Long Chain Fatty Acid Affects Excited State Branching in Bilirubin-Human Serum Protein Complex†

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After binding to human serum albumin, bilirubin could undergo photo-isomerization and photo-induced cyclization process. The latter process would result the formation of a product, named as lumirubin. These photo induced behaviors are the fundamental of clinical therapy for neonatal jaundice. Previous studies have reported that the addition of long chain fatty acids is beneficial to the generation of lumirubin, yet no kinetic study has revealed the mechanism behind. In this study, how palmitic acid affects the photochemical reaction process of bilirubin in Human serum albumin (HSA) is studied by using femtosecond transient absorption and fluorescence up-conversion techniques. With the addition of palmitic acid, the excited population of bilirubin prefers to return to its hot ground state (S₀) through a 4 ps decay channel rather than the intrinsic ultrafast decay pathways (<1 ps). This effect prompts the Z-Z to E-Z isomerization at the S₀ state and then further increases the production yield of lumirubin. This is the first time to characterize the promoting effect of long chain fatty acid in the process of phototherapy with femtosecond time resolution spectroscopy and the results can provide useful information to benefit the relevant clinical study.

Key words: Bilirubin, Human serum albumin, Femtosecond transient absorption, Excited state dynamics

I. INTRODUCTION

Bilirubin (BR) is a neurotoxic compound formed in mammals through the catabolism of heme. It can undergo photo-isomerization and photo-cyclization when exposures under light irradiation are key for clinical treatment of neonatal jaundice using blue light [1-3]. Binding with human serum albumin (HSA) can promote those photochemical processes, and BR-HSA complex is the most suitable model system for studying phototherapy in vitro [4]. The promotion effect for the photochemical reaction of BR by HSA may come from the inhibition of the molecular structure of BR due to its rigid protein environment, which eliminates the un-
trafast excited state deactivation pathways of BR in solutions [5–8]. Moreover, the distribution of amino acid residues at specific binding sites in HSA may limit the accessible BR configurations, which can also regulate its physical and chemical properties.

It has been reported that the conformation of HSA is different when pH, temperature, salt concentration, and fatty acid concentration are altered [9, 10]. There are six major binding domains in HSA for the transportation of various endogenous (e.g., bilirubin, heme/exogenous (e.g., drug) substances that participate in the physiological cycle in vivo [11], and HSA conformation has a great influence on its binding ability. Among them, BR is mainly bound to the subdomain IA and IIA (FIG. 1, structural model, red circle) with extremely high affinity, and secondary bound in heme pocket (subdomain IB) [12]. Under physiological environment (pH=7.4, 36.5 °C), the type and concentration of fatty acids are the main factors affecting the structure of HSA. Fatty acids with chain length of 10 or more carbon atoms (e.g., palmitic acid, with chain length of 16, use PA as abbreviation in the following text) were found to increase by as much as 3-fold the quantum yield for the formation of lumirubin [4]. However, this result is only statistically significant because no study has revealed the reaction mechanism behind.

As mentioned above, BR owns multiple ultrafast excited state relaxation pathways in solution [5–8, 13], and the first time-resolved spectroscopy study on BR-HSA complex was carried out in 2004. Utilizing the single-wavelength fluorescence up-conversion data, Zietz et al. made a simple identification of the excited state relaxation of BR-HSA complex but no information about photochemical reaction was mentioned [14]. Two recent
studies on model systems of bilirubin dipyrrinones have distinguished the characteristic spectra of each photoreaction product, providing a strong reference for the research [15, 16].

In this work, excited state dynamics of BR-HSA complex with different concentrations of PA were studied by using steady-state and femtosecond time-resolved spectroscopy techniques. While the steady-state spectra results suggest the photochemical quantum yield (QY) for BR-HSA complex increased by 83% after adding 4 mol equivalent PA, ultrafast spectroscopy data revealed that the addition of PA branches excited state dynamics of BR to a 4 ps decay pathway rather than altering the excited state lifetimes. To the best of our knowledge, this is the first time to reveal the affection of fatty acid on the excited state dynamics of BR-HSA complex and our results could provide a strong theoretical basis for relevant clinical medical research on phototherapy of jaundice [17, 18].

II. EXPERIMENTS

A. Sample preparation

HSA (Sigma-Aldrich, essentially fatty acid free) was dissolved in phosphate buffer (0.1 mol/L, pH=7.4) as an original solution with high concentration solute. PA (Sigma-Aldrich) was dissolved in methanol containing equimolar NaOH and aliquots were dried under nitrogen to a film on glass tubes [4]. PA was added into the HSA solutions and mixed by either vortexing or stirring until optically clear. One control group and three experimental groups (three concentrations of PA were studied: 1, 2, and 4 equivalents per mole of albumin) were settled based on a previous research result [4]. BR (Sigma-Aldrich) was dissolved in NaOH solution (0.01 mol/L) first and then added into HSA-PA solutions. Equivalent HCl solution (0.01 mol/L) was added to neutralize the solutions. Samples with 60 μmol/L BR and 80 μmol/L HSA were used in most of experiments for this work, and samples were diluted 5 times to avoid the internal filtration and self-absorption effect for steady-state fluorescence test.

All samples were strictly excreting oxygen and protected from light before spectroscopy measurements.

B. Experimental techniques

An UV-Vis spectrophotometer (TU1901, Beijing Purkinje General Instrument Co. Ltd.) and a FluoroMax-4 spectrofluorometer (Horiba, Jobin Yvon) were used to record steady-state absorption spectra and fluorescence spectra, respectively.

Femtosecond transient absorption (TA) spectra with spectral range from 300 nm to 850 nm were acquired on a TA spectrometer (Helios-EOS Fire, Ultrafast Systems) with a 1 kHz Ti:sapphire amplifier (Astrella, Coherent Inc.) [19]. Samples were held in a 2 mm fused silica cuvette. The instrument response function was determined to be ~120 fs by measuring solvent responses under the same experimental conditions. All measurements were performed at room temperature.

Femtosecond time-resolved fluorescence decay curves were collected by a home-built fluorescence up-conversion setup which has been reported previously [20]. In brief, the pump pulse was generated by an optical parametric amplifier (OPerA Solo, Coherent Inc.) and spectrally filtered by a pair of UV fused-silica prisms. The gate pulse was a small portion of an 800 nm fundamental beam. Fluorescence was collected by a pair of parabolic focus mirrors and then focused into a 0.5 mm Beta-BaB_{2}O_{4} (BBO) crystal, which was used for sum frequency with the 800 nm gate pulse. Up-conversion signals were collected by a monochromator (Omnik500, Zolix) and detected by a photomultiplier tube (CR317, Hamamatsu). Time resolution was determined to be ~400 fs.

III. RESULTS

A. Steady-state spectroscopy

Steady-state absorption and emission spectra of BR-HSA complexes with different molar equivalent PA are shown in FIG. 1(a). Absorption band with a peak around 280 nm belongs to tryptophan in HSA and the other one ranges from 350 nm to 550 nm belongs to BR. A shoulder band at around 425 nm is clearly observed in PA free BR-HSA complex and this peak shows a red-shift to 470 nm with the addition of PA. An obvious isosbestic point is found at 438 nm, indicating the transformation of the intensity ratio of the two transition oscillators in this system. With the addition of PA, the lower energy transition vibration mode with the increasing proportion seems to have a higher fluorescence emission quantum yield. With the excitation at 500 nm, a series of emission spectra peaked at 540 nm with varying intensity but uniform shape are observed for BR-HSA complexes.

Illumination treatment tests were carried out to show
the effect of PA on the photochemical reaction quantum yield of BR-HSA complexes. The changes of absorption spectra of the four samples at different illuminate times are displayed in FIG. S1 in Supplementary materials. The ratio of the maximum absorption value at time $t$ to the value before illumination is used as the ordinate to eliminate the extinction coefficient and directly show the change of the amount of substance after illumination (FIG. 1(b)). Clearly, the purple line (BR-HSA combined with 4 equivalent PA) shows the highest photochemical QY and it has an 83% increasing compared with that in the BR-HSA complex one without PA (details in Supplementary materials).

The addition of PA changes relative oscillator strengths of two major transition modes in the complexes. Excitation wavelength dependent fluorescence emission spectra were measured to verify this ground state modulation. The 3-D fluorescence excitation-emission spectra are given in FIG. S2 (Supplementary materials). With two dash lines serving as a guide to eyes, it can be concluded that the spectra of BR-HSA complex with 4 mol equivalent PA have the greatest distortion. Representative spectra are normalized and displayed in FIG. 2. The isoemissive point can be observed both in FIG. 2 (a) and (b), indicating that there are two emissive species in BR-HSA complex solutions. The larger emission red shift of the BR-HSA complex with 4 mol PA indicates that the proportion of long-wavelength emission species is increased after the addition of PA.

### B. Time-resolved spectroscopy

Femtosecond TA spectra were recorded to investigate the effect of PA addition on the excited state dynamics of BR-HSA complexes (FIG. 3(a–d)). Three positive (with peaks at 390, 518, and 800 nm) and two negative bands (with peaks at 460 and 565 nm) are observed in all samples. The 390 and 800 nm bands could naturally be identified to the excited state absorption (ESA) signals of initial excited states as they show up in the initial decay. The decay-associated spectra (DAS) from global fitting and the corresponding evolution-associated difference spectra (EADS) are given in FIG. S3 in Supplementary materials and FIG. 3(e–h), respectively. Four components with lifetime less than 1 ps (red), $\sim$4 ps (yellow), $\sim$30 ps (cyan) and 0.2 ns (purple) were determined for all four samples. Clear blue shift from red to cyan curve is observed and it is shown in FIG. 3 (e) and (f), while no shift can be found in FIG. 3(h). Another difference is observed for the 518 nm band. The amplitude of this band is increasing in samples with 0 and 1 PA (yellow EADS to red EADS in FIG. 3(e–f)), but this spectra evolution is invisible in sample with 4 PA (FIG. 3(h)). More interestingly, at the end of the spectral evolution, the strength of the purple component with two new ESA bands at 425 nm and 640 nm is observable in FIG. 3(e–f), but is extremely weak in FIG. 3(h). For the negative bands in the TA spectra, the 460 nm band stands for ground state bleach (GSB) and the 565 nm band is originated from stimulated emission (SE).

Four kinetic curves representing ESA, GSB, SE and ESA-SE mixed signal are exhibited in FIG. 4. Under the influence of PA, BR-HSA complexes show a relatively flatter decay curve in the first 100 ps. The ultrafast decay processes with lifetime less than 1 ps are inhibited, and the main decay channels turn into the 4 and 30 ps pathways. Moreover, ESA signal strength build-up appears in the sample with 0 PA (red curve, probe at 640 nm) while the one with 4 PA (purple) only
gives negative SE signal. This could be due to the overlapping between the ESA and SE signal in TA spectra. In order to acquire a clear signal from excited state of BR-HSA complexes, femtosecond fluorescence up-conversion method was used and the results are shown in FIG. S4 in Supplementary materials. The four decay time constants observed in TA are confirmed. It is clear that the amplitude of the 4 ps component increases significantly after adding PA while the proportion of the 30 ps component increases slightly, and the less than 1 ps decay channel is inhibited (Table I).

<table>
<thead>
<tr>
<th>PA</th>
<th>Up-conversion$^b$/%</th>
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<tbody>
<tr>
<td>0$^a$</td>
<td>55.9</td>
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<tr>
<td>1</td>
<td>54.4</td>
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<td>2</td>
<td>48.3</td>
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<td>4</td>
<td>48.2</td>
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$^a$ '0 PA' stands for samples without PA.
$^b$ Value is the percentage of each component in the overall amplitude.

IV. DISCUSSION

$Z$-$E$ and $E$-$Z$ isomerization can easily occur in $4Z$, $15Z$-bilirubin under light irradiation, and the latter isomer could rapidly further evolve to lumirubin [1–3, 21]. These water-soluble products generated by light can be efficiently excreted by the body through body fluid, which is the reason for clinical efficacy [1, 22, 23]. Therefore, there are two main types of bilirubin photochemical products in clinical practice, $Z$-$E$ isomer and

FIG. 3 (a–d) Broadband TA spectra of BR-HSA complexes with 0, 1, 2, 4 molar equivalents of PA under 500 nm excitation. (e–h) Corresponding evolution-associated difference spectra (EADS) extracted from global fitting of TA spectra.

FIG. 4 Kinetics of BR-HSA complexes with 0 PA (red solid circle), 2 PA (cyan solid circle) and 4 PA (purple solid circle). Four probe wavelength (385, 465, 565, 640 nm) kinetics are shown. Lines are best global fitting curves.

TABLE I Global fitting results of fluorescence up-conversion (t in ps).

<table>
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<tr>
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$^a$ '0 PA' stands for samples without PA.
$^b$ Value is the percentage of each component in the overall amplitude.
lumirubin. The former is the most efficient in mice and the latter is found in human, in terms of their metabolic efficiency [2, 23]. According to the study by Malhotra et al., it was found that the promotion effect of fatty acids on the formation of lumirubin depended on the chain length and the molar ratio binding to HSA [4]. Significantly promoted effects have been observed in fatty acid with chain length larger than 10 atoms and the molar ratio larger than 2. It was proposed that the structural changes of HSA caused by fatty acid addition can further affect the dihedral angle of BR, which determines the generation rate of lumirubin. The destruction of cotton effect on circular dichroism spectra of BR after the addition of fatty acid also indicates the change of structure [24]. In this work, the promotion effect of PA on BR photochemical QY was directly shown in FIG. 1(b) and Table S1 in Supplementary materials. Compared with the fatty free sample, the total BR photochemical reaction QY of the sample with 4 PA was enhanced by 83% and the aim of this study is to reveal the mechanism behind.

From the area normalized fluorescence emission spectra, it is clear that there are isoemissive points in both 0 and 4 PA BR-HSA complexes. This suggests that there are two emissive species in the complexes. According to literature, the Z-1 sample of bilirubin dipyrrinone has a red shift absorption, stronger luminescence, and higher photochemical reaction efficiency than the E-1 [15, 16]. Linkage of the two bilirubin dipyrrinone subunits by carbon-carbon single bond could lead to the generation of the absorption shoulder band in BR similar to the one seen in FIG. 1(a) (0 PA, red line). This spectra change is attributed to the degree of exciton coupling between the two dipyrrinone subunits that can be regulated by the solvent environment [5, 8, 25]. Therefore, we believe that it is a combination of both Z-Z and Z-E BR when it forms complex with HSA. As shown in FIG. 2(b), the long wavelength emission is more intense after the addition of PA, suggesting BR should prefer the Z-Z conformation when PA concentration is high (FIG. 1, structural model).

Femtosecond time-resolved spectroscopy was used to explore the excited state dynamics of BR-HSA complexes with different content of PA. For the complexes with 0 and 1 PA (FIG. 3(e−f)), a blue shift of the 390 nm ESA band at the first 3 ps after excitation clearly shows the vibration relaxation of S0 and the GSB recovery indicates the highly effective depopulation of the S1 state in this ultrafast timescale. This process was inhibited in complex with 4 PA and the 4 ps decay component contributes to larger amplitude. Similar decay process has been observed and it is assigned to internal conversion back to the hot ground state (hot-S0) [15]. The 4 ps component was then evolved into the 30 ps component, which further turns into a component with a lifetime of 200 ps (at 425 nm and 640 nm). The 30 ps component originates from ring torsion related structural relaxation process [26] and it can eventually lead to formation of a small amount of Z-E or E-Z isomer. Considering that the calculation results show that E-Z isomer has a relatively high potential energy surface compared with Z-E isomer and the previous results of photo isomerization yield study [8, 15, 21, 27–30], we assign the 200 ps lifetime to the S1 state of Z-E isomer. The excited state dynamics of BR-HSA complexes observed in TA is verified by fluorescence up-conversion data (Table I and FIG. S4 in Supplementary materials).

The most important discovery is that the addition of PA can branch the excited state dynamics from the subpicosecond decay pathway to the 4 ps one in BR-HSA complex rather than altering the excited state lifetimes. This means that PA does not change the electronic transitions of BR-HSA but it affects the constituent of BR isomers that bind to HSA. As mentioned above, higher proportion of BR should be in the Z-Z conformation in the BR-HSA complex when PA concentration is high and the main excited state decay pathway for this species should be the 4 ps channel. After it returns to the hot S0 state, it can evolve to the E-Z isomer more effectively and increase the yield of lumirubin.

V. CONCLUSION

In summary, excited state relaxation mechanism of BR-HSA complex was studied and the key reason to enhance the lumirubin formation by adding PA is revealed. As shown in Scheme 1, BR mainly undergoes an ultrafast excited state relaxation process with less than 1 ps lifetime in the fatty acid free sample. Another 4 ps decay channel as well as a 30 ps torsion related decay pathway also exists in the BR-HSA complex. With the addition of PA, the ultrafast decay is inhibited and excited state population of BR now decay back to hot S0 state through the 4 and 30 ps channels. Z-Z to E-Z isomerization would take place in the hot S0 state with higher probability and it can enhance lu-
mirubin formation. Our work is the first complete characterization and modeling of the excited state dynamics of BR-HSA complex, and also provides confirmation of the time scale for BR photoisomerization. More importantly, we demonstrated the reason for the promoting effect of fatty acid on BR photochemical reaction, which is significance for relevant clinical medical research.

Supplementary materials: Additional experimental method for illumination experiments, the relative generation rate of lumirubin and global fitting are given. FIGs. S1–S4 show additional steady-state and time-resolved spectroscopy data.

VI. ACKNOWLEDGMENTS

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