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Vitamin B_2 in Nanoscopic Environments under Visible Light: Photosensitized Antioxidant or Phototoxic Drug?

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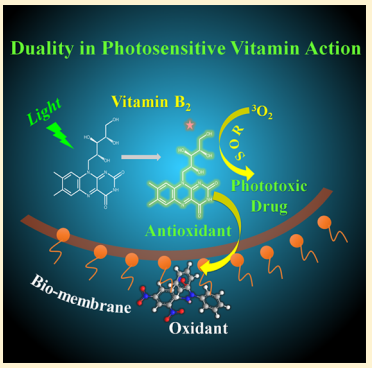
ABSTRACT: Vitamin B_2 has been studied as a conventional antioxidant (in the dark) since its discovery in 1926. The effect of visible light on vitamin B_2-containing food has a long history of scientific investigation. Although photodegradation of the vitamin producing several photoproducts is evident in certain experimental conditions, phototoxicity revealing an additional oxidative stress in the medium is also clear from some reports. Here we report the photosensitized antioxidant effect of the vitamin, which is found to be greater than 2 orders of magnitude more efficient than that in the dark condition. The photoinduced antioxidant property is apparently paradoxical compared to the reported phototoxic effect of the vitamin. Our present study unravels a unified picture underlying the difference in character of vitamin B_2 under visible light irradiation. UV−vis absorption and fluorescence studies in a number of physiologically relevant nanoscopic environments (micelles and reverse micelles) reveal the antioxidant activity to a well-known oxidative stress marker 2,2-diphenyl-1-picrylhydrazyl (DPPH) as well as a phototoxicity effect resulting in self-degradation of the vitamin. Picosecond-resolved Förster resonance energy transfer (FRET) from the vitamin to the marker DPPH in the biomimetic environments clearly reveals the role of proximity of an oxidizing agent in the photoinduced effect of the vitamin. Our systematic and detailed studies unravel a simple picture of the mechanistic pathway of the photosensitized vitamin in the physiologically important environments leading to the antioxidant/phototoxicity effect of the vitamin. The excited vitamin transfers its electron to the oxidizing agent in proximity for the antioxidant effect, but otherwise it employs oxygen to generate reactive oxygen species (ROS), resulting in phototoxicity/self-degradation.



INTRODUCTION

A vital micronutrient, riboflavin (vitamin B_2) is an essential vitamin found in milk, cheese, vegetables, yeast, mushrooms, eggs, and meat products.[1,2] It is a crucial precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).[3−6] These coenzymes with dehydrogenase and oxidase activities play a key role in the electron transport chain for cellular metabolism in human beings.[7,8] The use of the vitamin as antioxidant drug is conventional in medicine,[9,10] and the activity of the vitamin under visible light has a long history of scientific reports.[11,12] It is well-known that upon photoexcitation, the isoalloxazine ring system undergoes intramolecular photoreduction in which the ribityl side chain donates an electron in the absence of an external reductant.[13] A few photoproducts are formed due to oxidation of the side chain. It is converted to lumichrome upon photolysis in neutral aqueous solutions and into lumiflavin in alkaline conditions.[1,14−16] The vitamin under visible light can also generate reactive oxygen species (ROS), including superoxide anions and singlet oxygen.[17,18] Upon photoexcitation, the vitamin (^{1}Rf*) is converted to excited triplet riboflavin (^{3} Rf*) through intersystem crossing, which is reduced by abstraction of electrons or the hydrogen ion to form riboflavin radical.[19] The riboflavin radical eventually reacts with atmospheric triplet oxygen to form the superoxide anion and singlet oxygen.[8,20]

The density of oxygen, the concentration of the vitamin riboflavin, and the presence of other oxidizable reactants or quenchers determines the distribution of the reactive oxygen species (ROS) formed in a particular system.[18,21,22] The excited state relaxation of the vitamin in protein environments is found to be ultrafast (femtosecond-picosecond) in nature and due to the electron transfer from the vitamin to the proximal amino acids in the protein.[23,24] Antioxidant activity of the vitamin is generally estimated by monitoring free radical scavenging of a stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH; violet color) in the absence of visible light.[25] It is well-established that the vitamin supplement in the diet can enhance the scavenging kinetics in cellular medium.[26] The vitamin is found to donate a proton/electron to the radical DPPH, eventually reducing the radical to $DPPH_2$ form, revealing decolourization (from violet to yellow) of the medium.[27,28] Although antioxidant property of the vitamin is estimated using DPPH as model oxidizing agent in absence of light,[29] very little is known about the photosensitized antioxidant activity of riboflavin,[30] which is the focus of the present study. In a recent report, antimicrobial activity of riboflavin under visible light irradiation is illustrated.[31]

Here we have studied the antioxidant activity of vitamin B_2 under visible light irradiation (blue light, λ_{max} = 450 nm) in a variety of nanoscopic physiologically important environments.

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We have compared the experimental observation of the antioxidant activity with that in the dark in an ethanol−water (1:1) solution. Our observation of photoantioxidant activity of the vitamin in a number of micelles with different charges (anionic, cationic, and neutral) reveals the importance of the location of the oxidizing agent for the efficacy of antioxidant activity of the vitamin in the physiologically relevant environments. For instance, the cationic hexadecyltrimethylammonium bromide (CTAB) micelle is capable of mimicking histone protein,[32] the neutral (polar) TritonX-100 (TX-100) resembles a protein cavity,[33] and the anionic sodium dodecyl sulfate (SDS) micelle can be a good substitute of the negatively charged DNA surface.[34,35] On the other hand, our studies of the photoantioxidant property in AOT reverse micelles with various degrees of hydration (different hydrodynamic diameters of the water pool) clearly show the importance of the proximity of the oxidizing agent with respect to the excited vitamin. Reverse micelles are nanopools of polar solvent surrounded by a monolayer of surfactant molecules at the periphery with polar head groups pointing inward toward the polar solvent and the hydrocarbon tails are directed toward the nonpolar organic solvents.[36] Reverse micelles (RMs) with water nanopools resemble the water pockets found in various bioaggregates such as proteins, membranes, and mitochondria.[37] Thus, these systems are very often considered as excellent biomimetics for exploration of biological membranes and biologically confined water molecules.[38] Although the antioxidant property of the vitamin is followed by observing decolourization kinetics of DPPH in the environments, in order to quantitatively estimate the distance between the oxidizing agent (DPPH) and the excited vitamin prior the antioxidant activity, we have employed picosecond resolved Förster resonance energy transfer (FRET) in the nanoscopic environments of micelles and RMs with various degrees of hydration. We have also analyzed the probability distribution of the RF-DPPH distances in the nanoenvironments. Overall, our studies unravel a mechanistic pathway for the photoinduced antioxidant property of the important vitamin in a number of physiologically important nanoenvironments.

2. MATERIAL AND METHODS

Riboflavin, sodium dodecyl sulfate (SDS), disodium hydrogen phosphate dehydrate, sodium dihydrogen phosphate dehydrate and sodium azide were purchased from Sigma (99%). Hexadecyltrimethylammonium bromide (CTAB) (Fluka), sodium bis[2-ethylhexyl] sulfosuccinate (AOT) (Fluka), TX-100 (Romil), and isooctane (Spectrochem) were of the highest commercially available purity and were used without further purification. Millipore water was used throughout the experiments for the aqueous solution. Aqueous micelles (SDS, TX-100, and CTAB) of 100 mM concentration were prepared by adding calculated amounts of surfactant in a known volume of phosphate buffer (pH 7) and stirring the solution for about 45 min. AOT RMs containing riboflavin solution of different w_0 (= [water]/[AOT]) values were prepared by injecting a known amount of riboflavin solution into a measured volume of AOT-isooctane solution ([AOT] = 100 mM) followed by continuous stirring to obtain a clear solution.

The absorption spectrum was measured with a Shimadzu UV-2450 spectrophotometer using a quartz cell of path length of 1 cm. Concentrations of the samples were calculated from the absorbance using the extinction coefficient values of 12 200 M^{-1} cm^{-1} at 450 nm[39] and 11 500 M^{-1} cm^{-1} at 520 nm[40] of riboflavin (RF) and DPPH, respectively. In order to measure the blue-light-induced degradation processes with spectroscopic precision, an indigenously developed and a versatile fiber-optic-based system[41] was used for sensitive optical measurements. The characteristic absorption of DPPH at 520 nm was chosen in order to monitor the degradation process with DPPH in the presence of blue light at room temperature. The absorption spectra of DPPH at 30 s intervals were recorded using SPECTRA SUITE software supplied by Ocean Optics. Finally, the DPPH absorption at 520 nm was plotted against the time of photoirradiation. All fluorescence measurements were performed on a JobinYvon Fluorolog fluorometer. Concentrations of riboflavin and DPPH in the experiments were kept at 80 and 40 μM for absorbance and 2 and 1 μM for fluorescence experiments, respectively. For performing the fluorescence measurement, the bandwidth of excitation and emission slits were kept at 2.0 nm. The quantum yield was calculated according to the following equation,[42] $Q = Q_R\ (I/I_R)\ (OD_R/OD)\ (n^2/n_R^2)$, where Q and Q_R are the quantum yields of the riboflavin and the reference, I and I_R are the integrated fluorescence intensities of riboflavin and the reference, OD and OD_R are the optical densities of riboflavin and the reference at the excitation wavelength, and n and n_R are the refractive indices of samples and the reference solutions. The absolute quantum yield of riboflavin in ethanol was taken to be 0.3.[43] Refractive indices of the solutions were measured by using a Rudolph J357 automatic refractometer.

Time-resolved studies were performed with a time-correlated single-photon counting (TCSPC) setup from Edinburgh Instruments. A picosecond pulsed laser diode was used to excite the sample at 409 nm with an instrument response function (IRF) of 80 ps. The excitation was vertically polarized, and the emission was recorded through a polarizer oriented at 55° from the vertical position. Incorporation of a long-pass filter with a cut off at 420 nm in the emission channel effectively eliminates possible scattered excitation light. The picosecond-resolved fluorescence transients were fitted with multi-exponential (n) function, $\sum_{i=1}^{n} A_i \exp(-(t/\tau_i))$ where, A_i's are weight percentages of the decay components with time constants of τ_i. The average excited state lifetime is expressed by the equation $\tau = \sum_{i=1}^{n} A_i \tau_i$, when $\sum_{i=1}^{n} A_i = 1$.

The Förster distances of donor−acceptor pair were calculated using the equation[42] $R_0 = 0.211 \times [\kappa^2 n^{-4} \Phi_D J(\lambda)]^{1/6}$ in Å, where R_0 is the distance between the donor and the acceptor at which the energy transfer efficiency is 50%, and κ^2 is a factor which describes the relative orientation in space of the transition dipoles of the donor and acceptor. The magnitude of κ^2 is assumed to be 0.66[42] for random orientation of donor and acceptor pair. The refractive index (n) of the medium (biological systems) is assumed to be 1.4. $J(\lambda)$, the overlap integral, which expresses the degree of spectral overlap between the donor emission and the acceptor absorption, is given by $J(\lambda) = (\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda)/(\int_0^\infty F_D(\lambda)d\lambda)$, where $F_D(\lambda)$ is the fluorescence intensity of the donor in the wavelength range of λ to $\lambda + d\lambda$ and is dimensionless. $\varepsilon_A(\lambda)$ is the extinction coefficient (in M^{-1} cm^{-1}) of the acceptor at λ. If λ is in nm, then $J(\lambda)$ is in units of M^{-1} cm^{-1} nm^4. Once the value of R_0 is known, the donor−acceptor distance (r) is calculated using the formula, $r^6 = [R_0^6(1 - E)]/E$, where E is the efficiency of energy transfer, which can be expressed as follows, $E = 1 - (\tau_{DA}/\tau_D)$, where τ_D and τ_{DA} are lifetimes of the donor in the absence and in the presence of the acceptor.

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Distance distribution between donor and acceptor was estimated according to the procedure described in the literature.[42,44] The observed fluorescence transients of the donor (riboflavin) in absence of acceptor (DPPH) in various surfactants complex were fitted using a nonlinear least-squares fitting procedure (software SCIENTIST) to the following function, $I_D(t) = \int_0^t E(t')P(t' - t)dt'$, which comprises the convolution of the IRF ($E(t)$) with exponential $P(t) = \sum_i \alpha_{Di}\exp(-t/\tau_{Di})$. The distance distribution function $P(r)$ in the fluorescence transients of donor in the presence of acceptor in the system under studies is estimated using the same software (SCIENTIST) in the following way. The intensity decay of the D−A pair, spaced at a distance r, is given by $I_{DA}(r, t) = \sum_i \alpha_{Di}\exp[-(t/\tau_{Di}) - (t/\tau_{Di})\ (R_0/r)^6]$ and the intensity decay of the sample considering $P(r)$ is given by, $I_{DA}(t) = \int_{r=0}^{\infty} P(r)I_{DA}(r, t)dr$, where $P(r)$ consists of the following terms, $P(r) = (1/(\sigma(2\pi)^{1/2}))\exp[-(1/2)((\bar{r} - r)/\sigma)^2]$, where $\bar{r}$ is the mean of the Gaussian with a standard deviation of σ. Usually distance distributions are described by the full width at half maxima, and half width is given by hw = 2.354σ.

RESULTS AND DISCUSSION

The molecular structures of riboflavin (RF; vitamin B_2) and model free radical (DPPH) are shown in the upper panel of Figure 1. Riboflavin (nitrogen circled red) tranfers electrons to

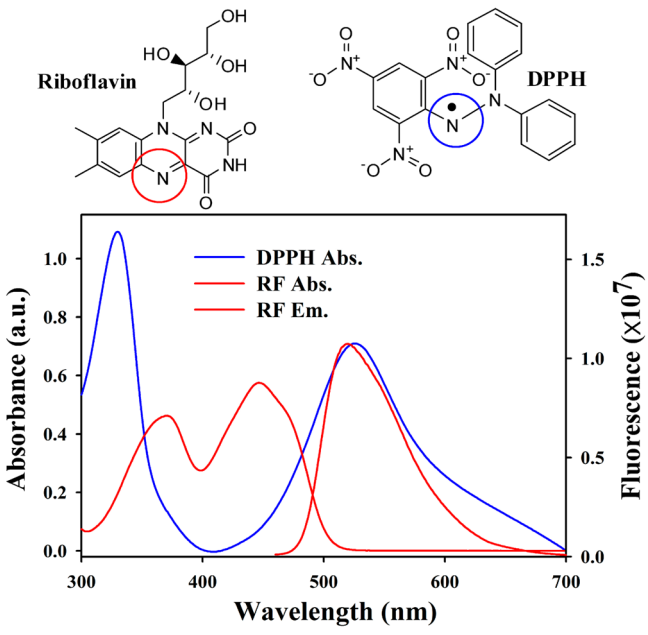

Figure 1. Upper panel: circled nitrogen in red denotes the electron donor of riboflavin which transfer its electrons to appropriate hydrogen acceptors in photochemical reactions, and circled nitrogen in blue depicts the nitrogen centered free radical (see text). Lower panel: absorption and emission spectra of riboflavin (λ_{ex} = 450 nm, λ_{em} = 520 nm) and DPPH (absorption peak at 520 nm).

the appropriate hydrogen acceptors in photochemical reactions,[13] whereas the nitrogen centered (circled blue) free radical accepts an electron or a hydrogen radical to form a stable diamagnetic molecule in the antioxidant mechanism.[45,46] The absorption and emission spectra of RF in ethanol−water (1:1) solution are shown in the lower panel of Figure 1. The absorption spectrum of DPPH in the figure clearly reveals a significant spectral overlap with the emission spectrum of RF. The large spectral overlap is also indicative of the possibility of energy transfer from the excited RF to the free radical DPPH prior to the radical scavenging. Thus, the presence of DPPH in the RF solution quenches emission intensity of the vitamin. It has to be noted that the emission spectrum of riboflavin in the presence of DPPH remains unaffected with respect to the shape and position of the emission maximum (data not shown). Figure 2 shows the kinetics of the free radical scavenging

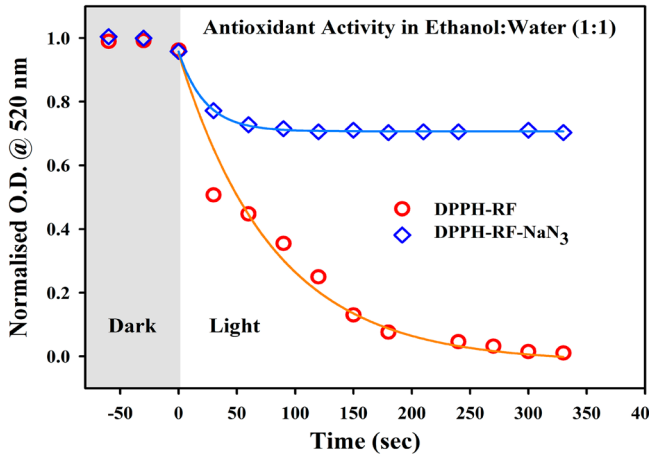

Figure 2. Depicts the absorption kinetics (degradation monitored at 520 nm) of riboflavin−DPPH in the presence and absence of sodium azide (50 mM) in ethanol−water (1:1) solution. Blue light irradiation is given from the point "0" on the X-axis.

activity of RF in ethanol−water (1:1) solution in the dark compared with that in the presence of blue light (λ_{ex} = 450 nm). The free radical scavenging kinetics of RF in the absence and presence of blue light have been fitted with single exponential decay functions, and the time constants are found to be to 2051 to 66 s, respectively. A significant enhancement in the rate of radical scavenging of the vitamin in the presence of blue light is clearly evident from Figure 2. It is also evident from the figure that a significant retardation of the scavenging activity in the presence of sodium azide (NaN_3), a potential ROS quencher.

Earlier reports state that the proximal radical scavenging activity of most antioxidants occurs by donating H atoms either by H atom transfer (HAT) or stepwise electron transfer−proton transfer (ET−PT) or sequential proton loss−electron transfer (SPLET).[47−50] Our observation on reduction of photoantioxidant activity of the vitamin RF in the presence of ROS quencher NaN_3 clearly reveals the formation of ROS from the excited RF following the mechanism detailed in the earlier literature.[8] In the absence of sodium azide, ROS generated from excited vitamin quenches DPPH proximally as well as through distance resulting in enhanced yield of scavenging (∼100% in our time window). By addition of NaN_3, only proximal DPPH can access riboflavin for radical scavenging, which initially causes higher rate (time constant of 23 s) of degradation but low yield (about 30% scavenging in our time window). The structural integrity of the vitamin in the presence of NaN_3 has been confirmed through absorption and emission spectra of RF (data not shown). Our observation indicates that scavenging of the DPPH free radical in the solution is essentially governed by the generation of ROS from the excited vitamin and thus proximity between RF and DPPH in solution has minimal effect on the photoinduced antioxidant

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property of the vitamin. In earlier studies,[45,51] the redox reaction mechanism of DPPH with ROS has been elaborated. Although several reaction products in the redox processes involving DPPH convert oxygen anion ($O\bullet_2^-$) into oxidants (OH•, HOO•), formation of a minor amount of reaction products has been found by using TLC techniques.[51]

Figure 3a shows antioxidant activity of the vitamin in aqueous micellar solutions, namely, SDS (anionic), CTAB (cationic), and TX-100 (neutral) in both dark and light conditions. Although a significant enhancement of the activity upon irradiation is evident, the rate of antioxidant activity in the presence of light is observed to depend on the nature of charges in the head groups of the micelles. RF shows almost similar affinity to all the micelles under investigation, disrediting the role of specific RF localization in the different antioxidant activity. Our studies indicate that RF essentially stay outside the micelles in the bulk solution. Our experiments were performed with equal concentrations of RF and DPPH in micelles with various charges on the head groups. From Figure 3a, it is clear that the scavenging activities in the micelles follow the sequence of SDS > TX-100 > CTAB. The degradation time (in sec) of DPPH in SDS, TX-100, and CTAB were found to be 54.5, 275, and 300 respectively after fitting with the first-order decay function. The highest activity in the SDS micelle with anionic headgroup among other varieties can be rationalized in the following way. The free radical DPPH, which has a large hydrophobic moiety, is expected to lead the localization of the radical in the hydrophobic core of the micelles independent of the nature of the charges at the headgroup.[52] DPPH is found to be sparingly soluble in bulk water. However, it has been reported earlier that the free radical in the DPPH is cationic in nature[49,53] and is expected to reside near the interface of the anionic SDS micelles, offering more accessibility to the excited RF in the bulk solution. In a recent study,[52] enhanced DPPH radical scavenging activity of Rutin in anionic SDS micelle compared to other micellar charged varieties (CTAB, TX-100, etc.) has been reported. Convenient localization of Rutin in the micellar interface in SDS and DPPH in the hydrophobic core are concluded to be reason for the enhanced activity.

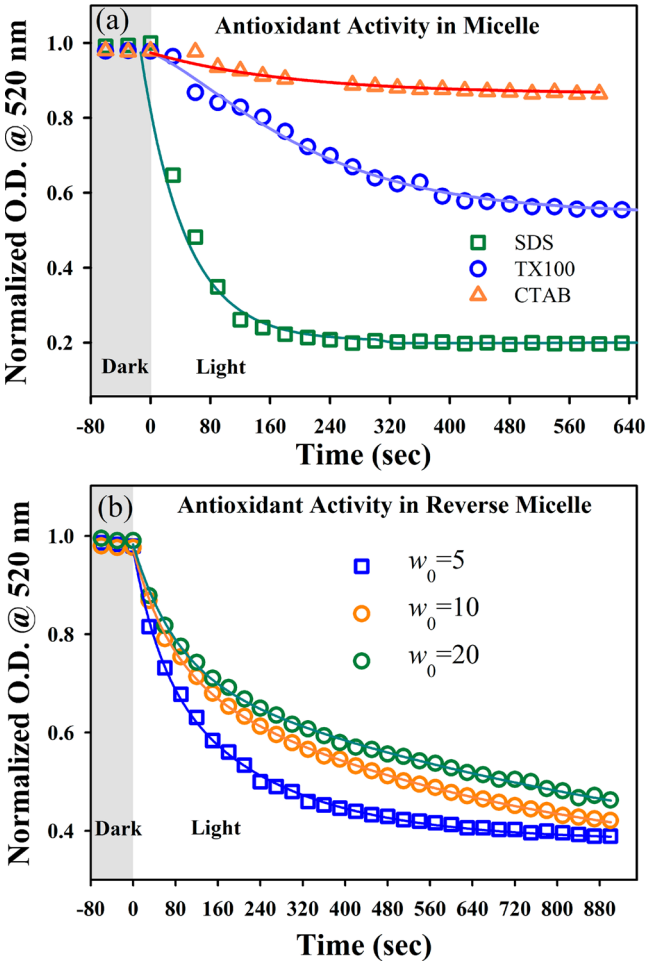

Figure 3. (a) Depicts the absorption kinetics of DPPH (degradation monitored at 520 nm) in three micellar systems of SDS, TX-100, and CTAB, and (b) shows the absorption kinetics of DPPH (degradation monitored at 520 nm) in AOT-reverse micelles with $w_0$5, $w_0$10, and $w_0$20 in the presence of blue light (450 nm) and in the dark. Blue light irradiation is given from the point "0" on the *X*-axis.

Figure 3b shows the antioxidant activity of RF in RMs with various degrees of hydrations. It has to be noted that RF, because it is polar, would reside in the water pool of the RMs,[24] whereas the hyphophobic DPPH is expected to reside in the bulk isooctane. However, the cationic charge in the free radical[53] is expected to compel the radical DPPH at the anionic interface of the AOT-RMs. A clear decrease in the antioxidant activity with the increase in the degree of hydration (from w_0 = 5 to w_0 = 20) is evident from Figure 3b. It is also noted that the decay time (in sec) of DPPH in AOT-RMs with w_0 = 5, w_0 = 10, and w_0 = 20 were found to be 50, 70, and 79, respectively, after fitting with first-order decay function. The observation shows the importance of the proximity of the model oxidizing agent DPPH with the excited RF, which also reveals less importance of ROS in the reaction pathway that may lead to a distal effect.[54] The negligible role of ROS in the photoinduced antioxidant activity of the vitamin in the nanoenvironments, including SDS micelles, is also clear from Figure 4a, where a very minimum effect of NaN_3 (ROS quencher) in the antioxidant kinetics is observed (40 s degradation time after fitting with first-order decay function). In this context, it is worth mentioning that generation of ROS from the photo-excited RF should be indiscriminative and it is expected to oxidize the RF itself, leading to photodegradation of the vitamin.

In order to investigate the photodegradation of the RF in various environments, emission of the vitamin at 520 nm (DPPH has no emission) is found to be a good marker rather than the absorption at 450 nm (peak absorption of the vitamin B_2), because the interference of the absorption due to DPPH (absorption peak at 520 nm) is unavoidable at the detection wavelength. It has to be noted that the photodegradation of the parent RF may result in some luminescent photoproducts, including lumichrome or lumiflavin and may interfere with the emission of RF. However, the fluorescence quantum yields of the major photoproducts (lumichrome, 0.008 in water;[16] lumiflavin, 0.14 in water)[16] are significantly lower than that of the parent RF (0.26 in water).[14] Thus, photodegradation of the parent molecule (RF) would always reduce the fluorescence intensity. Although final intensity of emission may be convoluted with the photoproducts, the kinetics of fluorescence quenching is expected to reflect the rate of photoconversion of RF to other products, that is, photodegradation. The photodegradation of RF in ethanol–water (1:1) solution shows first-order kinetics (Figure 4b), and the rate of

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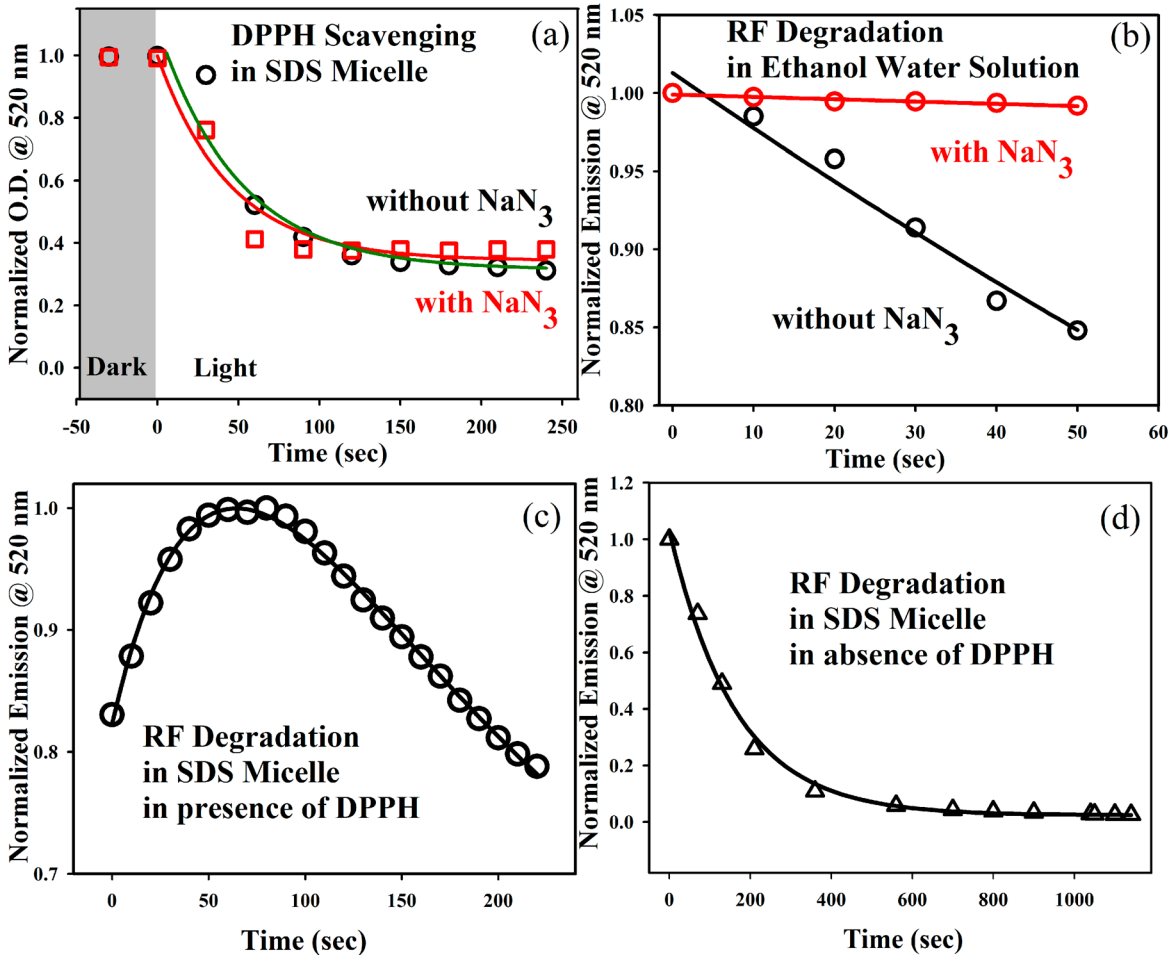

Figure 4. (a) Depiction of the absorption kinetics of riboflavin-DPPH in SDS micelles with and without 50 mM sodium azide, in the presence of blue light and in the dark. Blue light irradiation is given from the point "0" on the *X*-axis. (b) Shows the emission spectra of riboflavin with and without 50 mM sodium azide in ethanol–water (1:1) solution. Panels (c) and (d) show emission spectra of riboflavin in SDS micelles in the presence and absence of DPPH, respectively.

deterioration decreases (from 281 to 6000 s) in the presence of NaN_3, revealing the role of ROS in the self-degradation of the vitamin. Figure 4c,d show kinetics of RF emission in SDS micelles in the presence and absence of DPPH, respectively. As shown in Figure 4c, the emission kinetics of RF in SDS micelles in the presence of DPPH show a rise of the time constant of 63 s followed by a decay of the time constant of 144 s. Existence of multiple excited state events is clear from the kinetics pattern. The rise component of the emission is consistent with the DPPH scavenging (Figure 4a) time constant of 40 s and thus can be assigned as the loss of energy transfer from RF to DPPH in the micelle leading to temporal absorption quenching at 520 nm. The decay time constant (Figure 4c) of 144 s is similar to the self-degradation time constant of 163 s, as shown in Figure 4d. Figure 4d shows monotonous (single exponential with time constant of 163 s) decrease of RF emission in the absence of DPPH in the micellar solution under blue light irradiation.

From our above observations on the photoinduced antioxidant properties of the vitamin RF in nanoscopic environments, the importance of the proximity of the model oxidizing agent DPPH with the excited RF is clearly revealed. Whereas proximity of the vitamin with the oxidizing agent (DPPH) in the nanoscopic environments of micelles and reverse micelles are found to be important, a quantitative estimation of the proximity leading to various degrees of photo-oxidation in the nanoenvironments is essential. In this regard, we have employed picosecond resolved FRET experiments, where the vitamin and the model oxidizing agent DPPH are considered to be energy donor and acceptor, respectively, for the spectral overlap of the donor emission with the absorption spectrum of the acceptor, as shown in Figure 1b. Figure 5a–c show picosecond resolved emission transients of RF (donor) in various micelles in the absence and presence of DPPH (acceptor). The details of the numerical fitting of the fluorescence transients are tabulated in Table 1. The probability distribution of the donor–acceptor distances in the micelles are shown in Figure 5d–f. The parameters related to FRET and the estimated donor–acceptor distances are shown in Table 2. From Figure 5 and Table 2, it is evident that the SDS micelle allows more proximity of the vitamin with the oxidizing agent DPPH than other varieties of the micelles prior to the photo-oxidation. The observation is consistent with the fact that in the SDS micelle RF shows highest rate of photo-oxidation kinetics (Figure 3a) compared to other micelles. It is also evident from Table 2 and Figure 3a that the donor–acceptor distances in the micelles essentially dictate efficacy of the photo-oxidation kinetics rates. The calculated half width (hw), revealing heterogeneity or fluctuation[55] in the observed distance between the donor and the acceptor, is shown in Figure 5d–f. It is worth mentioning here that DPPH is completely insoluble in water

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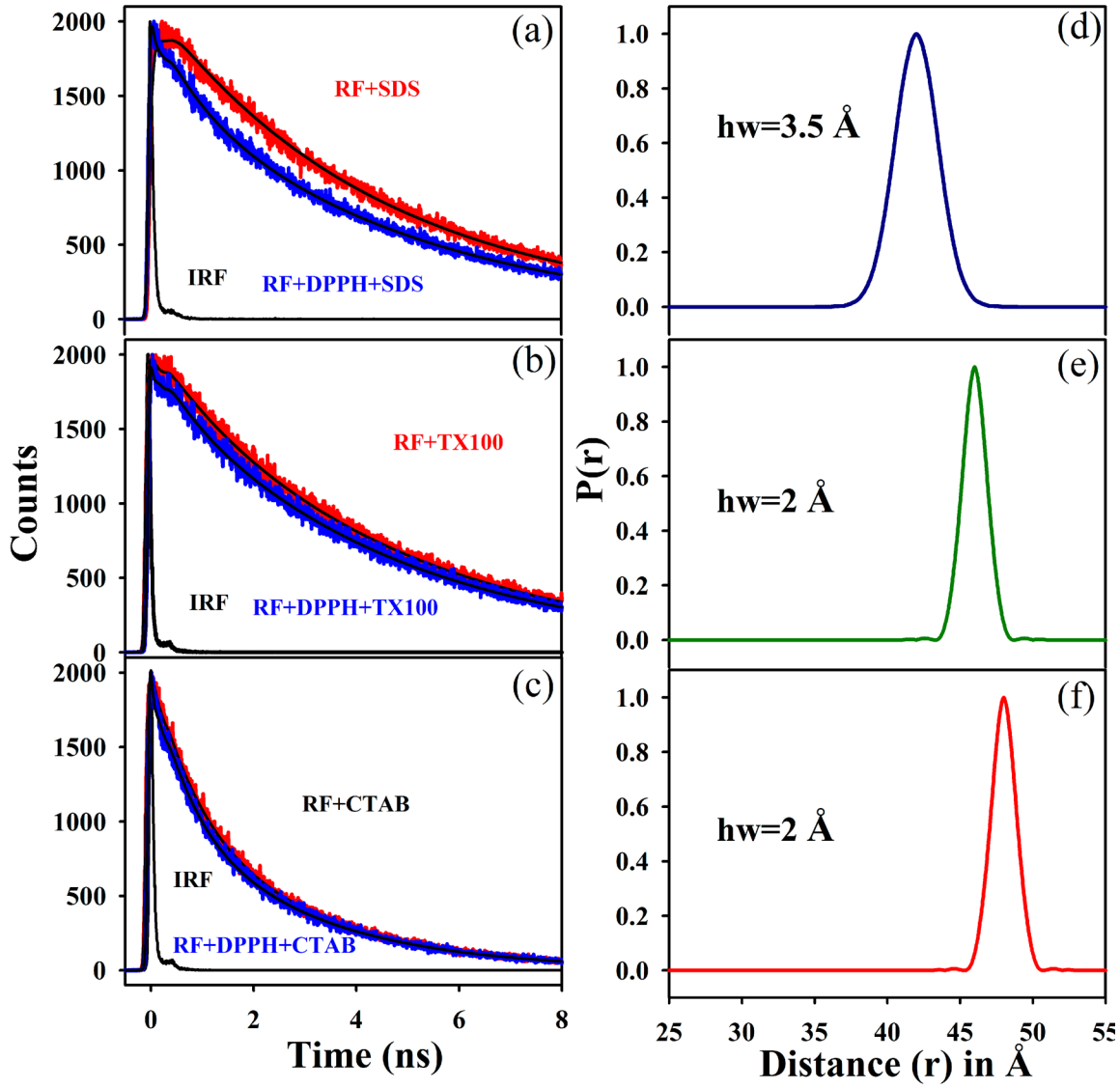

Figure 5. Panels (a) to (c) depict the fluorescence lifetime quenching of riboflavin in SDS, TX-100, and CTAB micelles, respectively, considering the distance distribution between donor (riboflavin) and acceptor (DPPH). Panels (d) to (f) show the corresponding probability of distance distribution ($P(r)$) with respect to mean donor–acceptor distance.

Table 1. Picosecond-Resolved Fluorescence Data Fitting of Riboflavin with DPPH in Micelles[a]

system	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{avg} (ns)
		SDS micelle		
RF		0.11 (20%)	4.61 (80%)	3.7
RF-DPPH	0.03 (40%)	0.50 (14%)	4.54 (46%)	2.2
		TX-100 micelle		
RF		0.22 (20%)	4.43 (80%)	3.6
RF-DPPH	0.10 (26%)	1.23 (10%)	4.59 (65%)	3.1
		CTAB micelle		
RF		0.45 (40%)	2.38 (60%)	1.6
RF-DPPH	0.10 (18%)	0.50 (38.7%)	2.70 (43.3%)	1.4

[a]Numbers in parentheses denote the relative weightage in percentage. The time constants are within ± 5% error.

Table 2. Förster Resonance Energy Transfer (FRET) Parameters of Riboflavin-DPPH with Measured FRET Efficiencies (E_{FRET})

system	Q_Y	$J(\lambda)$	R_0 (Å)	E_{FRET} (%)	r (Å)
		micelles			
SDS	0.250	7.91×10^{14}	35.49	41.0	37.70
TX-100	0.235	7.89×10^{14}	35.10	12.5	48.54
CTAB	0.220	7.97×10^{14}	34.70	14.0	46.90
		AOT reverse micelles			
$w_0 = 5$	0.230	6.23×10^{14}	22.91	74.4	19.20
$w_0 = 10$	0.260	7.02×10^{14}	35.02	53.7	34.20
$w_0 = 20$	0.270	7.14×10^{14}	35.34	48.7	35.65

and can only reside in the hydrophobic core of the micelles; however, cationic charge in the free radical site[53] is expected to bring DPPH in closer proximity of the micellar surface. In the same argument in the CTAB micelle with the cationic surface, the free radical DPPH is expected to reside away from the surface leading to a higher donor–acceptor distance and the lowest photo-oxidation kinetics rate (Figure 3a). In the TX-100 micelle with the polar head groups, the photo-oxidation kinetics rate (Figure 3a) and donor–acceptor distance is between the other two micelles (SDS and CTAB).

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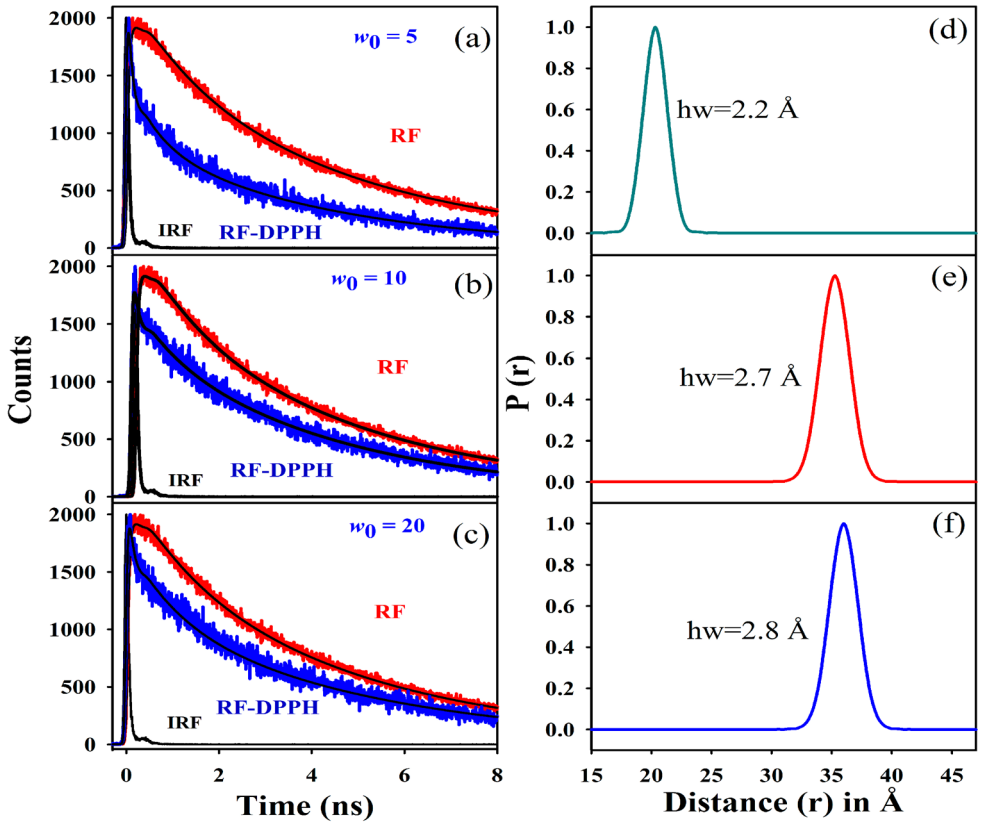

Figure 6. Panels (a) to (c) depict the fluorescence lifetime quenching of riboflavin in $w_0$5, $w_0$10, and $w_0$20 micelles, respectively, considering the distance distribution between donor (riboflavin) and acceptor (DPPH). Panels (d) and (f) show the corresponding probability of distance distribution ($P(r)$) with respect to mean donor–acceptor distance.

The experimental control of RF-DPPH distance can be achieved from the studies on the donor–acceptor pair in the reverse micellar system, where hydrodynamic radius of the nanopool of water is shown to be manipulated in nanometer precision.[56] Considering the case of the AOT reverse micelle with an anionic headgroup, the DPPH is expected to reside at the interface between polar (water) and nonpolar (isooctane) phases because of the cationic charge.[53] As observed in the case of the micellar system, the vitamin RF is expected to have negligible affinity to the interface and prefers to reside in the central water pool. Thus, the RF-DPPH distance can be controlled precisely with an increase in the hydrodynamic radius of the water pool and the addition of more water in the surfactant solution. As shown in Figure 6, RF-DPPH distances gradually increase with the increase in the hydration number (w_0) of the AOT reverse micelle. Figure 6a–c show picosecond resolved emission transients of RF (donor) in RMs with various degrees of hydration in the absence and presence of DPPH (acceptor). The details of the numerical fitting of the fluorescence transients are tabulated in Table 3. The probability distribution of the donor–acceptor distances in the micelles are shown in Figure 6d–f. The parameters related to FRET and the estimated donor–acceptor distances are shown in Table 2. From Figure 6 and Table 2, it is evident that proximity of the vitamin with the oxidizing agent DPPH increases with w_0 values prior to the photo-oxidation. The observation is consistent with the fact that in the RM with w_0 = 5, RF shows the highest rate of photo-oxidation kinetics in Figure 3b compared to that in w_0 = 10 and 20. It is also evident from Table 2 and Figure 3b that the donor–acceptor distances in the reverse micelles essentially dictate the efficacy of the rates of photo-oxidation kinetics. This observation is consistent with the case in micellar solutions. The calculated half width (hw) values, revealing heterogeneity or fluctuation[55] in the observed distances between the donor and the acceptor, are shown in Figure 5d–f. Although a direct correlation of such fluctuations in the photo-oxidation turnover is not clear from the present study, the hw values in the case of micelles and RMs show opposite trends with respect to the donor–acceptor distances. From our studies in the nanoscopic systems, a unified molecular picture of the phototoxicity and photosensitized antioxidant properties of the vitamin is distinct and given in Scheme 1.

Table 3. Picosecond-Resolved Fluorescence Data Fitting of Riboflavin with DPPH in Reverse Micelles[a]

system	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{avg} (ns)
AOT reverse micelles (w_0 = 5)				
RF		1.34 (25%)	4.80 (75%)	3.9
RF-DPPH	0.05 (63%)	0.63 (14%)	4.15 (23%)	1.0
AOT reverse micelles (w_0 = 10)				
RF		1.11 (20%)	4.84 (80%)	4.1
RF-DPPH	0.05 (48%)	0.86 (11%)	4.38 (41%)	1.9
AOT reverse micelles (w_0 = 20)				
RF		0.62 (20%)	4.69 (80%)	3.9
RF-DPPH	0.07 (48%)	1.00 (16%)	5.00 (36%)	2.0

[a]Numbers in parentheses denote the relative weightage in percentage. The time constants are within ± 5% error.

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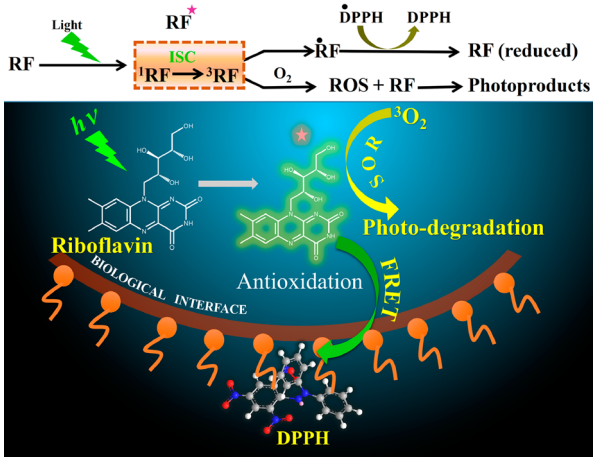
Scheme 1. Mechanism and Pathway[a]



[a]Upper panel: Mechanistic pathway illustrates the excited singlet riboflavin, which is converted to triplet riboflavin after absorbing energy from light. Excited triplet riboflavin transfers electron in the presence of an oxidizing agent (DPPH) or interacts with triplet oxygen to form photoproducts by ROS generation. Excited singlet and triplet states of riboflavin are depicted by the "*" symbol in the scheme. Lower panel: Radical-scavenging of Vitamin B_2 (riboflavin) in the nanoscopic systems by proximity with the oxidizing agent DPPH. The mechanism of ROS generation leading to the self-destruction employing dissolved oxygen is also shown.[8,57,58].

CONCLUSION

The effect of visible light on vitamin B_2 present in food has a long record of empirical evidence. In these studies, we have deduced the antioxidant activity of photosensitized riboflavin in ethanol−water (1:1) solution and in various nanoscopic systems. The photoinduced antioxidant activity of the vitamin is significantly more efficient than in the dark condition. We have suggested a new mechanistic pathway where the excited vitamin quenches the free radical DPPH without being facilitated by the reactive oxygen species prior to electron transfer, which is pictorially represented in Scheme 1. Moreover, our experiments reveal that the radical-scavenging mechanism is strikingly different in solvents from that in confined environments. Our studies also confirm that pico-second resolved Förster resonance energy transfer (FRET) in the biomimetic nanoscopic environments catches the proximity of an oxidizing agent (DPPH) with the excited vitamin (riboflavin) just prior to the photosensitized antioxidant activity. In summary, vitamin B_2 under visible light irradiation may play a dual role of "photosensitized antioxidant" and "phototoxic drug" depending upon the proximity of an oxidizing species. Photoinduced electron transfer from the vitamin to the oxidizing agent in proximity is the key for the antioxidant effect. On the other hand, the excited vitamin undergoes intersystem crossing and eventually employs dissolved oxygen to generate reactive oxygen species (ROS), resulting in phototoxicity/self-degradation. As the generation of ROS and the photosensitized antioxidant activity of the vitamin has a general appeal in the cellular milieu, our studies on the physiologically relevant nanoscopic environments would find importance in the design of a new strategy in the phototherapy of several diseases.

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Notes

The authors declare no competing financial interest.

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