

Ultrafast photochemistry of bilirubin-albumin complex reveals the molecular origin of jaundice neonatal blue-green phototherapy

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Abstract

Blue-green light is clinically more effective than blue light at clearing bilirubin in neonatal jaundice phototherapy, yet the molecular origin of this wavelength dependence remains unclear. Here, we determine how excitation wavelength controls the initial photochemistry of the bilirubin-human serum albumin (HSA) complex, in physiologically environment, by combining ultrafast spectroscopies and theoretical calculations. We show that HSA creates an asymmetric reaction environment that differentiates the photochemistry of bilirubin's two non-conjugated asymmetrical dipyrinone subunits, denoted as the AB and CD moieties. Blue-light excitation drives Z→E isomerization through the CD-side pathway, yielding the long-lived, non-metabolizable 4Z,15E photoproduct. Conversely, blue-green-light excitation selectively accesses an AB-side pathway where ultrafast proton transfer coupled isomerization, generating a deprotonated 4E,15Z ground-state intermediate preorganized for subsequent photocyclization to lumirubin, the excretable bilirubin photoproduct in humans. Our results reveal the physical basis underlying the superior efficacy of blue-green phototherapy and provide a mechanistic basis for optimizing next-generation phototherapy devices.

Introduction

Neonatal jaundice is a common condition in both preterm and term infants and arises when bilirubin (BR) production exceeds the infant's metabolic capacity¹. Because neurotoxic free BR is lipid-soluble and can cross the blood-brain barrier, excessive serum bilirubin can cause irreversible neurological injury and even life-threatening complications². Phototherapy, which uses continuous blue light exposure on the skin to reduce hyperbilirubinemia, has therefore remained the standard treatment since its clinical introduction in 1958, saving tens of millions of infants worldwide^{3, 4, 5}.

Native 4Z,15Z-BR comprises two non-conjugated asymmetrical dipyrinone subunits (denoted as AB and CD moieties) connected by a flexible methylene bridge (Fig. 1b). Although BR contains multiple polar groups, intramolecular hydrogen bonds stabilize a ridge-tile structure that makes it overall hydrophobic. Phototherapy converts lipid-soluble 4Z,15Z-BR into more water-soluble photoproducts, including reversible configurational isomers (4E,15Z-BR, 4Z,15E-BR, and 4E,15E-BR) and lumirubin (LR), an excretable structural isomer formed by photocyclization¹. The configurational isomers readily revert to the native 4Z,15Z-BR in bile and undergo enterohepatic reabsorption⁶, whereas LR is the major human metabolite because of its relatively irreversible formation, enhanced water solubility and metabolic stability⁷. In vivo, BR binds non-covalently to human serum albumin (HSA, Fig. 1a), which defines the primary photochemical environment and favors structural isomerization⁸. However, the BR-binding structure in HSA remains debated. Crystallography assigned a 4Z,15E-BR species to subdomain IB⁹ (Fig. 1a), while multiple biochemical studies instead support subdomain IIA as the physiologically relevant high-affinity site for native 4Z,15Z-BR^{10, 11, 12, 13, 14, 15, 16}. This unresolved structural issue complicates the mechanistic interpretation of BR photochemistry in HSA.

Phototherapy is now entering a critical stage of optimization. Improving efficacy is a priority not only for faster bilirubin clearance but also for reducing treatment-associated risks like dehydration, thermal injury, and DNA damage^{17, 18}. Current clinical guidelines recommend blue-green light (460–490 nm)^{19, 20}, a recent clinical studies show that, at matched irradiance, blue-green light (478 ± 10 nm) produces LR more efficiently than conventional blue light (459 ± 10 nm), with therapeutic gains of up to 31%^{21, 22, 23, 24}. Yet the molecular basis of the enhanced efficacy of blue-green light remains elusive. Existing explanations mainly invoke tissue optics, including reduced hemoglobin absorption and wavelength-dependent skin penetration^{25, 26}. Nevertheless, in vitro irradiation studies of BR-HSA complex solutions show that the same spectral range directly enhances BR clearance rate even without skin optics^{27, 28, 29, 30}. These observations point to an additional photochemical mechanism intrinsic to BR-HSA complex, which remains to be identified.

While time-resolved studies have clarified the excited-state relaxation, ultrafast isomerization and hydrogen-bond cleavage of free BR^{31, 32, 33}, the corresponding photochemistry of the BR-HSA complex remains much less understood³⁴. Yet it is precisely within this complex that the clinically relevant reaction pathway is defined. Because LR is generated through photocyclization rather than simple configurational isomerization, the central mechanistic problem is not merely how BR relaxes after excitation, but how the BR-HSA complex accesses the productive LR-forming pathway.

Here, we combine time-resolved electronic and vibrational spectroscopic techniques with molecular dynamics (MD) simulations and time-dependent density functional theory (TD-DFT) calculations to resolve the initial photochemical dynamics of the BR-HSA complex at the molecular level. We aim to define the wavelength-dependent early photochemical evolution of BR in HSA, focusing on the blue-green-light-driven channel that generates a deprotonated 4E,15Z ground-state intermediate as the precursor to LR formation. This work provides a mechanistic framework for understanding wavelength-dependent therapeutic efficacy and guiding rational design of next-generation phototherapy devices.

Result and discussion

Wavelength-dependent transient absorption reveals exciton-selective photochemical branching

The key photochemical events in neonatal jaundice phototherapy occur within the BR-HSA complex in the bloodstream. To probe the intrinsic photoreaction mechanism within the HSA binding pocket, we employed a simplified binary BR-HSA model at a mass ratio of ~ 8mg/1g (molarity ratio around 1:1.1), which mimics severe hyperbilirubinemia^{35, 36}. Under these conditions, the BR concentration approaches, but remains below, the saturation limit of the primary high-affinity HSA binding site¹⁰, so the observed photochemistry predominantly reflects this clinically relevant binding environment. The absorption spectra of the BR-HSA complex and free 4Z,15Z-BR (Supplementary Fig. 2a) reveal broad visible absorption band arising from excitonic coupling between its two dipyrnone chromophores. This

coupling produces Davydov splitting and yields two localized Frenkel excitons^{37, 38} (Supplementary Fig. 1b), manifesting as an absorption profile that can be approximated by two partially overlapping high- and low-energy Gaussian bands³⁹. Upon HSA binding, the BR absorption profile broadens, with two localized excitonic states centered at 415 and 463 nm, respectively (Supplementary Fig. 2a). These states are central to the wavelength-dependent BR photochemistry⁴⁰. In free 4Z,15Z-BR, shifting irradiation from blue to green light progressively decreases the yield of the major photoproduct 4Z,15E-BR isomer, while increasing that of the minor product 4E,15Z-BR⁴⁰. In BR-HSA complex, however, HSA imparts distinct regiospecificity, blue light preferentially generates 4Z,15E-BR⁴¹, while 4E,15Z-BR remain comparatively undetectable even upon blue-green light excitation⁴². Yet once formed, HSA-bound 4E,15Z-BR can undergo photocyclization to lumirubin (LR), the major excretable photoproduct responsible for therapeutic bilirubin clearance. Notably, even in the BR-HSA complex, where the highest LR yield has been reported, the overall quantum efficiency remains as low as 0.3%⁴³. One of the key factors limiting this yield is thought to be the availability of the 4E,15Z-BR isomer, a critical intermediate in the cyclization pathway⁴². Therefore, the central unresolved question in elucidating the physical picture of phototherapy is not merely why the yield of LR is so low, but, more fundamentally, how BR isomerizes within the HSA binding pocket to access the 4E,15Z pathway.

To address this question, we performed excitation-wavelength-dependent fs-TA measurements from 400 to 500 nm, together with decay-associated difference spectra (DADS) global fitting (Fig. 1c-1f and Supplementary Figs. 3, 4 and 5). For free BR, four exponential decay components were obtained, with lifetimes essentially unchanged across the excitation range²⁴ (Supplementary Fig. 5). In sharp contrast, as shown in Supplementary Fig. 3, 4, the BR-HSA complex reveals four decay components and one non-decay component, with τ_1 and τ_2 progressively shortening as the excitation wavelength is red-shifted (summarized in Supplementary Table. 1).. HSA binding therefore breaks the kinetic equivalence of the two excitonic states, indicating that, the two localized excitonic states experience distinct amino-acid and water interactions, which impose different local chemical constraints and lead to different relaxation pathways.

The ground state bleaching (GSB) line shape further tracks the exciton population distribution. The τ_3 component, whose GSB is minimally affected by overlapping excited state absorption (ESA) signals, shows a double-minimum GSB at 419 and 463 nm under 400 nm excitation, indicating population of both excitonic states (Fig. 1e). With progressive red-shifting of the excitation wavelength, the GSB associated with the higher-energy excitonic state steadily diminishes, and the τ_3 GSB evolves into a single-minimum profile. By 500 nm excitation, the GSB is dominated by the low-energy excitonic state (Fig. 1f).

The τ_4 component further identifies a distinct low-energy pathway, characterized primarily by a GSB profile confined to the low-energy excitonic range, along with an excitation-wavelength-independent lifetime of 150 ps that is accompanied by a absorption band centered at 520 nm (Fig. 1e, 1f). We assign τ_4 to a short-lived intermediate state relaxation channel associated with the low-energy exciton state. Given that HSA-bound 4E,15Z-BR has been proposed as a key productive LR precursor but does not

accumulate detectably in steady state measurement⁴², τ_4 component likely reflects a transient population that does not accumulate to a detectable level but remains available for subsequent photocyclization²⁹.

By contrast, τ_5 is a non-decaying absorption feature centered at ~ 490 nm within the fs-TA window (Fig. 1e). Its relative contribution decreases with red-shifted excitation. Under 400 nm excitation, it accounts for $\sim 9.7\%$ of the ground-state population (Supplementary Fig. 6), consistent with the reported $\sim 11\%$ quantum yield of 4Z,15E-BR under blue irradiation⁴¹. This assignment is supported by Microsecond transient absorption (μ s-TA), where the ~ 490 nm band persists throughout the μ s window under 400 nm excitation but is absent under 500 nm excitation (Supplementary Fig. 7), and by absorbance difference spectra showing a stable 470–550 nm photoproduct band only after 400 nm LED irradiation (Supplementary Fig. 8). Thus, blue excitation predominantly channels BR-HSA complex into the long-lived but non-metabolizable 4Z,15E configurational pathway.

The photocyclization of AB moiety of BR requires proton assistance⁴⁴, whereas the excited state of BR may have the ability to promote deprotonation⁴⁵. We next examined whether proton mobility is enhanced in the low-energy pathway. Replacing H₂O with D₂O markedly enhances BR-HSA complex fluorescence (Supplementary Fig. 2a, 2b), and this enhancement becomes stronger at longer excitation wavelength (Supplementary Fig. 2c), indicating that proton transfer is more significant in the low-energy excitonic state and competes with fluorescence emission. fs-TA isotope effects support this conclusion (Fig. 1g, 1h and Supplementary Fig. 9). Under 500 nm excitation, D₂O slows BR-HSA complex relaxation with kinetic isotope effect (KIE) values of 1.68, 1.72 and 1.67 for τ_1 , τ_2 and τ_3 , respectively. Under 400 nm excitation, the KIE is much weaker (~ 1 , 1.13 and 1.38). Notably, τ_4 showcases an unchanged KIE of 1.47 at different excitation wavelengths, indicating that it originates from the same intrinsic low-energy-state channel rather than from a wavelength-dependent mixture of states.

These results demonstrate that the excited-state dynamics of BR are governed by wavelength-selective excitation. Specifically, blue light excitation, which populates the high-energy exciton, predominantly generates the 4Z,15E-BR isomer localized on the CD moiety. In contrast, blue-green light excitation selectively populates the low-energy exciton and promotes pronounced proton transfer on the AB moiety, a behavior closely associated with the requirements for photocyclization.

The wavelength-dependent excited-state dynamics observed above point to an asymmetric chemical reaction environment for BR in HSA. This shifts a problem from kinetic alone to structure: which HSA subdomain defines the local binding environment responsible for the observed BR photodynamic? Answering this question, we combined spectroscopic experiments and theoretical calculations to identify which HSA binding pocket best accounts for the photophysical behavior of the BR-HSA complex.

MD simulations and TD-DFT calculations identify subdomain IIA as the asymmetric photochemical pocket

HSA protein (Fig. 1a) is composed of 585 amino acids and folds into three identical domains (Domain I, II, and III), each divided into A and B sub-domains⁴⁶. Crystallographic captured a 4Z, 15E-BR species with M-type enantiomer (Supplementary Fig. 1a) binding to the sub-domain IB site of HSA in a non-covalent form (PDB ID: 2VUE)⁹. However, ligand competition^{10, 11, 12}, single-site residue mutation^{13, 14}, and other biochemical studies^{15, 16} instead supported sub-domain IIA as the physiologically relevant high-affinity site for native 4Z,15Z-BR with a P-type enantiomer.

To address which of these two proposed sub-domains most accurately represents the binding environment to governing the photodynamics of the BR-HSA complex, we resorted to the theoretical approaches, combining molecular dynamics (MD) simulations and quantum chemical calculation (DFT for the ground-state optimization and the TD-DFT for the Frank-Condon excited-states, details are provided in the Supplementary section 1). We evaluated the two candidate sites, the crystallographic IB site and the proposed IIA site, using three critical directly relevant to the photophysical dynamics observed in BR-HSA complex: the local hydration environment required to support proton transfer, the excited-state localization patterns that govern wavelength-dependent photochemistry, and the chirality of BR reflected in circular dichroism spectra. The hydration environment was assessed through three independent 1.5 μ s MD simulations, which reconstructed the dynamic distribution of transient water molecules around BR (the RMSD and RMSF shown in Supplementary Fig. 10), and the excited-state properties were analyzed by TD-DFT calculations on cluster models derived from MD snapshots with explicit inclusion of nearby residues and water molecules.

We first examined the crystallographic IB model (PDB ID: 2VUE), in which BR is assigned as 4Z,15E configuration⁹. MD simulations reveal that the AB moiety remains embedded in a hydrophobic cleft formed between helices within the IB pocket, even when transient waters are considered⁹, indicating that this site lacks a hydrophilic micro-environment required to support efficient proton transfer (Fig. 2a and Supplementary Fig. 11). TD-DFT calculations further show that BR in the IB pocket exhibits two domain excited states S_1 and S_2 , with transition energies of ~ 2.86 eV (433.51 nm) and ~ 3.07 eV (403.86 nm), respectively (Fig. 2d and Supplementary Table. 2). Electron-hole analysis reveals that the $S_0 \rightarrow S_1$ transition is predominantly localized on the CD moiety (86.52%), whereas the $S_0 \rightarrow S_2$ transition is localized on the AB moiety (78.98%) (Fig. 2b, 2c, Supplementary Fig. 12a and Supplementary Table. 3). Furthermore, the calculated circular dichroism spectra for IB model fail to reproduce the experimental result with positive couplet located at 460(+)/410(-) consistent with the P-type enantiomer (Fig. 2e)¹⁰. In stark contrast, all three calculated spectra for IB model display a completely different lineshape (negative couplet or only negative bands). Thus, the IB model predicts an M-type-like enantiomer. These results can not account for the BR-HSA complex photophysics under physiological conditions, owing to insufficient AB moiety hydration, incorrect low-energy excited-state location, and an incorrect chiral response.

We therefore examined subdomain IIA as a more plausible native 4Z,15Z-BR binding site. Because no experimental structure of native 4Z,15Z-BR bound to HSA is available, likely due to high flexibility of this high-affinity binding sites⁴⁷, molecular docking was first used to generate the initial BR-HSA complex interaction model⁴⁸. The highest-scoring pose was used for the following MD simulations. In particular, the IIA pocket contains multiple positively charged residues with aliphatic side chains, capable of stabilizing BR anions in serum. Among them, residues R218, R222 and R257, as well as K199 and K195, have hydrogen bonds or salt bridges with BR (Fig. 2f and Supplementary Fig. 11).

Within sub-domain IIA site, the AB moiety is situated in a highly favorable hydrophilic environment. It interacts with residues R218, R222, R257 and K195, and is further surrounded by transient water molecules, together forming a well-defined hydrogen-bond network, while the CD moiety remains embedded in the hydrophobic region of the pocket. TD-DFT calculation identify two dominant excited states at ~ 2.93 eV (423.15 nm) and ~ 3.18 eV (388.89 nm), respectively (Fig. 2i and Supplementary Table. 2). In contrary to the IB site, the low-energy $S_0 \rightarrow S_1$ transition is predominantly localized on the AB moiety (85.31%), whereas the higher-energy $S_0 \rightarrow S_2$ transition is mainly localized on the CD moiety (86.95%) (Fig. 2g, 2h, Supplementary Fig. 12 and Supplementary Table. 4). This result implies that excitation into the lower-energy excitonic band preferentially addresses the cyclization-active AB moiety, while higher-energy excitation primarily drives the dynamics associated with the CD moiety. In addition, the two calculated circular dichroism spectra for IIA model showcase a positive couplet feature that is consistent with the experimental results. Although one simulated conformer fails to reproduce the experimental circular dichroism spectrum, this discrepancy arises from the large dihedral angles ϕ_1 and ϕ_2 , which induce an in-line conformation of BR molecule⁴⁹, as discussed in the Supplementary Fig. 13.

Taken together, these functional analyses indicate that BR likely binds to the IIA pocket in the 4Z,15Z configuration as the P-enantiomer. TA and computational results establish a site-specific excitonic framework for the BR-HSA complex, wherein the asymmetric IIA pocket differentiates the photochemical dynamics of the two dipyrinone moieties. However, these approaches mainly provide population dynamics and static structure assignments, the detailed structural dynamics along the reactions are unresolved. In particular, the isomerization coordinates of the two dipyrinone moieties remain unclear, especially whether the short-lived low-energy pathway associated with the AB moiety corresponds to the formation of an intermediate containing the 4E,15Z-BR configuration. To address this, we turned to femtosecond stimulated Raman spectroscopy (FSRS) to directly probe the excited-state structural dynamics of the BR-HSA complex^{50, 51, 52, 53}. Although the ground state vibrational spectra of AB and CD moieties are difficult to separate because of their similar intrinsic frequencies⁵⁴, FSRS can exploit the excited state location to selectively enhance the vibrational signature of different excitonic channels by tuning the excitation wavelength, as shown in Fig. 3a, 3b and Supplementary Fig. 17 (baseline and the selected raw data of FSRS are shown in Supplementary Fig. 18–20). Raman assignments were made using DFT harmonic vibrational analysis of the BR structure bound in the HSA IIA site. In this cluster model, key residues and proximal explicit water molecules were embedded within an implicit aqueous

continuum solvent (Supplementary Fig. 14). Meanwhile, the assignments also referred to previous studies^{33, 54, 55, 56}.

FSRS resolves proton-transfer-coupled AB-side isomerization

Under 500 nm excitation, FSRS predominantly probes the AB moiety. Analysis using the sequential evolution model of species-associated difference spectra (SADS) reveals three exponential components: 0.12 ps, 1.0 ps, and 20 ps (see the bottom panel of Fig. 3a). By contrast, 420 nm excitation produces mixed AB/CD vibrational dynamics (Supplementary Fig. 17a). For mixed vibrational modes, such as the 1627 cm^{-1} double bonds stretching mode ($C_4 = C_5$ and $C_{15} = C_{16}$). Under 420 nm excitation, this mode shows altered kinetics together with an additional long-lived component beyond the experimental time window (Fig. 4b), indicating an extra contribution from the CD moiety.

Direct evidence for CD-side dynamics under 420 nm excitation comes from two fingerprint bands at 816 and 1542 cm^{-1} , neither of which appears under 500 nm excitation (Fig. 4a). The 1542 cm^{-1} mode reveals relatively pure CD-moiety relaxation with lifetimes of 0.62 ps, 7.1 ps, 21 ps and a long-lived component beyond the detection window (in Fig. 4c). The 816 cm^{-1} , assigned to the C_{15} -H hydrogen-out-of-plane (HOOP) motion of the CD moiety, decays mono-exponentially with a time constant of $\sim 500\text{ fs}$ (Fig. 4d). Because HOOP modes are well-established marker of double-bond twisting and photoisomerization³³, this feature provides direct structural evidence that 420 nm excitation drives C_{15} -center Z-to-E photoisomerization, leading to the formation of 4Z,15E-BR.

An AB-side HOOP mode is also observed. The 866 cm^{-1} band, which coexists with the 816 cm^{-1} mode at 420 nm excitation and appears alone under 500 nm excitation, is assigned to the C_5 -H HOOP motion. This mode obtained from both excitation wavelength decay mono-exponentially with a time constant of $\sim 120\text{ fs}$ (Supplementary Fig. 21), indicating rapid AB-moiety distortion from a planar to a twisted structure and supporting assignment of the short-lived low-energy pathway to a 4E,15Z-like transient product.

Because TA independently revealed proton-transfer dynamics in the low-energy pathway, we next examined whether AB-side isomerization and proton transfer are coupled. Such coupling would require a local structure that can simultaneously support double-bond twisting and proton transfer. This possibility is strongly suggested by the MD results for the IIA-bound model, which show that the ring-B pyrrole N-H group and the ring-C propionic acid-derived COO^- group, together with surrounding water molecules, form a local $\text{N-H}\cdots\text{COO}^-$ hydrogen-bonding network, providing a plausible structural basis for such coupling (Supplementary Fig. 11d-f). Isotope-dependent FSRS under 500 nm excitation in H_2O and D_2O (pH/D = 7.4) further supports this assignment. As shown in Fig. 3a, b, several vibrational modes are initially red-shifted in D_2O , and SADS resolves three decay components with pronounced KIE. These features provide strong evidence that proton motion contributes substantially to the AB-side excited-state dynamics.

Charge-density-difference analysis reveals that photoexcitation to the Franck-Condon region drives electron redistribution toward the nitrogen of the pyrrole ring B (Supplementary Fig. 15), which weakens the N-H bond and promotes deprotonation toward the stabilized COO⁻ acceptor. Consistently, FSRS directly resolves B-ring N-H vibrational marker bands, including the out-of-plane bending mode at 703 cm⁻¹ in H₂O (693 cm⁻¹ in D₂O) and the in-plane bending mode at 969 cm⁻¹ in H₂O (963 cm⁻¹ in D₂O)⁵⁶. Both modes exhibit a transient red shift of ~ 15 cm⁻¹ with strong isotope effects (dashed lines in lower panels of Fig. 3a,b). For the in-plane bending mode (in Fig. 3c), the red-shift kinetic proceeds with time constants of $\tau_1 = 0.12$ (0.21) ps and $\tau_2 = 1.1$ (2.3) ps in H₂O (D₂O) with KIE values of 1.75 (τ_1) and 2.09 (τ_2). Importantly, both the relaxation kinetics and the transient red shifts display clear isotope effects, reflecting progressive softening of the N-H bending potential as the proton moves away from its equilibrium geometry toward the acceptor.

Notably, the biexponential N-H bending dynamics further indicate that the proton-transfer process is not an isolated proton-motion event, but is closely coupled to concurrent structural evolution. Direct evidence for this coupling is provided by the AB moiety HOOP mode at 866 cm⁻¹, which also exhibits a pronounced KIE effect (see Fig. 3d). Because HOOP modes directly report double-bond twisting, the isotope sensitivity of this mode strongly suggests that proton transfer and AB-side Z-to-E isomerization are closely coupled in the excited state. We therefore suggest that low-energy excitation launches the wavepacket of 4Z,15Z-BR along a coupled isomerization/proton-transfer coordinate on the AB moiety. In the first step, ultrafast C₄ = C₅ torsion drives the excited-state population through an S₁/S₀ conical intersection, producing a vibrationally hot 4E,15Z ground-state intermediate. At this stage, the original excited-state hydrogen-bonding geometry has already been disrupted by the Z-to-E isomerization, but the proton has not yet fully dissociated from the B-ring N-H donor.

This picture is directly supported by the low-frequency hydrogen bond mode assigned to N-H...COO⁻ out-of-plane bending motion at 260 cm⁻¹ in H₂O (red shift to 253 cm⁻¹ in D₂O). Unlike the N-H bending modes, this low-frequency hydrogen exhibits only single-exponential decay with a time constant of 150 fs in H₂O (240 fs in D₂O) (Fig. 3e)⁵⁵. The absence of second decay component indicates the initial excited state hydrogen bond configuration collapse on the ultrafast time scale of the first structural step, that is, concomitantly with AB-side Z-to-E isomerization. We therefore assign τ_1 to the formation of a hot 4E,15Z-like ground-state intermediate in which the hydrogen-bond framework has been reorganized but proton transfer remains incomplete. The slower τ_2 process, observed in the N-H bending vibrational markers, is then assigned to the subsequent full dissociation of the proton from the B-ring N-H site.

Further support for this assignment comes from a low-frequency skeletal mode at 187 cm⁻¹, which is attributed to a collective carbon-backbone motion of BR. Its kinetics contain three KIE-sensitive components: 120 fs, 1.1 ps and 20 ps in H₂O, slowed to 210 fs, 2.3 ps and 35 ps in D₂O (Fig. 3f). This behavior indicates that the carbon skeletal motion remains dynamically coupled to proton transfer and hydrogen-bond reorganization. In particular, the τ_2 component coincides with completion of proton transfer, indicating that skeleton rearrangement facilitates full proton dissociation and stabilizes a

deprotonated 4E,15Z ground-state species. The slower τ_3 component of the 187 cm^{-1} mode further shows that the BR framework relaxation continues even after proton transfer. The persistence of a KIE in τ_3 component indicates that, although no further proton displacement occurs, the subsequent skeletal evolution remains influenced by the reorganized hydrogen-bonding network^{57, 58}.

Thus, the AB-side reaction proceeds through a coupled cascade of isomerization, proton transfer, and structural relaxation. Ultrafast proton transfer and Z-to-E isomerization first drives the 4Z,15Z excited state through an S_1/S_0 conical intersection, generating a vibrationally 4E,15Z-BR like ground-state intermediate with a reorganized hydrogen-bond geometry. Proton dissociation is then completed on the picosecond timescale, yielding a hot deprotonated 4E,15Z ground-state BR species. The resulting deprotonated product continues to undergo $\sim 20\text{ ps}$ slower skeleton-wide structural relaxation under the influence of the reorganized local hydrogen-bonding network. Notably, the additional $\sim 150\text{ ps}$ lifetime observed in TA spectra for the AB-side pathway suggests that the deprotonated 4E,15Z ground-state species can relax back toward the native 4Z,15Z configuration through ground-state proton back transfer, accompanied by continued structural rearrangement of the BR framework and reorganization of the surrounding hydrogen-bond network.

Under continued irradiation, however, the same deprotonated 4E,15Z ground-state species is the most plausible precursor for photocyclization. Proton dissociation localizes electron density on the B-ring dipyrinone unit and enhances its nucleophilicity, while Z-to-E isomerization deplanarizes the AB chromophore, disrupts A/B π -conjugation, and further concentrates the reactive electron density on the B-ring side. Meanwhile, the MD model suggests that nearby basic residues, particularly arginine and lysine side chains around the A-ring region (Fig. 2f), may polarize the A-ring carbonyl and adjacent vinyl fragment, thereby increasing the electrophilic character. In addition, C_4 -center Z-to-E photoisomerization is more consistent with the reactive geometry required for cyclization because it preserves the proximity of the $C-3^2$ vinyl group to the C-7 position of the B ring (Fig. 1b)^{59, 60, 61}. Together, these coupled structural and electronic changes preorganized the deprotonated 4E,15Z species into a cyclization-competent state.

We therefore propose that lumirubin formation is most consistently described as an electrocyclization proceeding from the activated deprotonated 4E,15Z-BR intermediate. This ring-closure mode is geometrically feasible and governed by the Woodward-Hoffmann rules⁶². Nevertheless, the overall photocyclization efficiency remains low. This low yield likely reflects the combined effects of a finite cyclization barrier that still requires additional energy input, and a narrow reactivity window limited by the short lifetime of the 4E,15Z active intermediate before it relaxes back toward the native (4Z,15Z) configuration. Moreover, the photophysics of lumirubin imposes an additional constraint on net product accumulation. TD-DFT calculations indicate that the cyclized structure of LR leads to a higher transition energy (Supplementary Fig. 16 and Table 5), placing its main absorption in the blue region, consistent with previous HPLC-based analyses⁷. Thus, under blue-light phototherapy, lumirubin itself can be re-excited and undergo reverse photoconversion⁴⁴.

These considerations provide a mechanistic basis for the superior efficacy of blue-green illumination^{21, 22, 23, 24}. In addition to previously proposed factors to favor lumirubin production, including higher lumirubin quantum yield at longer wavelengths and reduced competition from hemoglobin and melanin^{25, 26}, our results suggest that blue-green light has a dual advantage: it more efficiently accesses the productive 4E,15Z-to-lumirubin pathway while reducing re-excitation and loss of lumirubin once formed. Thus, the clinical advantage of blue-green phototherapy arises from both enhanced lumirubin formation and reduced lumirubin depletion.

Conclusion

Previous clinical and photochemical studies have shown that blue-green light is more effective than shorter-wavelength blue light for neonatal phototherapy, and this advantage has generally been attributed to more efficient lumirubin formation. Our results provide a molecular-level explanation for this wavelength effect. As summarized in Fig. 5, blue-green light excitation of the BR–HSA complex preferentially accesses the AB-side reaction channel, in which ultrafast Z-to-E isomerization is coupled to proton transfer and yields a deprotonated 4E,15Z ground-state intermediate. This species is not merely a transient configurational isomer, but a structurally and electronically preorganized precursor for subsequent photocyclization. By contrast, blue-light excitation predominantly populates the CD-side pathway and produces the long-lived but non-metabolizable 4Z,15E photoproduct. In this sense, the superior efficacy of blue-green phototherapy can be understood not only in terms of product distribution, as previously recognized, but also in terms of more efficient dynamical access to the productive lumirubin-forming pathway. This provides a molecular-level rationale for the reported superiority of blue-green illumination and offers mechanistic guidance for future optimization of phototherapy spectra and device design.

Methods

Sample preparation. In this study, the BR-HSA complex was prepared at a molar ratio of 1.1(HSA):1 (BR) to ensure exclusive binding to the highest affinity site. HSA lyophilized powder (Sigma-Aldrich, A1887, purity > 96%, fatty acid content ~ 0.005%) was used. A total of 60 mg of HSA was dissolved in 3 mL of phosphate-buffered saline (PBS, pH 7.4 or pD 7.4, prepared with ultrapure water). Separately, 1 mg of bilirubin (Cayman Chemical, purity > 98%) was dissolved in 0.4 mL of 0.02 M NaOH aqueous solution and then filtered through a 0.22 µm membrane. Subsequently, 0.2 mL of the filtered BR/NaOH solution was titrated into the PBS-HSA solution. The resulting BR-HSA solution had an absorbance of approximately 1.25 OD/mm and served as the stock solution for subsequent dilutions. To prevent deterioration and oxidation, the solution was stored light-protected at 2–8°C and used for all spectroscopic measurements within two days.

Time-resolved spectroscopy experiments.

BR-HSA complex samples at pH 7.4 and pD 7.4 were placed in a sample cell made of fused silica with an optical path of 2 mm.

The fs transient absorption (fs-TA) spectra were collected by a commercial femtosecond transient absorption spectrometer (Helios Fire, Ultrafast System). The 800 nm fundamental beam was generated from a Ti: Sapphire laser amplifier system (Astrella, Coherent, Inc., 35 fs, 7 W, and 1 kHz). Wavelength-tunable actinic pump pulses from 400 to 500 nm, with a pulse energy of approximately 30 nJ, were generated using an optical parametric amplifier (OPerA Solo, Coherent Inc.). A broadband supercontinuum white light with a wavelength range from 330 ~ 680 nm serves as the probe pulse by focusing the fundamental beam into a CaF₂ crystal plate. The instrument response function (IRF) of this system is about 120 fs. Global analysis of the fs-TA data was performed using OPTIMUS⁶³.

The Microsecond transient absorption (μ s-TA) spectra were collected using a commercial transient absorption spectrometer (EOS, Ultrafast System). The actinic pump (300 nJ per pulse) was generated by an optical parametric amplifier (OPerA Solo, Coherent Inc.). A broadband probe continuum spanning 400–700 nm was generated from a sub-nanosecond white-light source. IRF of the μ s-TA is approximately 100 ps.

The femtosecond stimulated Raman spectroscopy (FSRS) experiments were performed in a home-built set-up. The fundamental laser pulses (Astrella, Coherent, Inc, 35 fs pulse duration, 7 mJ pulse energy, and 1 kHz repetition rate) were split into three beams to generate a tunable narrowband ps Raman pump, a broadband fs Raman probe, and an fs actinic pump. The actinic pump beams centered at 420 nm and 500 nm (average power of 240 nJ per pulse) were generated by an optical parametric amplifier/OPA (OPerA Solo, Coherent, Inc.). Approximately 3 W of the 800 nm fundamental output was directed through a second-harmonic bandwidth compressor (SHBC, Coherent Inc.) and a picosecond optical parametric amplifier (TOPAS-400, Coherent Inc.) to generate a narrowband Raman pump centered at 580 nm, with a pulse energy of approximately 300 nJ. To improve spectral resolution and suppress spectral background, the Raman pump was further cleaned using a home-built grating-based spectral filter^{33, 64}. A broadband Raman probe was generated by focusing approximately 15 mW of the 800 nm fundamental beam into a 2-mm-thick single-crystal sapphire plate, together with a 580 nm Raman pump at the anti-Stokes side, covering $\sim 120\text{--}1900\text{ cm}^{-1}$ for the FSRS experiment.

Declarations

Author contributions

W.L conceived and supervised the project. R.P and W.Y conducted all the transient absorption and femtosecond stimulated Raman spectroscopy measurements and performed the fitting and analysis of the data to make the associated figures and tables. Y.L and Z.Z performed the TDDFT calculations, and made the associated figures and tables. Y.X and B.W performed molecular dynamics (MD) simulations

and interpreted the results. R.P, W.Y, and W.L wrote the manuscript with contributions from B.W and Y.L. All authors have approved the final version of the manuscript.

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Molecular dynamics simulations and quantum chemical calculations

Details are provided in the Supplementary Information.

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Figures

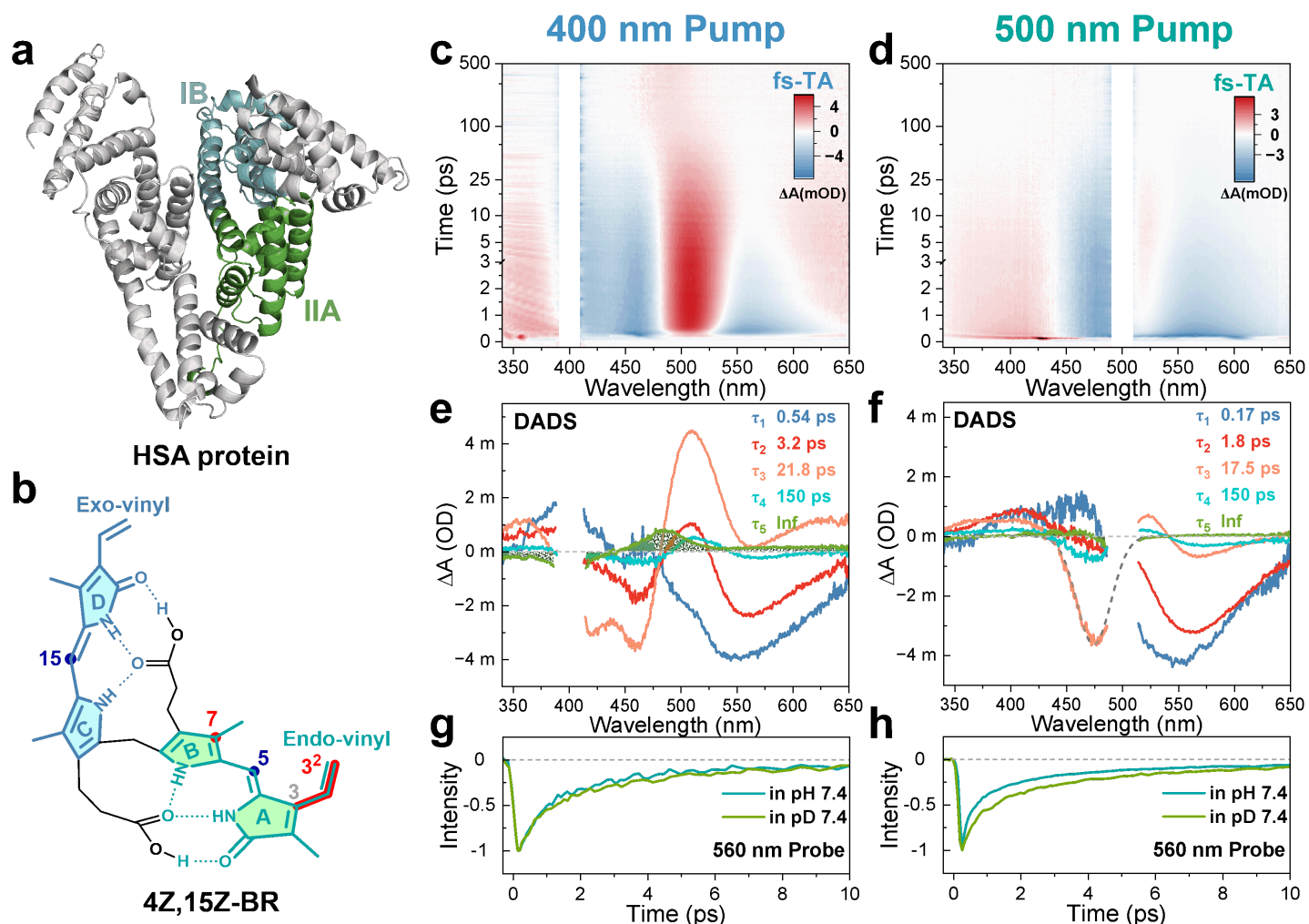


Figure 1

Structural features of BR and HSA and fs-TA dynamics of the BR-HSA complex. **(a)** The structure of human serum albumin (HSA, PDB ID:1AO6), highlighting the two BR binding sub-domains, IB and IIA. **(b)** Chemical structure of free 4Z,15Z-BR, the C₄=C₅ and C₁₅=C₁₆ double bonds involved in configurational isomerization are marked with blue dots at C₅ and C₁₅, and sites associated with photocyclization are highlighted in red at C₇ and C₃² vinyl group. **(c, d)** Two-dimensional fs-TA spectra of BR-HSA complex under 400 and 500 nm pump. **(e, f)** Decay-associated difference spectra (DADS) obtained from global analysis of fs-TA under 400 and 500 nm pump. **(g, h)** The single-wavelength stimulated emission decay curve at 560 nm under 400 and 500 nm pump for the BR-HSA complex in H₂O and D₂O.

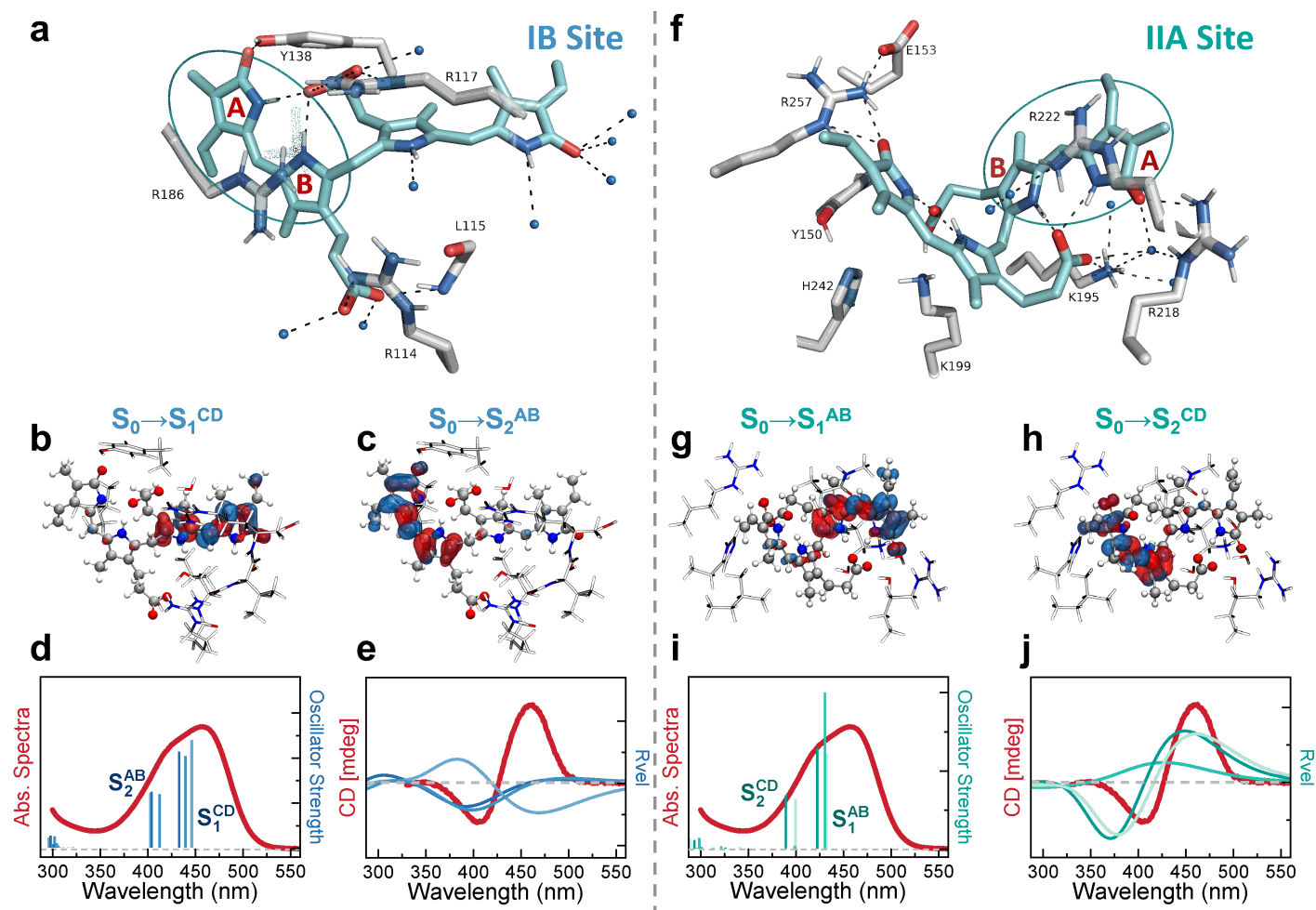


Figure 2

Theoretical calculations of two proposed BR-HSA binding sites. (a) and (f), Representative BR-HSA binding structures extracted from equilibrated MD trajectories for the (a) sub-domain IB site and (f) sub-domain IIA site, respectively. Three representative conformations from independent MD trajectories (present in Supplementary Fig. 11) were used for subsequent quantum-chemical calculations. Hydrogen bonds are shown as black dashed lines, water molecules as blue spheres, and the cyan shaded region highlights the AB dipyrri- none moiety of BR. Surrounding residues are partially displayed for clarity. (b, c) and (g, h), TD-DFT-based electron-hole analysis of the two lowest excited states of BR in the IB and IIA binding sites. Electron-hole analysis based on TD-DFT calculations reveals the localization characteristics of the first two excited states of BR. Red and blue isosurfaces represent hole and electron distributions, respectively, with an isovalue of 0.002. For BR bound in the IB site (b, c), the S_1 and S_2 states are primarily localized on the CD and AB moieties, respectively. In contrast, for BR bound in the IIA site (g, h), the localization order is reversed, with S_1 mainly localized on the AB moiety and S_2 on the CD moiety. (d) and (i) Comparison between the calculated oscillator strengths of the first six excited states and the experimental absorption spectrum (red curves) of the BR-HSA complex. All three TD-DFT datasets are included, with the corresponding transition energies and oscillator strengths listed in Supplementary Table 2. The calculated S_1 and S_2 transitions dominate the visible absorption region,

consistent with the exciton-coupling model. The key distinction between the IB and IIA binding models lies in the spatial localization of the two bright exciton states. **(e) and (j)** Comparison between calculated and experimental CD spectra. The red curves represent the experimental CD spectrum of the BR-HSA complex.

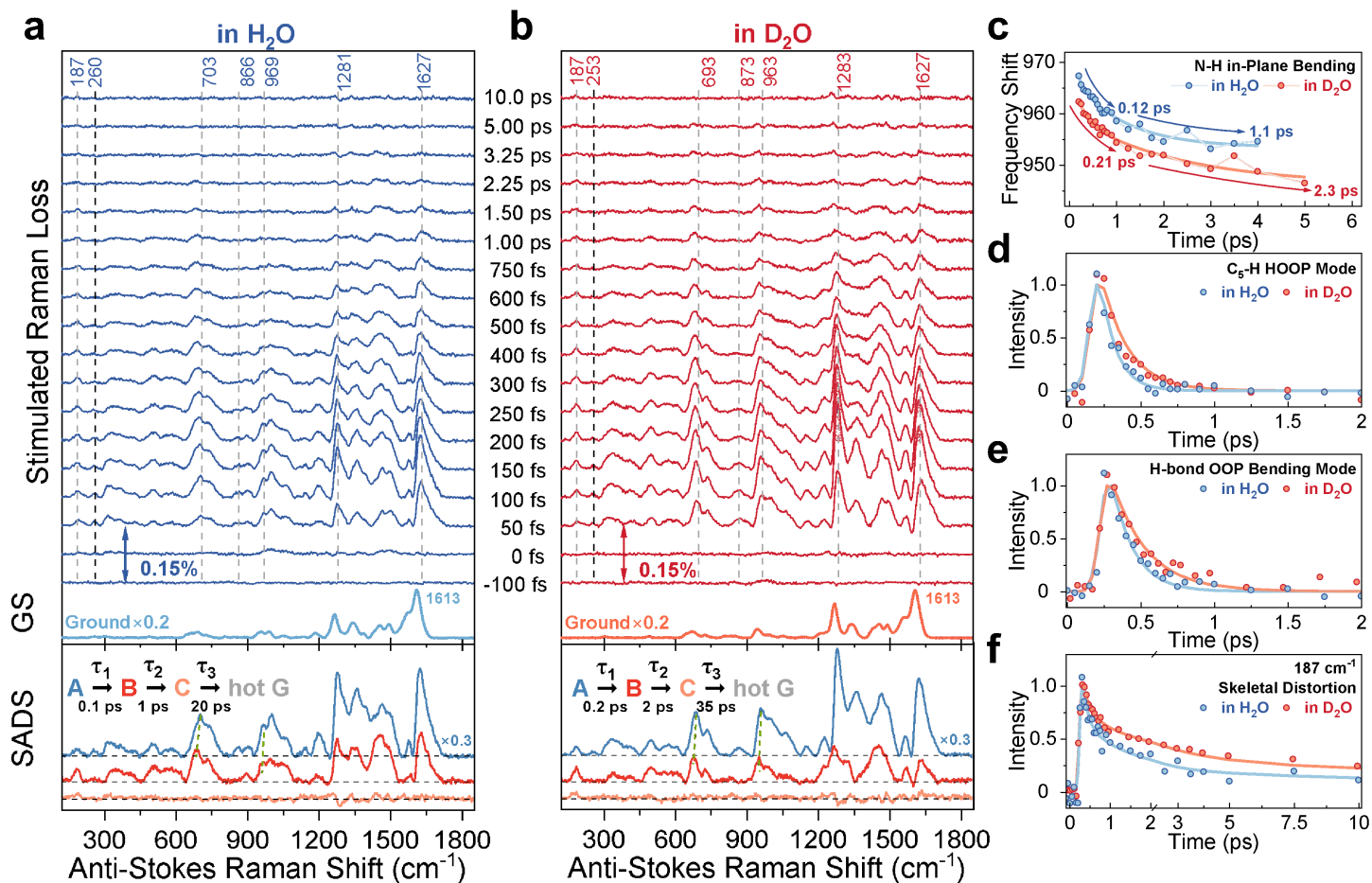


Figure 3

Isotope-dependent FSRS dynamics of the BR-HSA complex under 500 nm excitation. FSRS spectra of BR-HSA complex in the frequency range of 120-1850 cm^{-1} under 500 nm pump in **(a)** H_2O and **(b)** D_2O . The ground-state Raman spectra are shown at the bottom of each spectral map, and the corresponding species-associated difference spectra (SADS) are shown in the lower panels. The lifetimes change from 0.1, 1, and 20 ps in H_2O to 0.2, 2, and 35 in D_2O , respectively. **(c)** Transient frequency shift of the B-ring N-H in-plane bending Raman mode in H_2O and D_2O . **(d, e, f)** Time-dependent integrated Raman peak areas of selected vibrational modes in H_2O and D_2O . Symbols represent the experimental peak-area amplitudes, solid lines represent the corresponding kinetic fits. **(d)** Transient amplitude of the Raman mode of $\text{C}_5\text{-H}$ HOOP mode in H_2O and D_2O . **(e)** Transient amplitude of the Raman mode of H-bond out-

of-plane bending mode in H₂O and D₂O. **(f)** . Transient amplitude of the 187 cm⁻¹ skeletal motion mode in H₂O and D₂O.

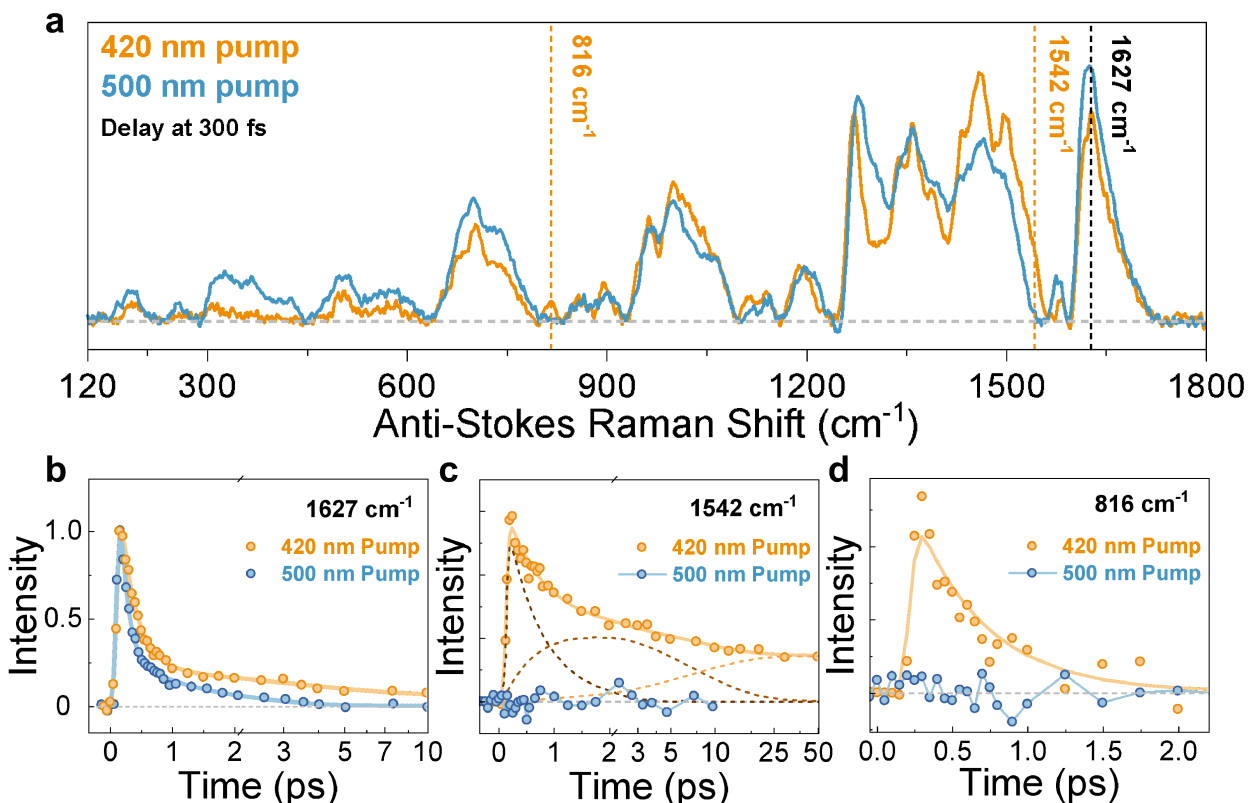


Figure 4

Excitation-wavelength-dependent FSR dynamics of BR-HSA complex. **(a)** The FSR spectra of the BR-HSA complex at 300 fs after 420 and 500 nm excitation. The yellow dashed lines mark vibrational modes at 816 cm⁻¹ and 1542 cm⁻¹ that can be assigned to the CD moiety. **(b, c, d)** Time-dependent integrated Raman peak areas of selected vibrational modes at 420 and 500 nm excitation. Symbols represent the experimental peak-area amplitudes, solid lines represent the corresponding kinetic fits. **(b)** Dynamics of the mixed C=C stretching mode involving both C₄=C₅ and C₁₅=C₁₆ coordinates. Consistent with the TA results, the relaxation dynamics change as the excitation wavelength is shifted from 420 to 500 nm. **(c) and (d)** CD-specific vibrational dynamics extracted from the mixed FSR response. These modes are observed under 420 nm excitation but are absent under 500 nm excitation, indicating selective activation of the CD moiety.

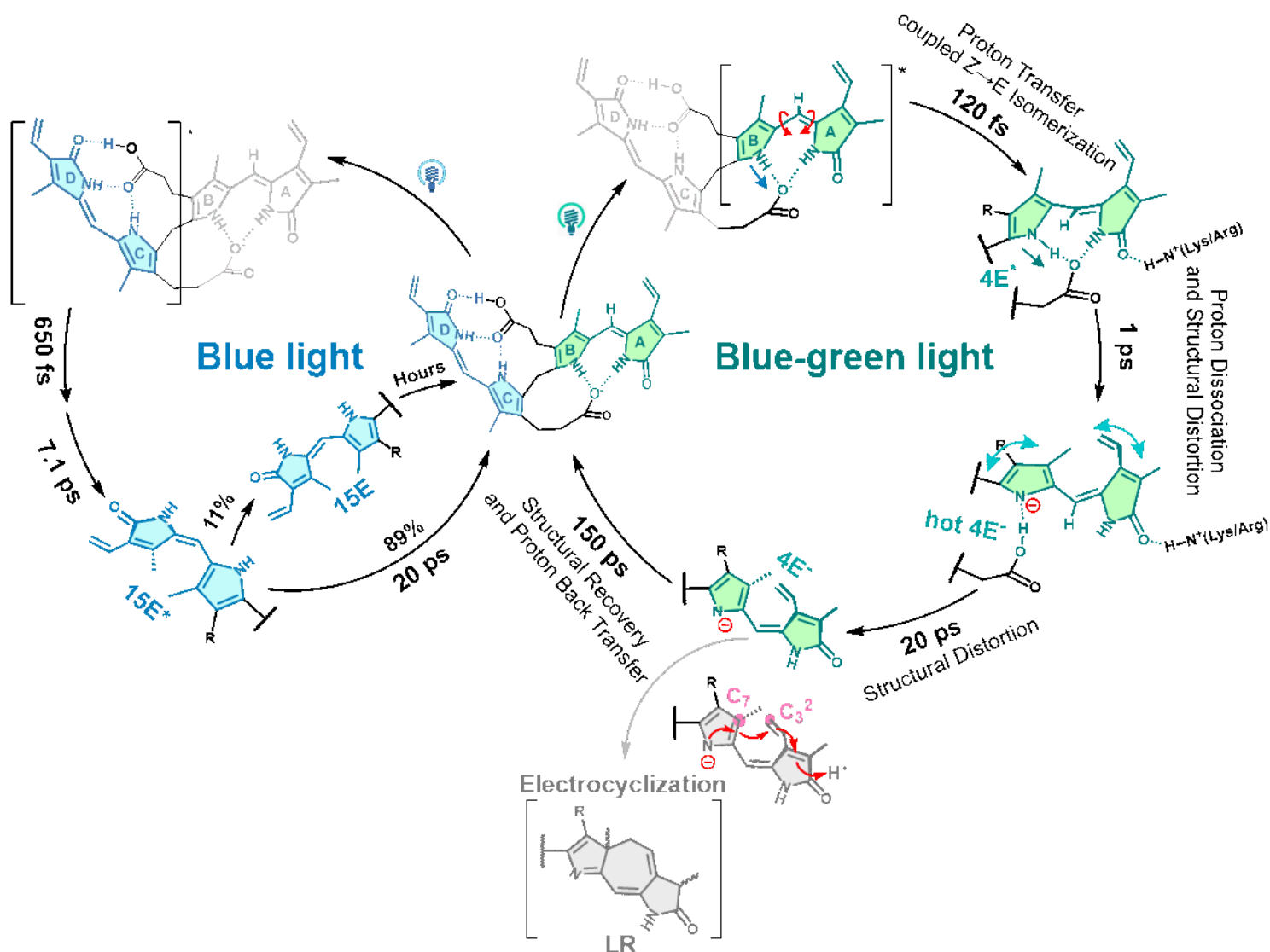


Figure 5

Proposed excited-state reaction pathways underlying neonatal jaundice phototherapy. Exciton coupling in native 4Z,15Z-BR gives rise to distinct wavelength-dependent reaction channels in the BR-HSA complex. Blue light irradiation preferentially activates the CD moiety, generating a 15E* intermediate on the picosecond timescale, which subsequently evolves into the stable 4Z,15E-BR photoproduct on a longer timescale. This pathway is therapeutically non-productive. In contrast, blue-green excitation selectively activates the AB moiety and drives ultrafast proton-transfer coupled Z-to-E isomerization within ~ 120 fs. Subsequent N-H bond cleavage and structural reorganizations within 1 ps, followed by ~20 ps skeletal relaxation to a hot deprotonated 4E-,15Z-BR ground-state intermediate. This species represents a cyclization-competent precursor for lumirubin formation. Under continued irradiation, the deprotonated 4E-,15Z-BR intermediate can undergo electrocyclization to form lumirubin, the major excretable photoproduct in neonatal phototherapy. However, most of the population relaxes back toward native 4Z,15Z-BR on the ~150 ps timescale through proton back-transfer and structural recovery, accounting for the low overall lumirubin quantum yield.

Supplementary Files

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