemoglobin under 4.6-mW excitation after photodamage. (Video 1, MPEG, 8.5 MB) [URL: <http://dx.doi.org/10.1117/1.JBO.20.10.105014.1>].

It is well known that neither heme nor hemoglobin emits detectable fluorescence under single-photon excitation, because when heme is excited, the ultrafast charge transfer from the porphyrin ring to the unfilled  $d$  orbital of Fe quenches its fluorescence emission.<sup>26,27</sup> Obviously, the theory does not explain the strong Soret fluorescence emission from heme and hemoglobin via two-photon excitation. The discrepancy must originate from the unique molecular structure of heme. Because porphyrin dianion belongs to the  $D_{4h}$  point group, it has an inversion center and is centrosymmetric. Its electronic states can be cataloged into groups of gerade ( $g = \text{even}$ ) and ungerade ( $u = \text{odd}$ ), respectively. Although the porphyrin with conjugating peripheral substituents, as in the case of heme, may not strictly belong to the  $D_{4h}$  point group, the electronic states retain a large amount of “ $D_{4h}$ -like” character.<sup>9</sup> According to the Laporte parity-selection rule, electronic transitions of centrosymmetric molecules with conservation of parity are forbidden. For a centrosymmetric molecule, the  $g-g$  and  $u-u$  electronic transitions are not allowed in the single-photon excitation process. However, the  $g-g$  and  $u-u$  transitions are allowed when a centrosymmetric molecule absorbs two photons simultaneously, indicating that the single- and two-photon absorption spectra of centrosymmetric molecules could be significantly different. This difference has been reported in the previous study of tetrapyrrolic compounds of centrosymmetry.<sup>28</sup> The quantum-chemical calculations of the porphyrin system revealed that its Soret-band absorption attributed to the gerade ground state  $S_0$  to the ungerade  ${}^1E_u^*$  symmetry state  $S_2$  transition of  $\sim 3.1$  eV energy, much lower than the energy of the two-photon excitation of heme and hemoglobin from 550 to 750 nm, equivalent to 4.5–3.3 eV.<sup>9</sup> This indicates that heme must be excited from the gerade ground state to the higher-energy electronic state  $S_n$  ( $n > 2$ ), which is theoretically predicted to have a gerade  ${}^1A_{1g}^*$  symmetry, and this  $g-g$  transition is allowed only via two-photon process.<sup>9</sup> The experimental study also confirmed that porphyrin and its derivatives have strong two-photon allowed  $g-g$  transition from  $S_0$  to  $S_n$  ( $n > 2$ ) of energy higher than the single-photon-allowed  $g-u$  Soret-band transition.<sup>28</sup> More importantly, in the study of other metalloporphyrins, it has been experimentally demonstrated that the gerade-excited states  $S_n$  ( $n > 2$ ) produced by two-photon excitation have quantitatively different relaxation

dynamics from the ungerade states excited via single-photon process.<sup>27,29</sup> For example, in the study of single- and two-photon excited fluorescence from zinc tetraphenylporphyrin, it was found that a molecule excited to a high-energy electronic state  $S_n$  ( $n > 2$ ) via two-photon process almost exclusively relaxes to an electronic state designated  $S_2'$  in the Soret band.<sup>29</sup> The  $S_2'$  state is weakly coupled with the conventional second-excited singlet state  $S_2$ , and the characteristics of fluorescence emission from  $S_2'$  to  $S_0$  transition are significantly different from the conventional single-photon-excited Soret-band emission attributed to  $S_2$  to  $S_0$  transition.<sup>29</sup> The results suggest that a similar  $S_2'$  state could also exist in heme and be excited mainly via two-photon process. If the charge transfer from the  $S_2'$  to the unfilled  $d$  orbital of Fe is not as strong as the single-photon-excited  $S_2$  state, the Soret-band emission from  $S_2'$  to  $S_0$  transition could be detectable, though it has an extremely short lifetime due to ultrafast charge transfer.

## 4 Conclusion

In conclusion, we found that heme is the sole fluorophore of hemoglobin. The absorption and fluorescence properties of heme and hemoglobin are almost identical, providing evidence that the interactions between globin and heme subunits are not strong enough to cause the electronic states of hemoglobin to be different from heme.<sup>10</sup> The monomer form is critical for heme to efficiently emit fluorescence, and heme, as a coenzyme in biology, is usually surrounded by proteins, meaning that heme is in monomer form in its natural condition. The centrosymmetric structure of heme could result in the fact that hemoglobin fluorescence can only be efficiently excited via two-photon process. However, further investigations are required to understand the detailed excitation and relaxation dynamics.

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