Rose Bengal Binding to Collagen and Tissue Photobonding

Emilio I. Alarcon,*†‡∥§ HeeGwang Roh,†∥ Jean-François Couture,‡ Jeffrey Comer,*∥§ and Irene E. Kochevar†

1Division of Cardiac Surgery, University of Ottawa Heart Institute, 40 Ruskin Street, K1Y 4W7 Ottawa, ON, Canada
2Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, K1H 8M5 Ottawa, ON, Canada
3Center for Bioinformatics and Molecular Simulation, Universidad de Talca, 2 Norte 685, Casilla 721, Talca 3460000, Chile
4Institute of Computational Comparative Medicine, Nanotechnology Innovation Center of Kansas State, and Department of Anatomy and Physiology, Kansas State University, Manhattan, Kansas 66503, United States
5Wellman Center for Photomedicine, Massachusetts General Hospital and Harvard Medical School, 40 Blossom Street, Boston, Massachusetts 02114, United States

ABSTRACT: We investigated two critical aspects of rose Bengal (RB) photosensitized cross-linking that may underlie recently developed medical applications. Our studies focused on the binding of RB to collagen by physical interaction and the effect of this binding and certain amino acids on RB photochemistry. Molecular dynamics simulations and free-energy calculations, complemented with isothermal titration calorimetry, provided insight into the binding between RB and a collagen-like peptide (CLP) at the atomic level. Electrostatic interactions dominated, which is consistent with the finding that RB bound equally well to triple helical and single chain collagen. The binding free energy ranged from −5.7 to −3 kcal/mol and was strongest near the positively charged amino groups at the N-terminus and on lysine side chains. At high RB concentration, a maximum of 16 ± 3 bound dye molecules per peptide was found, which is consistent with spectroscopic evidence for aggregated RB bound to collagen or the CLP. Within a tissue-mimetic collagen matrix, RB photobleached rapidly, probably due to electron transfer to certain protein amino acids, as was demonstrated in solutions of free RB and arginine. In the presence of arginine and low oxygen concentrations, a product absorbing at 510 nm formed, presumably due to dehalogenation after electron transfer to RB. In the collagen matrix without arginine, the dye generated singlet oxygen as well as the 510 nm product. These results provide the first evidence of the effects of a tissue-like environment on the photochemical mechanisms of rose Bengal.

1. INTRODUCTION

Novel medical treatments are currently being developed based on the photosensitized formation of protein–protein cross-links using rose Bengal (RB), a member of the xanthene family of dyes. For example, sutureless wound sealing is achieved by applying RB to the wound surfaces, followed by a short green light irradiation. This application is known as photochemical tissue bonding or PTB. A major advantage of PTB is the minimal scarring and inflammation, known as photochemical tissue passivation.2 Despite the increasing number of medical uses of RB-mediated protein cross-linking, the underlying molecular mechanisms remain largely unknown.

The photochemistry and photophysics of rose Bengal have been investigated extensively in aqueous solution.13,14 At pH 7.0, RB has an absorption maximum of 550 nm with a shoulder at 525 nm, a fluorescence quantum yield of 0.02, and a triplet quantum yield of 0.75.14,15 Rose Bengal is often used to generate singlet oxygen (1O2), which has been suggested to mediate formation of protein–protein bonds.6,17 In addition, both reductive and oxidative quenching of the RB triplet state are favorable with many electron donors and acceptors. In aqueous solution at pH 7.0, the one electron reduction potential is 1.09 V (vs standard calomel electrode (SCE)) and the oxidation potential is −0.78 V (vs SCE).18 The rose Bengal anion radical forms dehalogenated and/or dihydro products.14,18–20 However, RB forms dimers and higher multimers in aqueous solution that show substantially reduced photochemical reactivity.21–24

Received: May 25, 2017
Accepted: September 11, 2017
Published: October 11, 2017

DOI: 10.1021/acsomega.7b00675
ACS Omega 2017, 2, 6646–6657
The in vivo molecular environment in tissues is expected to strongly influence the photochemical reactions of rose Bengal because the dye binds to proteins including collagen, human serum albumin, and silk fibroin. Our previous studies using a combination of changes in absorption spectra of RB and molecular dynamics revealed the formation of rose Bengal–collagen ground state complexes. The dye–collagen complexes show a red-shifted absorption maximum at 560 nm, a lower fluorescence yield, and a reduced rate of photo-degradation. Interestingly, similar spectral changes are observed when rose Bengal is applied to tissues. Ground state association between RB and proteins within the tissue limits the dye mobility, which might reduce triplet excited state quenching. In addition, in tissue, the oxygen supply is lower than that in air, thus reducing O₂ formation and potentially favoring electron transfer processes for protein cross-linking.

Our long-range goal is to increase the efficiency of PTB to extend its range of medical applications. As steps to reach this goal, we have carried out studies on two fundamental aspects of the association. A more detailed molecular level picture of the association between RB and proteins within the tissue limits the dye mobility, which might reduce triplet excited state quenching. In addition, in tissue, the oxygen supply is lower than that in air, thus reducing O₂ formation and potentially favoring electron transfer processes for protein cross-linking.

2. RESULTS

2.1. Association of Rose Bengal with Collagen and a Collagen-Like Peptide. Collagen (type I) is the major structural protein in the connective tissues, for example, the skin, cornea, and tendon, and tissue repair using PTB is believed to result from cross-links between collagens. To begin to understand the association of RB with collagen, we examined whether the normal triple helical collagen conformation was required, investigated the interaction of RB with a tissue-mimetic collagen matrix, and oxygen on rose Bengal photochemistry.

Figure 1. Effect of collagen-like peptide on 10 μM rose Bengal absorption. (a) Spectral changes and (b) changes in absorption at 550 nm. All measurements were carried out in 10 mM MES buffer (pH 5.0).

Circular dichroism experiments for rose Bengal measured at a ratio of 4:1 (rose Bengal/collagen) showed optically active features in the 520–600 nm region (Figure S2a), which is a further indication of the ground state association.

Experiments carried out at a lower dye concentration, 1.25 μM, and decreasing collagen concentrations showed that the initial shift in the absorption spectrum is present at a low ratio of 1:2 RB/collagen and becomes more distinct at higher dye loading with a broadening of the absorption spectra (Figure S2b). Over this concentration range, RB fluorescence decreased with a 50% lower fluorescence for the rose Bengal/collagen ratio of 0.5 (Figure S2c).

To examine the association between RB and single chain collagen in a simpler system, absorption spectra were recorded in the presence of varying concentrations of a collagen-like peptide (CLP) that contains high proportions of glycine (G), proline (P), and hydroxyproline (O), similar to the composition of collagen (≈81% homology; CG-(PKG)₄(POG)₄(DOG)₄), and was also used for the simulations described below. As the concentration of CLP increased, the RB absorption maximum at 550 nm shifted to longer wavelengths (≈570 nm) and decreased in amplitude to an even greater degree than that observed in the presence of collagen (Figure 1a). Higher concentrations of CLP (230 μM, see Figure 1b) were required to produce the maximal change in RB absorption maximum compared to that of collagen (≈1.25 μM, see Figure S1c).

We then studied the calorimetry for the binding of rose Bengal to the CLP. Figure S3a shows the enthalpy changes resulting from addition of increasing concentrations of CLP to a dye solution. At peptide concentrations larger than 200 μM, there was a plateau in the enthalpy, which can be interpreted as the endothermic assembly of the single unit peptide into a triple helix supramolecular structure. Next, we evaluated the changes in enthalpy for the binding of rose Bengal to CLP at concentrations at which the peptide is assembled (450 μM, Figure S3b). The enthalpy of formation for the RB–CLP complex is exothermic, in the range of −10 ± 2 kcal/mol, which roughly agrees with the free energies estimated by molecular simulations, vide infra.

We had initially attempted to measure heat changes for the association of RB to type I collagen by adding RB to collagen solutions. However, even adding even small quantities of the dye led to precipitation of collagen (not shown). Independent
experiments measuring the protein surface charge, $\zeta$ potential measurements, for solutions of collagen showed a 50% decrease of the protein charge upon association of the dye Figure S4.

2.1.2. Rose Bengal Association with Collagen Matrices. To investigate the binding of RB to collagen in a more tissue-like environment, we generated 3D collagen hydrogel matrices and evaluated the RB absorption spectra for three different RB concentrations (5, 10, and 40 $\mu$M; Figure S5). Control experiments indicated that the denaturation temperature of the matrices with and without the dye ($54 \pm 2 ^\circ C$, not shown) did not show a statistical difference. Rose Bengal within the collagen matrix has a maximum absorption centered at 555 nm, similar to that for collagen in solution (Figure 2). The relative absorptions at 525 and 550 nm for 40 $\mu$M RB in collagen hydrogels (right panel) were comparable to that seen for 1000 $\mu$M RB in solution (left panel), indicating that the RB concentration for aggregation was lower in the collagen hydrogels.

2.1.3. Molecular Dynamics Simulations of RB–Collagen Association. To determine the nature of RB binding to the CLP at the atomic level that might provide information for modeling RB protein photo-cross-linking in tissue, we carried out molecular dynamics simulations. The structure of the CLP triple helix was modeled based on an experimentally derived collagen structure. RB was modeled with the predominant protonation state for pH > 4.3, with $-2$ charges (Figure 3a). The simulation system is illustrated in Figure 3b. The resulting affinity free-energy map is shown in Figure 3c. Owing to electrostatic interactions, the RB molecule appears to be attracted to the positively charged N-terminus (including three NH$_3^+$ groups) and the 12 Lys residues containing NH$_3^+$ on their side chain in the N-terminal region of the CLP. It is repelled from the negatively charged C-terminus (including three carboxylate groups) and the 12 carboxylate-containing Asp residues present near this terminus. A minimum of free energy of $-5.7$ kcal/mol appears at the N-terminus. A prominent local minimum with a free energy of $-5.5$ kcal/mol occurs about 7 Å further along the CLP axis, in the region of the CLP containing Lys residues. Another local minimum occurs nearer to the center of the peptide, at the interface between the region containing Lys residues and that containing Hyp residues, having a depth of $-3.4$ kcal/mol.

The free-energy landscape shown in Figure 3c was calculated with only a single RB molecule in the system and, therefore, estimates the free energy in the limit of low RB concentration. To better understand the affinity of RB for the CLP at the higher RB concentrations that are used in vivo for PTB, we performed five independent equilibrium simulations in systems containing 20 RB molecules. Averaging over the last 200 ns of the five independent simulations (1000 ns in length), we found $16 \pm 3$ (mean $\pm$ SD) molecules within 6 Å of the center of mass of the CLP. The free-energy landscape is shown in Figure 3d along with a snapshot from one of the simulations in Figure 3e. Despite its negative charge, RB formed aggregates both in contact with the CLP and in free solution. Note that in Figure

Figure 3. Molecular simulation of RB binding to the CLP. (a) Molecular model of RB in the charge state used in the simulations. The carboxylate and phenolate moieties each have charges of $-1$. Atom color code: H, white; C, cyan; O, red; Cl, green; I, purple. (b) Snapshot of a simulation of the CLP triple helix with one molecule of RB. The CLP is shown as a green tube, Na$^+$ and Cl$^-$ ions are shown as yellow and cyan spheres. For clarity, the explicit water molecules are shown as a transparent surface. (c) Free-energy landscape for RB in the vicinity of the CLP triple helix at low RB concentration, calculated by the adaptive biasing force method. The potential of mean force is mapped as a function of the position of the RB molecule along the CLP axis ($\text{disZ}$) and distance from this axis ($\text{disXY}$). The geometric contribution to the free energy along $\text{disXY}$ has been removed. (d) Free-energy landscape at a higher RB concentration calculated from a set of equilibrium simulations including a CLP triple helix and 20 RB molecules. (e) Snapshot from the simulation with 20 RB molecules.
Plotted are the number of H-bonds involving all CLP groups (CLP), backbone amide nitrogens (BB), N-terminal NH₃⁺, and Lys NH₃⁺. Dominant contribution from N-terminal and Lys NH₃⁺ groups. Most H-bonding occurs at and near the N-terminus, with the approximate locations of CLP side chains relevant for binding energy landscape are indicated in Figure 4e, along with the locations of the conformational microstates of the RB. An ionic Lys–O⁻ contact is also apparent. The free-energy landscape of RB near the CLP with labels (a–d) indicating the location of RB in the corresponding panels of this figure. The ranges of disZ values occupied by the N-terminus and Asp, Hyp, and Lys side chains are also indicated. The prevalence of H-bonding between RB and different groups of the CLP as a function of position along the CLP axis. Plotted are the number of H-bonds involving all CLP groups (CLP), backbone amide nitrogens (BB), N-terminal NH₃⁺ groups (Nter), Hyp side chain OH groups, and Lys side chain NH₃⁺ groups. (g) The prevalence of H-bonding between the CLP and different groups of RB as a function of position along the CLP axis.

3d, the electrostatic shielding due to the presence of many RB molecules reduces the affinity at the N-terminus, leading to a relatively uniform strong binding over the N-terminal half of the CLP.

We then sought to determine the atomic-scale interactions that underlie the RB affinity of different regions of the peptide model. The highest affinity was seen at the N-terminus where the N-terminal NH₃⁺ and NH₃⁺-containing Lys side chains make contact with the negatively charged groups of RB. Figure 4a shows an exemplary snapshot including an ionic interaction and H-bonding between an N-terminus and the carboxylate moiety of RB. Although a majority of configurations associated with the global free-energy minimum included ionic contacts, a few, such as that shown in Figure 4b, exhibited no close contact between charged groups. We find no water molecules between the three-ring motif of RB and the Pro side chains, indicating that hydrophobic collapse may drive RB binding, in addition to longer-range electrostatic interactions. Farther from the N-terminus, binding of RB was dominated by ionic contacts with the NH₂⁺ of Lys residues, an example of which is shown in Figure 4c. It might be noted that considerable nonplanarity of the three-ring motif of RB is sometimes observed when the phenolate O⁻ atom of RB makes contact with NH₂⁺ groups, as in Figure 4c. A local free-energy minimum at (disXY, disZ) = (10.1, –17.9 Å) appears to correspond to contact between the phenolate O⁻ atom of RB and NH₂⁺ of Lys residues of the collagen-like peptide, as well as H-bonding between the carboxylate of RB and OH groups of Hyp (Figure 4d). The locations of the configurations in Figure 4a–d on the free-energy landscape are indicated in Figure 4e, along with the approximate locations of CLP side chains relevant for binding RB.

The prevalence of H-bonds between RB and various groups of the collagen-like peptide are quantified in Figure 4f,g. The most H-bonding occurs at and near the N-terminus, with the dominant contribution from N-terminal and Lys NH₃⁺ donors (Figure 4f). Comparison to Figure 4g demonstrates that these terminal H-bonds predominantly involve the carboxylate and phenolate oxygens of RB. Near disZ = –40 Å, H-bonds involving the backbone of the CLP or the carboxyl oxygen of RB are also present. In the region of the CLP for disZ > 10 Å, few H-bonds are observed, and instead it is dominated by contacts between the carboxylate group of RB and the OH groups of the Hyp residues of CLP (exemplified in Figure 4d).

In the experiments, it was noted that a small fraction of the peptide did not form the conventional collagen triple helix, but remained free in solution. This motivated us to simulate a single collagen-like peptide chain together with a high concentration of the dye (20 molecules) to understand the binding mode of RB when the peptide does not form a triple helix. We found that the peptide adopted an ensemble of disordered compact structures, which contrasted with the well-defined linear arrangement of the triple helix. However, formation of RB aggregates both close to the peptide and in solution was observed. Averaging over the last 10 ns of an independent simulation (100 ns in length), we found 8 ± 1 (mean ± SD) molecules within 5 Å of the peptide (not shown).

2.2. Photochemical and Photophysical Behavior of Rose Bengal in Solution and 3D Collagen Matrix. In our previous study, the RB photodegradation rate was lower in collagen-containing solutions, indicating that the dye photoreactivity in tissue might be influenced by its association with collagen. To begin to understand the RB photochemistry that initiates collagen cross-linking, we studied the dye photodegradation at high concentrations in aqueous solution where aggregates dominate, roughly mimicking the aggregates formed with collagen. These results were compared to RB photodegradation at low concentrations, where mainly monomeric species are present, in solutions containing collagen, and in the 3D collagen hydrogels. We also evaluated the effects of certain amino acids on RB excited triplet state lifetime and photodegradation in solutions and hydrogels to...
further characterize potential pathways initiating protein cross-linking.

2.2.1. Photodecomposition of Rose Bengal Alone in Solution. The absorption spectrum of 125 μM RB in phosphate-buffered saline (PBS) showed the typical absorption maximum at 550 nm with a ratio of absorption at 550–520 nm as approximately 2, an indication that rose Bengal exists as dimers and aggregates at this concentration. Monomeric RB shows a ratio of approximately 3. In O2 and air, the absorbance at 550 nm decreased without a change in the spectral shape and product(s) absorbing at approximately 450 nm formed (Figure S6a,b). In a N2 atmosphere, the RB absorption decreased with a slight broadening of the 550 nm peak to longer wavelengths and without formation of a 450 nm absorbing product (Figure S6b). Photodecomposition was less efficient in N2, as shown by the greater RB absorption at the end of the irradiation period.

2.2.2. Influence of Arginine, Lysine, and Ascorbic Acid on Rose Bengal Photodegradation in Solution. Arginine and lysine are potential electron donors to the rose Bengal triplet state and, because they are positively charged at pH 7, are present in collagen chains at reasonable levels; they are also likely to be association sites for RB with collagen, as indicated by the simulations described above. Irradiations carried out on 125 μM RB solutions containing 25 mM Arg showed that the dye photodegraded more rapidly in N2-saturated compared to O2-saturated solutions. Significantly, the absorption maximum under N2 blue-shifted to 510 nm (Figure 5a), which is consistent with the formation of a deiodinated fluorescein product that would be formed after initial electron transfer to the RB excited triplet state. In an air atmosphere, only a 10 nm red shift was observed, and in O2-saturated solutions, total photobleaching occurred without an absorption maximum shift (Figure 5b).

A similar set of irradiations carried out using 25 mM Lys had little effect on RB photodecomposition, except under N2 where it decreased the degradation rate by about 3.5 fold. Ascorbate (1.25 mM), another biological electron donor, increased the RB photodegradation rate in air and N2 atmospheres compared to the dye alone, and the absorption maximum shifted toward a 510 nm absorbing product (Figure S7).

The photobleaching rate for monomeric rose Bengal (10 μM) was greater under N2 than in an air-saturated solution (Figure 6a), the opposite of that found for RB aggregates. However, similar to what is seen for the rose Bengal aggregates, Arg enhanced the dye photodegradation. Azide blocked the RB photodegradation in air, which suggests singlet oxygen mediated dye degradation, and carrying out the irradiation in deuterium oxide buffer had little effect initially but appeared to increase the RB photodecomposition after 15 min irradiation, which may suggest participation of singlet oxygen in the photodecomposition mechanism.

2.2.2.1. Transient Absorption Measurements in Solution. Because we have now shown that arginine enhances the rate of rose Bengal photodecomposition, we asked whether this might result from direct interaction of the RB excited triplet state with Arg. The triplet decays for rose Bengal at 620 nm in the absence and presence of 0.5 mM Arg showed quenching with a rate constant of 0.17 × 108 M−1 s−1. The tryptophan quenching rate constant for rose Bengal triplet was considerably higher than that for Arg (3.0 × 106 M−1 s−1) in the same buffer system, see Figure S8, which is consistent with our previous report. No RB triplet state was observed when the solutions contained 2.5 μM collagen, the conditions under which RB appears to be entirely bound as aggregates to collagen.

2.2.3. Photodegradation of Rose Bengal in Collagen Solutions. To examine the effect of binding to collagen on RB photodegradation, we irradiated solutions containing 2.5 μM native type I collagen and 10 μM RB. As shown in Figure 6b, the absorption spectrum indicated that the dye was aggregated in association with collagen as previously reported. Photodegradation was enhanced by Arg (18%), D2O as solvent.
found for solutions of dye and collagen. Irradiation in an air atmosphere decreased the dye absorption and shifted the absorption maximum to the blue, which is similar to the found for solutions of dye and collagen. RB was irradiated at pH 7.4 with a green LED centered at 525 nm. As shown in Figure 7 (left), the RB absorption maximum is ≈556 nm as

2.2.4. Photodegradation of Rose Bengal in Collagen Hydrogel Matrices. In the tissue-like environment of hydrogels prepared from native type I porcine collagen, RB was irradiated at pH 7.4 with a green LED centered at 525 nm. As shown in Figure 7 (left), the RB absorption maximum is ≈556 nm as

Figure 7. Photodegradation of 40 μM RB incorporated within collagen hydrogels. Left: Absorption spectra for the RB–collagen hydrogel composite measured at different irradiation times in an air atmosphere. Right: Changes in absorption intensities measured at 556 nm for RB solutions recorded in the presence of different additives [Azide: 10 mM, Trp: 2 mM, Arg: 10 mM]. All data collected in PBS buffer pH 7.4 at room temperature. Data correspond to the average calculated from three independent experiments each repeated in triplicate (n = 9).

Figure 8. Triplet transient lifetime and quenching of RB embedded in collagen hydrogels in the presence of different quenchers. (a) RB triplet decay monitored at 620 nm measured in air (black circles) or nitrogen (blue circles) saturated solutions. Insets correspond to decay residuals obtained from the exponential fit for the decays shown in the figure. Measured lifetimes for: (b) RB triplet, (c) RB anion radical, and (d) singlet oxygen under different conditions or additives. Concentrations of additives were: tryptophan: 2.0 mM, Arg: 10 mM, and sodium azide: 10 mM. Effect of additives for laser flash photolysis experiments were carried out in nitrogen saturated solutions. For singlet oxygen measurements, samples were equilibrated with air prior to laser excitation. Measurements for triplet and anion radical were carried out in 10 mM pH 5.0 MES buffer. For singlet oxygen phosphorescence, a 10 mM MES buffer was prepared with a pH of 5.0. In all cases, photodegradation was kept lower than 10%. Time traces correspond to the average of 12 separate decays from 3 independent samples.

2.2.5. Transient Absorption Measurements in Collagen Matrices. An investigation of the RB-derived transient species in the hydrogels showed the presence of a short-lived dye triplet (620 nm absorption maximum; lifetime = 3.5–4.0 μs; Figure 8a), which is significantly shorter than that in the collagen-free solutions (∼100 μs, see Figure S8). Thus, in the matrices, the triplet lifetime appears to be limited by competitive processes such as electron transfer to the protein or intra-aggregate excitation decay routes. The rose Bengal triplet lifetime was not influenced by tryptophan although Arg and azide both significantly quenched the RB triplet (Figure 8b). The RB anion radical was detected at 420 nm, which is consistent with electron transfer from collagen or ground state RB. It was quenched by O2 and partially quenched by Arg, Trp, and azide (Figure 8c). Interestingly, O2 luminescence at 1270 nm was detected for oxygenated samples, and this emission was readily quenched by sodium azide (Figure 8d), a well-known charge transfer deactivator for singlet oxygen.

2.2.6. Tryptophan-Mediated Photodegradation in Rose Bengal Containing Collagen Matrices. To further assess the photoreactivity of RB in collagen matrices, we studied its ability to promote degradation of tryptophan. Collagen matrices containing RB were incubated with 2.0 mM Trp and then

exposed to the green LED. Changes in Trp fluorescence intensity at 370 nm upon excitation at 295 nm are shown in Figure S9. In the absence of Trp, no emission was detected. Figure S9a shows a fast decrease for the Trp emission intensity within the first 5 min of irradiation, which then remains largely unchanged (see Figure S9b). To assess the contribution of rose Bengal photoproducts toward the generation of photochemically active species, we pre-irradiated a set of samples of RB–collagen matrices for 30 min and we then incubated those samples with Trp followed by green light irradiation, see Figure S9b. For those samples, the emission values remained within experimental error, and practically unchanged when compared to time 0.

3. DISCUSSION

The results of these investigations suggest a first approximation of the processes involved in PTB for tissue healing. In this model, aggregates of rose Bengal bind to tissue collagen at positively charged amino acids and less avidly by hydrophobic forces at other sites. Triplet excited state RB accepts an electron from arginine side chains and possibly other sources when the O2 level is low. Subsequent reactions of the RB anion radical or arginine cation radical may initiate protein cross-linking. At higher O2 levels, cross-linking may be initiated by reactions of •O2−.

The two-dimensional free-energy landscapes as a function of distance along the triple helix and the distance from this axis shown in Figure 3, revealed two strong electrostatic interactions with energies from −5.7 to −3 kcal/mol between the two negative charges of rose Bengal and positively charged amino groups of the three N-termini and of the 12 Lys amino acids, visible in the lower half of Figure 4e. These electrostatic interactions are possible for both single chain and triple helical

DOI: 10.1021/acsomega.7b00675
ACS Omega 2017, 2, 6646−6657
collagen, and explain our observation that RB binding to native and denatured collagen was nearly identical (Figure S1). In addition to the electrostatic binding modes for the association, hydrophobic interactions between the three-ring portion of the dye structure and Pro side chains were seen (Figure 4c). Notably, a nonplanarity of the three-ring motif was also observed. This distortion in the ring planarity can be linked to changes in electronic density and uniformity of the dye molecular orbitals, which leads to perturbations in the dye absorption spectrum fingerprinting at 525 nm.

Multioccupation or cooperative binding of rose Bengal to collagen is a plausible explanation for the formation of rose Bengal aggregates with collagen.31 In fact, the multioccupation/cooperative binding of rose Bengal to the collagen structure, which results in a net change of protein surface charge, explains our unsuccessful attempts to carry out ITC by adding dye to collagen that resulted in precipitation of the protein due to changes in the protein charge (Figure S4). ITC measurements carried out for the CLP indicate that assembly of the peptide into a triplet helix occurs at >200 μM, see Figure S3, which is in good agreement with the data collected for binding of rose Bengal to CLP, see Figure 1. Consistent with the molecular dynamics simulations, association of rose Bengal to the peptide showed exothermic behavior, with values close to the calculated values (Figures 3 and 4). Similarly, for the experiments embedding rose Bengal within the collagen matrix, the presence of molecular aggregates was evident at 40 μM of dye, a concentration that does not show the presence of aggregates in aqueous solutions, see Figure 2.

The binding of rose Bengal to collagen differs from its previously reported association with other proteins. For example, binding in pockets on human serum albumin produced a similar red shift in the RB absorption spectrum. However, a 7-fold increase in the RB fluorescence emission was observed,25,31 whereas collagen binding produced a decrease in the dye fluorescence, see Figure S2c. For albumin−RB, the increase in emission was attributed to the transfer of the dye to a confined environment, wherein hydrogen bonding with water was no longer favored. For rose Bengal and collagen, the binding modes described for the CLP might lead to a more effective nonradiative deactivation, that is, intracomplex electron transfer, and consequently decreased fluorescence intensity. The collagen structure differs substantially from that for albumin. The triple helices are closely packed into fibrils that do not allow RB molecules to enter the fibril. Consequently, binding of rose Bengal to collagen in tissues occurs on the outer collagen fibril surfaces and involves the associations described by the molecular dynamics simulations.

Although almost all previous studies of RB photochemistry in aqueous solution have focused on monomeric RB, we investigated aggregated as well as monomeric rose Bengal because an ~1.0 mM solution is used for medical applications and, at this high concentration, the dye exists as aggregates. In fact, the absorption spectrum of rose Bengal applied to cornea showed the red-shifted absorption spectrum and the <2 ratio of absorbance at 560−525 nm characteristic of aggregates bound to collagen.8,22

Rose Bengal photochemistry in aggregates has differences and similarities with that for monomeric RB. For example, although both aggregated and monomeric RB photobleach in O2 without a spectral shift, only aggregated RB forms product(s) absorbing around 450 nm (Figure S6). These products may involve adjacent RB molecules in the aggregate, although their identity awaits further investigation. The mechanism for RB photobleaching in O2 also appears to differ. Irradiation of monomeric rose Bengal generates 1O2, which then oxidizes the xanthone ring to colorless products, a process supported by the enhancement by D2O and quenching by azide of RB photobleaching (Figure 6). In contrast, in a previous report, RB triplets were not detected in RB aggregates bound to collagen thus precluding a role for 1O2.22

Electron transfer from Arg to RB triplet may initiate collagen−collagen cross-linking because arginine enhanced the RB photodegradation rates of both aggregated and monomeric dye (Figures 5 and 6) and quenched the RB triplet (Figures S8 and 8). Early studies of photoreduction of monomeric RB by amines identified dehalogenation products, especially the 510 nm absorbing 2,7-diiodotetrachlorofluorescein.20 Our results show the appearance of this product clearly for aggregated RB in the presence of Arg under N2, considerably less formed in air, and practically none in oxygen-saturated solutions (Figure 5). Significantly, this result suggests that triplet quenching within the aggregates does not preclude reactions with external electron donors. Dehalogenation results from initial electron transfer from Arg to the dye triplet producing the RB anion radical and the amine cation radical. Subsequent deprotonation of the amine cation radical and rearrangement steps produce radical intermediates that could lead to protein−protein cross-linking. However, substantial yields of colorless products also form because the amount of dehalogenated product (based on the absorption coefficient for fluorescein) is much smaller than the amount of rose Bengal destroyed. These products may be the dihydro dye formed by initial electron transfer from ground state RB to RB triplet.19,32

Rose Bengal photodegradation, when associated with native type 1 collagen in hydrogels (Figure 7), may mimic the photoreactions occurring in tissues during photo-cross-linking. The decrease in RB absorption was accompanied by a blue shift of the peak (Figure 7) indicating dehalogenation, which is similar to the result obtained for RB aggregates in the presence of Arg and ascorbate (Figures S7 and 5). If so, it would suggest that the electron-donating amino acids in native collagen are able to act as reaction partners of aggregated rose Bengal triplet excited state to initiate a radical pathway potentially involved in collagen cross-linking. Because Arg but not Lys increased RB photobleaching, the electron donor may be arginine. Side chains of both amino acids are positively charged and capable of participating in binding RB through ionic interactions, as shown for lysine in the CLP (Figures 3 and 4). In fact, in a recent study by part of our team, we used a polymeric form of lysine (poly-1-lysine), in the rose Bengal cross-linking of acrylate-modified collagen, which resulted in an enhancement of the composite elasticity along with preventing the dye photodegradation.33 Those findings point toward possible intramolecular reactions between a rose Bengale reactive intermediate and the Lys residues, which resulted in the regeneration of the dye.

Arginine also effectively enhanced photobleaching of RB aggregates bound to collagen both in solution (Figure 6) and in collagen hydrogels (Figure 7). In hydrogels, triplet rose Bengal was readily quenched by Arg (Figure 8), and a further shift of the absorption spectrum toward the 510 nm deiodinated product was observed, see Figure 7. Thus, it appears that Arg reacts even more efficiently with triplet excited state rose Bengal in aggregates than the reducing amino acids in native collagen. If this observation is substantiated in tissues, it would
suggest that Arg might be used to enhance the efficiency of rose Bengal photosensitized medical treatments, assuming that electron transfer processes initiate protein–protein cross-linking. Interestingly, rose Bengal triplet lifetime was not influenced by tryptophan when bound to the collagen matrix, which suggests limited diffusion/accessibility of the quencher within the 3D matrix (Figure 8b).

Oxygen may play different roles in photosensitized protein cross-linking depending on the tissue being treated and the treatment conditions. The normal O2 level in tissues decreases during photosensitization because O2 is removed by reactions of 1O2 with proteins and lipids.34 Depending on the depth in tissue and the irradiance, O2 may not be replenished sufficiently rapidly by diffusion, and energy transfer from rose Bengal triplet to form 1O2 becomes inefficient. In this case, initial electron transfer to triplet state RB is favored and dehalogenation would be predicted as observed for the dye in collagen hydrogels (Figure 8). In the collagen hydrogels, 1O2 was observed but RB triplet lifetime was unchanged, suggesting that a process such as electron transfer dominates. Possibly, an initial electron transfer pathway for protein cross-linking could still show enhancement in O2 if intermediates trapped by O2 lead to cross-linking. Further studies to evaluate these possibilities are planned.

4. SUMMARY AND CONCLUSIONS

Binding of rose Bengal to collagen is a complex process that includes competitive binding of dye monomer and aggregate to the protein chains. Molecular dynamics simulation of RB binding to a collagen-like peptide indicated that up to 16 ± 3 rose Bengal molecules can bind to an assembled peptide triple helix, with many bound as aggregates. Ionic interactions between negatively charged RB and protonated amino groups dominated contributions to the global free-energy minimum. In solution at low oxygen level, Rose Bengal photodegradation produced a 510 nm species, likely a dehalogenation product in the presence of arginine. Within a tissue-mimetic collagen matrix, irradiation yielded the 510 nm product suggesting that amino acids in the collagen chains are able to donate electrons to RB triplet and potentially initiate a protein cross-linking mechanism. Surprisingly, nonquenchable RB triplets and singlet oxygen were detected in the collagen matrices.35 Although additional work is still needed, our work presents a comprehensive study on the role of rose Bengal–collagen interactions that may allow the design and engineering of novel dyes and materials for tissue photo-bonding.

5. MATERIALS AND METHODS

5.1. Materials. Rose Bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetra-iodofluorescein) sodium salt (≥99%), L-lysine, and L-ascorbic acid were obtained from Sigma-Aldrich. Type I medical grade porcine collagen (TheraCol) was purchased from Sewon Cellontech Co. Ltd. (Seoul, South Korea). Unless otherwise indicated, all solutions were prepared using Milli-Q water.

5.2. Effect of Collagen and Collagen-Like Peptides on Rose Bengal Spectroscopic Properties. The effect of macromolecule addition on the absorption spectrum of rose Bengal was studied in a manner similar to that previously described by part of our team.22 All measurements in solution were carried out in 10 mM MES buffer (pH 5.0) in which collagen remains soluble and assembled. Rose Bengal solutions (20 μM) prepared in MES buffer were mixed in a 1:1 ratio with collagen solutions with concentrations up to 5.0 μM. The absorption spectra were measured in 1.0 cm cuvettes (Luzchem Inc.) using a Cary-100-Bio UV–visible spectrophotometer at room temperature (300–700 nm). Measurements were carried out using either native or thermally denatured collagen (95 °C for 5 min). Data shown correspond to the average from three independent experiments, each one carried out in quadruplicate. A similar protocol was used for the collagen-like peptide (CLP), where a higher concentration (up to 460 μM) was required to observe spectral changes similar to those observed for type I collagen.

5.3. Changes in Collagen Surface Charge. Changes in ς potential for type I porcine collagen upon addition of the increasing concentrations of rose Bengal were carried out in a Malvern Zetasizer Nano ZS at 20 °C in 1.0 cm pathlength disposable ς potential cuvettes. Reported values correspond to the average of three independent batches, each measured in triplicate.

5.4. Isothermal Titration Calorimetry (ITC). ITC titrations were performed on a VP-ITC MicroCalorimeter from Malvern at 22 °C by using 10 μL injection with a total of up to 10 injections. Measurements were carried out in 10 mM MES buffer at pH 5.0. Data were processed using the Microcal Origin software. Two different sets of experiments were carried out using ITC. In the first set of experiments, increasing concentrations of CLP were added from a 10 mM concentrated solution up to 600 μM. In the second set of experiments, RB was added to a 450 μM CLP solution prepared in 10 mM, pH 5.0 MES buffer.

5.5. Collagen Matrix Preparation and Characterization. Briefly, type I medical grade porcine collagen was mixed with phosphate buffer saline pH 7.4, and cross-linked with 1.5% glutaraldehyde in ice. Unreacted aldehydes were deactivated with glycine. Rose Bengal was added to the mixture at a final concentration of 40 μM. The resulting viscous matrix, which cross-links in ≤30 min at 37 °C, has pore sizes in the range of 60 ± 10 μm as indicated by CRYO-SEM measurements for the collagen matrices.

The micromorphology of the collagen matrices was assessed using low temperature scanning electron microscopy (Cryo-SEM) in a Tescan (model: Vega II–XMU) equipped with a cold stage sample holder at −50 °C using a backscattered electron detector (BSE) and a secondary electron detector (SED). Pore sizes were measured from at least 400 individual pores using ImageJ software, similar to that described for other collagen matrices.36

Differential scanning calorimetry measurements were carried out for the hydrogels with and without the different concentrations of RB. Once chemically cross-linked, the hydrogels were thoroughly washed with PBS buffer pH 7.4 for 12 h prior to measuring their glass-transition temperature (Tg) in a Q2000 differential scanning calorimeter (TA Instruments). Heating scans were recorded within the range of 8–80°C at a scan rate of 10°C/min. Tg is defined as the onset of the endothermic peak.

5.6. Computer Simulations. 5.6.1. Generation of Molecular Models. As a first step, the SWISS-model server was used to generate the homology model of the collagen-like peptide (CLP) from the sequence CG(PKG)4(POG)4(DOG)4 (where O represents the amino acid hydroxyproline). On the basis of the sequence identity of 81.48% between collagen and
this collagen-like peptide, we used an experimentally derived collagen structure (PDB ID: 1NAY) as a template. As the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%, the structure generated for the percentage of coverage of the target sequence with respect to the template structure was 81%. The adaptive biasing force was applied on RB in the plane orthogonal to this axis. The adaptive biasing force as a function of position along the axis of the triple helix was applied to calculate the free energy as a function of two collective variables, disZ and disXY, that is, disZ = ρ = (X² + Y²)^(1/2). This produced a two-dimensional potential of mean force as a function of position along the axis of the triple helix and in the plane orthogonal to this axis. The adaptive biasing force method was implemented through the Colvars module of NAMD 2.12. The first collective variable was sampled using a multiple window scheme, with a total of 7 windows on the interval −70 ≤ disZ ≥ 70 Å. The second collective variable was sampled in a single window on the interval 0 ≤ disXY ≥ 25 Å for each window in disZ. Each window was simulated for at least 340 ns, for a total of 2400 ns over all windows. The deviation of the mean force in this work are shown with this geometric contribution removed, that is, the free energy far from the peptide axis is uniform rather than decreasing as −kB T ln ρ, due to the increasing amount of phase space for larger radii, 2πρ dρ.

5.6.3. Classical Molecular Dynamics Simulation. All simulations were performed in the molecular dynamics software NAMD 2.12 and the all-atom CHARMM36 force field. Using the Langevin thermostat and Langevin piston method, we maintained the temperature and pressure at 300 K and 101.325 kPa (1.0 atm), respectively. A smooth 8 Å cutoff of van der Waals forces was employed. The particle-mesh Ewald algorithm was used to compute the electrostatic interactions with a mesh spacing of <1.2 Å. The length of covalent bonds involving hydrogen atoms was constrained to the values prescribed by the CHARMM force field. The masses of nonwater hydrogen atoms were increased by a factor of 3 (to 3.0240 Da) by transferring mass from the heavy atom to which they were attached, allowing us to integrate the equation of motion with a time step of 4 fs with no appreciable loss of accuracy in thermodynamic quantities. All systems were relaxed for 25 000 steps of energy minimization followed by 20.0 ns of equilibration before beginning 250 ns of data production or free-energy calculations. For convenience, orientational restraints were applied to keep the long axis of the collagen-like peptide triple helix aligned along the Z axis using the Colvars module. As these restraints were applied as a torque to the entire triple helix, they do not lead to any bias in the protein conformation nor in its interaction with RB. H-bonds were identified using the criteria of donor acceptor distances <3.5 Å and donor−H−acceptor angles > 120°.

5.6.3.3. Free-Energy Calculations. Beginning with the equilibrated systems, the adaptive biasing force method was applied to calculate the free energy as a function of two transition coordinates: disZ, the position of RB along the Z axis with respect to the center of mass of the peptide, and disXY, the distance between the dye and the Z axis (passing through the center of mass of the peptide), that is, disXY = ρ = (X² + Y²)^(1/2). This produced a two-dimensional potential of mean force as a function of position along the axis of the triple helix and in the plane orthogonal to this axis. The adaptive biasing force method was implemented through the Colvars module of NAMD 2.12. The first collective variable was sampled using a multiple window scheme, with a total of 7 windows on the interval −70 ≤ disZ ≥ 70 Å. The second collective variable was sampled in a single window on the interval 0 ≤ disXY ≥ 25 Å for each window in disZ. Each window was simulated for at least 340 ns, for a total of 2400 ns over all windows. For systems with higher concentrations of RB, the free energy was estimated through straightforward equilibrium sampling, rather than by the adaptive biasing force method. Five systems containing the collagen-like peptide and 20 randomly placed RB molecules were created and simulated for 250 ns each with no biases. The free energy as a function of disZ and disXY was inferred from the average position distribution of RB molecules in the five systems during the last 200 ns of simulation (after 50 ns of equilibration). Because disXY is a cylindrical radial coordinate, it possesses a nonuniform geometric (Jacobi) contribution to the potential of mean force. The plots of the two-dimensional potentials of mean force in this work are shown with this geometric contribution removed, that is, the free energy far from the peptide axis is uniform rather than decreasing as −kB T ln ρ, due to the increasing amount of phase space for larger radii, 2πρ dρ.

5.7. Light-Mediated Degradation of Rose Bengal in Homogenous Solution and 3D Collagen Matrix. Sample solutions (570 μL) were irradiated in a plastic well (circular area = 1.9 cm², pathlength = 3 mm) in a cylindrical chamber (height = 2 cm, inner diameter = 4.5 cm) with removable poly(methyl methacrylate) windows and gas inlet and exit ports on the sides. Laser light (532 nm; CW KTP frequency-doubled laser, Oculight OR, IRIDEX Corporation, Mountain View, CA) was delivered via an optical fiber through the upper window and centered on the sample well with an irradiance of approximately 0.3 W/cm² at the sample surface.

The gas composition within the irradiation chamber was changed between N₂, O₂, and air (20% O₂). Before an irradiation, the chamber was purged with N₂ or O₂, then the flow was stopped for 5 min to allow diffusion into the solution. This process was repeated three times. During the irradiation, a moderate flow of gas was allowed. For experiments in an air atmosphere, the top chamber window was removed, and the power of laser was lowered by 8.0% to compensate for the loss of light when the window is used.

Prior to irradiation and periodically during the irradiation, spectra of the samples were recorded without opening the irradiation chamber. A polychromatic halogen lamp was placed above the chamber and the transmitted light was collected with an integrating sphere (model FOIS, Ocean Optics) and analyzed with an Ocean Optics QEPro spectrometer. The laser light delivery fiber was moved from the irradiation position during each transmission measurement. Changes in transmission at the selected wavelength as a function of irradiation time were analyzed. Absorbance at 550 nm was used unless the absorbance of the initial spectrum of the 125 μM RB solution was higher than 2 when absorbance at 515 nm was also analyzed. The absorbance was calculated by taking the average of values of three adjacent data points to minimize the effect of noise in signals.

Irradiation of collagen solutions and hydrogels was carried out using a custom-made irradiation system equipped with a 523 ± 5 nm LED (LZ4-00G110 LED unit, LedEngin, Inc.) mounted on a PAR25 LED Cooler 32 W Synjet that dissipates the heating produced by the light source. The light source was collimated with an aluminum tube (ø 10 mm) into a 1.0 cm optical pathlength cuvette holder (CUV-UV, Ocean optics). Total light fluence was matched to the amount delivered in the experiments using the continuous wave laser detailed above, but using half of the light irradiance, ≈0.15 W/cm².

5.8. Rose Bengal Laser Flash Photolysis. RB triplet transient absorption measurements were carried out in a LFP 111 laser flash photolysis system (Luzchem Inc., Ottawa, Canada) equipped with a Surlite OPO Plus (pump with a Nd:YAG 355 nm) operating at 550 nm and 10 mJ/pulse in 1.0 cm pathlength fused silica cuvettes (Luzchem Inc.). Triplet
absorption of RB was measured at 620 nm under conditions of minimal dye bleaching (less than 5%). Two different sets of experiments were carried out using time-resolved techniques: (1) Effect of amino acids on the triplet lifetime of RB in solution: Measurements were carried out in 10 mM MES buffer pH 5.0 using a 10 μM dye concentration. Samples were degassed using 99.99% pure N2 for 1 hour and increasing concentrations of L-arginine (Arg) and L-tryptophan (Trp) were added and the lifetime of RB triplet excited state was measured. Bimolecular rate constants were calculated from plotting k_{obs} = k_0 + k_{tr}c[Q], where k_{obs}, k_0, and k_{tr} correspond to the observed rate constant, rate constant in the absence of the quencher, and the bimolecular rate constant, respectively. (2) Effect of quenchers on RB reactive intermediates incorporated within collagen hydrogels: Collagen matrices were prepared as described above and the dye was incorporated within the 3D structure before solidification. The RB concentration was kept at 40 μM. Hydrogels were equilibrated for 3 hours prior to measurements in solutions of the quenchers (Arg [5.0 mM], Trp [1.0 mM], and sodium azide (NaN3 [10 mM]) prepared in phosphate buffer with pH 7.4. Samples were degassed using 99.99% pure N2 for 2 hours prior to measurements. In addition to the measurements for the triplet absorption at 620 nm, we monitored the bleaching of the ground state at 550 nm, and cation and anion radicals at 480 and 420 nm, respectively. Additional measurements for 1O2 emission upon excitation of the dye incorporated within the collagen matrix were also carried out. Singlet oxygen generation was quantified by following its phosphorescence decay at 1270 nm with a Hamamatsu NIR detector (peltier cooled at −62.8 °C operating at 800 V) after 550 nm laser excitation Surlite OPO Plus (pump with a Nd-YAG 355 nm), 10 mJ/pulse. Data were acquired and processed with a customized Luzchem Research software. Measurements were carried out in deuterated PBS buffer pH 7.0.

5.9. Statistical Analyses. Student’s t-test (unpaired data with unequal variance) using a confidence interval of p < 0.05 was considered to identify statistically significant differences. Analyses were carried out in Kaleida Graph 4.5.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00675.

Effect of type I porcine collagen, up to 2.5 μM, on the absorption spectra of 10 μM RB; representative circular dichroism spectra for a 1.25 μM rose Bengal solution with or without 5.0 μM type I collagen; isothermal titration calorimetry measurements for binding of rose Bengal to a collagen-like peptide (CLP); ζ potential measurements for 2.5 μM type I collagen in 10 mM pH 5.0 MES buffer in the presence of different rose Bengal concentrations; schematic representation for the preparation of RB containing collagen hydrogels; photodecomposition of rose Bengal (125 μM) under oxygen, air, and nitrogen saturated solutions; photodecomposition of RB (125 μM) in the presence of 1.25 mM sodium ascorbate in oxygen, air, and nitrogen saturated solutions; triplet transient lifetime and quenching of RB excited state in the presence of different quenchers; tryptophan degradation mediated by green light exposure in the RB containing collagen matrix (PDF)

AUTHOR INFORMATION

Corresponding Authors
*E-mail: ealarcon@ottawaheart.ca (E.I.A.).
*E-mail: jeffcomer@ksu.edu (J.C.).

ORCID

Emilio I. Alarcon: 0000-0001-5100-6179
Jeffrey Comer: 0000-0003-4437-1260

Present Address

*Department of Chemistry, Korean Advanced Institute of Science and Technology (KAIST), Yuseong-gu, Daejeon 305-701, South Korea (H.G.R.).

Author Contributions

The manuscript was written through contributions of all authors.

Funding

This work was made possible by funding from the Natural Sciences and Engineering Research Council (NSERC, RGPIN-2015-0632) and from the Canadian Institutes of Health Research (CIHR) to E.I.A. E.I.A. also thanks UOHI start-up grant 1255. E.I.A. and I.E.K. thank the Burroughs Wellcome Fund for a Travel Grant. H.G.R. thanks Núcleo Científico Multidisciplinario de la Universidad de Talca, Chile, and grant no. 1171155 from Fondecyt, Chile. J.C. was partially supported by the Kansas Bioscience Authority funds to the Institute of Computational Comparative Medicine (ICCM) at Kansas State University and to the Nanotechnology Innovation Center of Kansas State University (NICKS). Computing for this project was performed on the Beocat Research Cluster at Kansas State University, which is funded in part by NSF grants CNS-1006860, EPS-1006860, and EPS-0919443. This work also used the Extreme Science and Engineering Discovery Environment (XSEDE), which is supported by National Science Foundation grant number ACI-1053575. H.G.R. was supported by the Harvard-MIT Summer Institute at MGH, National Science Foundation Grant EEC-1358296.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to thank Professor Tito Sciaiano for the time-resolved measurements. E.I.A. thanks Michel Grenier from University of Ottawa for his assistance with the laser flash photolysis experiments. The authors would like also to express their gratitude to Dr. May Griffith from University of Montreal for providing a sample of the CLP peptide. I.E.K. and H.G.R. would like to thank Dr. Walfre Franco and Antonio Ortega-Martinez for technical support.

ABBREVIATIONS

Arg, L-arginine; CLP, collagen-like peptide; PTB, photothermal tissue bonding; RB, rose Bengal; Trp, L-tryptophan

REFERENCES

(1) Chan, B. P.; Amann, C.; Yaroslavsky, A. N.; Title, C.; Smink, D.; Zairis, B.; Kochevar, I. E.; Redmond, R. W. Photothermal repair of Achilles tendon rupture in a rat model. J. Surg. Res. 2005, 124, 274–279.
(2) Fairbairn, N. G.; Ng Glazier, J.; Meppelink, A. M.; Randolph, M. A.; Valero, I. L.; Fleming, M. E.; Kochevar, I. E.; Winograd, J. M.; Redmond, R. W. Light-Activated Sealing of Acellular Nerve Allografts following Nerve Gap Injury. J. Reconstr. Microsurg. 2016, 32, 421–430.
(3) Mulroy, L.; Kim, J.; Wu, I.; Scharper, P.; Melki, S. A.; Azar, D. T.; Redmond, R. W.; Kochevar, I. E. Photochemical keratodesmos for repair of lamellar corneal incisions. Invest. Ophthalmol. Visual Sci. 2000, 41, 3335−3340.

(4) O’Neill, A. C.; Winograd, J. M.; Zeballos, J. L.; Johnson, T. S.; Randolph, M. A.; J. E.; Kochevar, I. E.; Redmond, R. W. Microvascular anastomosis using a photochemical tissue bonding technique. Lasers Surg. Med. 2007, 39, 716−722.

(5) Senthil-Kumar, P.; Ni, T.; Randolph, M. A.; Velmahe, G. C.; Kochevar, I. E.; Redmond, R. W. A light-activated amnion wrap strengthens colonic anastomosis and reduces peri-anastomotic adhesions. Lasers Surg. Med. 2016, 48, 530−537.

(6) Tsao, S.; Yao, M.; Tsao, H.; Henry, F. P.; Zhao, Y.; Kochevar, J. J.; Redmond, R. W.; Kochevar, I. E. Light-activated tissue bonding for excisional wound closure: a split-lesion clinical trial. Br. J. Dermatol. 2012, 166, 555−563.

(7) Verter, E. E.; Gisel, T. E.; Yang, P.; Johnson, A. J.; Neckers, D. C. Rose Bengal aggregation in rationally synthesized quantum dots. J. Phys. Chem. B 2009, 113, 14501−14506.

(8) Chera, D.; Verter, E. E.; Melki, S.; Gisel, T. E.; Doyle, F. J.; Jr.; Scarrcelli, G.; Yun, S. H.; Redmond, R. W.; Kochevar, I. E. Colagen croslinking using rose bengal and green light to increase corneal stiffness. Invest. Ophthalmol. Visual Sci. 2013, 54, 3426−3433.

(9) Zhu, H.; Alt, C.; Webb, R. H.; Melki, S.; Kochevar, I. E. Corneal Crosslinking With Rose Bengal and Green Light: Efficacy and Safety Evaluation. Cornea 2016, 35, 1234−1241.

(10) Goldstone, R. N.; McCormack, M. C.; Goldstein, R. L.; Mallidi, S.; Randolph, M. A.; Watkins, M. T.; Redmond, R. W.; Austen, W. G., Jr. Photochemical Tissue Passivation Attenuates AV Fistula Intimal Hyperplasia Ann. Surg. 2016, 10.1097/SLA.0000000000002046.

(11) Goldstone, R. N.; McCormack, M. C.; Khan, S. I.; Salinas, H. M.; Meppelink, A.; Randolph, M. A.; Watkins, M. T.; Redmond, R. W.; Austen, W. G., Jr. Photochemical Tissue Passivation Reduces Vein Graft Intimal Hyperplasia in a Swine Model of Arteriovenous Bypass Grafting. J. Am. Heart Assoc. 2016, 5, No. e003856.

(12) Fernandes, J. R.; Salinas, H. M.; Broesch, G. F.; McCormack, M. C.; Meppelink, A. M.; Randolph, M. A.; Redmond, R. W.; Austen, W. G., Jr. Prevention of capsular contracture with photographic tissue passivation. Plast. Reconstr. Surg. 2014, 133, 571−577.

(13) Ludvikova, L.; Fris, P.; Heger, D.; Sebej, P.; Wiz, J.; Klan, P. Photocrosslinking of rose bengal in water and acetonitrile: a comprehensive kinetic analysis. Phys. Chem. Chem. Phys. 2016, 18, 16266−16273.

(14) Necker, D. C. Rose Bengal. J. Photochem. Photobiol. B 1999, 47, 1−29.

(15) Fleming, G. R.; Morris, J. M.; Morrison, R. J. S.; Robinson, G. W.; et al. Piezoelectric biosensors for the detection of xanthene dyes. J. Am. Chem. Soc. 1997, 99, 4306−4311.

(16) Kochevar, I. E.; Redmond, R. W. Photosensitized production of singlet oxygen. Methods Enzymol. 2000, 319, 20−28.

(17) Shen, H. R.; Spikes, J. D.; Kopeckova, P.; Kopecek, J. Photodynamic crosslinking of proteins. II. Photocrosslinking of a model protein-ribonucleic acid J. Photochem. Photobiol. B 1996, 35, 213−219.

(18) Lambert, C. R.; Kochevar, I. E. Electron transfer quenching of the rose bengal triplet state. Photochem. Photobiol. 1997, 66, 15−25.

(19) Zakrzewski, A.; Necker, D. C. Bleaching products of rose bengal under reducing conditions. Tetrahedron 1987, 43, 4507−4512.

(20) Oster, G.; Oster, G. K.; Karg, G. Extremely long-lived intermediates in photochemical reactions of dyes in non-viscous media. J. Phys. Chem. 1962, 66, 2514−2517.

(21) Lutter, D. K.; Valdes-Aguilera, O.; Linden, S. M.; Paczkowski, J.; Necker, D. C. Rose Bengal aggregation in rationally synthesized dimeric systems. Photochem. Photobiol. 1988, 47, 551−557.

(22) Simpson, M. J.; Poblete, H.; Griffith, M.; Alarcon, E. I.; Scaiano, J. C. Impact of dye-protein interaction and silver nanoparticles on rose bengal photophysical behavior and protein photocrosslinking. Photochem. Photobiol. 2013, 89, 1433−1441.
psi and side-chain chi(1) and chi(2) dihedral angles. J. Chem. Theory. Comput. 2012, 8, 3257−3273.

(41) Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K. Scalable molecular dynamics with NAMD. J. Comput. Chem. 2005, 26, 1781−1802.

(42) MacKerell, A. D.; Bashford, D.; Bellott, M.; Dunbrack, R. L.; Evanseck, J. D.; Field, M. J.; Fischer, S.; Gao, J.; Guo, H.; Ha, S.; Joseph-McCarthy, D.; Kuchnir, L.; Kuczera, K.; Lau, F. T.; Mattos, C.; Michnick, S.; Ngo, T.; Nguyen, D. T.; Prodhom, B.; Reiher, W. E.; Roux, B.; Schlenkrich, M.; Smith, J. C.; Stote, R.; Straub, J.; Watanabe, M.; Wiorkiewicz-Kuczera, J.; Yin, D.; Karplus, M. All-atom empirical potential for molecular modeling and dynamics studies of proteins. J. Phys. Chem. B 1998, 102, 3586−3616.

(43) Feller, S. E.; Zhang, Y.; Pastor, R. W.; Brooks, B. R. Constant pressure molecular dynamics simulation: The Langevin piston method. J. Chem. Phys. 1995, 103, 4613−4621.

(44) Darden, T.; York, D.; Pedersen, L. Particle mesh Ewald: An N·log(N) method for Ewald sums in large systems. J. Chem. Phys. 1993, 98, 10089−10092.

(45) Andersen, H. C. Rattle: A “velocity” version of the shake algorithm for molecular dynamics calculations. J. Comput. Phys. 1983, 52, 24−34.

(46) Miyamoto, S.; Kollman, P. A. Settle: An analytical version of the SHAKE and RATTLE algorithm for rigid water models. J. Comput. Phys. 1992, 100, 416−461.

(47) Hopkins, C. W.; Le Grand, S.; Walker, R. C.; Roitberg, A. E. Long-Time-Step Molecular Dynamics through Hydrogen Mass Repartitioning. J. Chem. Theory Comput. 2015, 11, 1864−1874.

(48) Fiorin, G.; Klein, M. L.; Hénin, J. Using collective variables to drive molecular dynamics simulations. Mol. Phys. 2013, 111, 3345−3362.

(49) Comer, J.; Gumbart, J. C.; Hénin, J.; Lelièvre, T.; Pohorille, A.; Chipot, C. The Adaptive Biasing Force Method: Everything You Always Wanted To Know but Were Afraid To Ask. J. Phys. Chem. B 2015, 119, 1129−1151.

(50) Darve, E.; Rodriguez-Gomez, D.; Pohorille, A. Adaptive biasing force method for scalar and vector free energy calculations. J. Chem. Phys. 2008, 128, No. 144120.

(51) Hénin, J.; Chipot, C. Overcoming free energy barriers using unconstrained molecular dynamics simulations. J. Chem. Phys. 2004, 121, 2904−2914.