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The detection sensitivity of commonly used singlet oxygen probes in aqueous environments

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ABSTRACT

The sensitivity for singlet oxygen ($^1O_2$) of two convenient $^1O_2$ probes, 1,3-diphenylisobenzofuran (DPBF) and 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA), has been investigated in different aqueous environments. Both probes are commercially available at reasonable cost and can be used with standard UV–vis spectrometers. Although DPBF is not soluble in neat water and is not specific to the detection of $^1O_2$, it has very high, essentially diffusion-limited, reactivity towards $^1O_2$; it can trap up to 50% of all $^1O_2$ created in alcohol/water or micellar solution, and even more when replacing H_2O by D_2O, which makes it highly useful when the process under investigation does not yield much $^1O_2$. On the other hand, ABDA has a much lower reactivity, reacting with only 2% of the singlet oxygen generated in H_2O, as well as a smaller extinction coefficient, resulting in a much smaller spectroscopic response, but is soluble in neat water and is specific for $^1O_2$, allowing for discrimination from other reactive oxygen species. The results presented here not only allow a comparative assessment of the usefulness of the two $^1O_2$ probes, but also provide a reference for an accurate absolute quantification of the amount of $^1O_2$ generated in an experiment from the observed absorbance bleach.

1. Introduction

The lowest electronically excited state of molecular oxygen, which unlike the triplet ground state has singlet spin character and is referred to as "singlet oxygen" ($^1O_2$), has a unique reactivity which makes it highly useful for a wide range of chemical and industrial applications and plays significant roles in many biological processes, such as intracellular signaling, protection of cells against bacteria, but also cell damage and ultimately induction of cell death [1,2]. The latter function is the basis of photodynamic therapy (PDT) [2,3], where a suitable photosensitizer is administered, ideally to a specific tissue location, which then produces $^1O_2$ upon light irradiation, thus allowing for doubly selective treatment based on the co-localization of administered drug and light.

In vitro, $^1O_2$ can be generated in chemical reactions [4], or by direct light excitation [5], although this is inefficient due to the spin-forbidden nature of the transition. More common is the use of a photosensitizing agent which absorbs light, undergoes intersystem crossing and transfers energy to molecular oxygen, generating $^1O_2$ [6,7]. Widely used photosensitizers are organic dye molecules with absorbance bands in the visible range of the spectrum; more recently, photosensitization has been reported using semiconductor nanocrystals [8], core/shell nanostructures [9] or plasmonic metal nanoparticles [10].

Any investigation of the mechanism of singlet oxygen induced reactions or biological responses, or of potential new photosensitizers, including those suitable for PDT, will require an appropriate method for detecting $^1O_2$, ideally quantitatively [2,4,11]. The most direct method for doing so is the observation of the characteristic $^1O_2$ emission band at 1270 nm [2,11–13]. However, this requires specialized equipment and can be difficult because of the low sensitivity of IR detectors and the extremely low quantum yield of the $^1O_2$ emission ($\sim 10^{-6}$ [2,7]), especially if the process under investigation produces $^1O_2$ with low yield or for investigations in water which rapidly quenches $^1O_2$ [6,13]. Therefore, it is more common to use molecular singlet oxygen probes whose properties are modified upon reaction with $^1O_2$. A variety of properties can be employed for detecting the reaction of the probe with $^1O_2$. Depending on the system under investigation, the question to be addressed, and the availability of equipment and expertise, detection

Abbreviations: $^1O_2$, singlet oxygen; ABDA, 9,10-anthracenediyl-bis(methylene)dimalonic acid; ADPA, 3,3′-anthracene-9,10-diyldipropanoic acid; DBB, 1,2-dibenzoylbenzene; DPBF, 1,3-diphenylisobenzofuran; EtOH, ethanol; PDT, photodynamic therapy; RB, rose bengal; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate

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can be based on slower, "off-line" analytical methods, such as electron paramagnetic resonance (EPR) [14], nuclear magnetic resonance (NMR) [15], or chromatography [16], but can also be achieved in real time and/or "*in situ*" (incl. microscopic methods) [2,11], normally by optical spectroscopic methods, which can be relatively easy to use and only require widely available simple spectrometers.

Although chemiluminescence probes have been developed [4], they suffer from the limited number of photons that are produced upon reaction with $^1O_2$. More widely used are probes whose fluorescence properties change upon reaction with $^1O_2$ [11,17]. Among such fluorescence probes is the widely used 1,3-diphenylisobenzofuran (DPBF), which however suffers from lack of solubility in water and is not specific to $^1O_2$ but also readily reacts with other reactive oxygen species (ROS), such as O_2^-, hydroxyl radical, or H_2O_2 [18–20]. These problems can be overcome by more recently developed anthracene-based fluorescence probes, such as the commercially available Singlet Oxygen Sensor Green (SOSG®) which has been used widely, in particular for biological research [2,15,21]. However, these probes carry significant costs; furthermore, they are able to photosensitize $^1O_2$ [21] and thus care needs to be taken in their application, especially in situations where the process under investigation produces $^1O_2$ with low yield [10]. Other $^1O_2$ probes are based on rare earth luminescence [11], but these require UV excitation which often makes them unsuitable, in particular for biological applications. In general, although fluorescence is a highly sensitive method, it can also easily suffer from artifacts, such as emission from other components present in the sample or contaminants, fluorescence quenching by other molecules, and dependence of the fluorescence yield on solvent conditions; these issues are a particular problem for biological work.

An alternative, and also widely used, approach for the detection of probe modification by $^1O_2$ is the use of UV–vis absorbance spectroscopy. Although this method is somewhat less sensitive than fluorescence to very small changes, it is also less prone to artifacts, since there is no absorbance quenching and absorbance spectra of a compound with high extinction cannot be dominated by small amounts of contaminants. One of the most widely used $^1O_2$ probes used with absorbance spectroscopy again is DPBF [2,5,22–26], which is commercially available for low cost, although, as pointed out above, it is not exclusively sensitive to $^1O_2$ and lacks solubility in water. The latter problem can be overcome by dissolving DPBF in micellar solutions [5,23–26] or in mixed alcohol/water solvents [10]. On the other hand, a range of water soluble anthracene-based $^1O_2$ probes have been reported [27–30], which do not react with other ROS species [3,17,27]. However, to the best of our knowledge, none of these are commercially available with the exception of 3,3′-anthracene-9,10-diyldipropanoic acid (ADPA), which has only low solubility in water [29], and 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), which is well soluble [29].

The basis of $^1O_2$ detection by DPBF and anthracene derivatives, independent of the use of fluorescence or absorbance spectroscopy, is their reaction with $^1O_2$ to form an endoperoxide *via* a [4 + 2]-cycloaddition, see Fig. 1, and the resulting loss of the extended π-electron system and its characteristic spectroscopic properties. DPBF forms endoperoxide **1** which then decomposes to 1,2-dibenzoylbenzene (DBB) [31]. Although formation of the anthracene endoperoxide **2** is partly reversible upon heating, at room temperature it can be regarded as irreversible [32].

In the context of our recent work on $^1O_2$ photosensitization by gold nanoparticles [10], we had to find a $^1O_2$ probe which can be used in combination with UV–vis spectroscopy, is soluble in aqueous samples and is commercially available at a reasonable price, which appears to restrict the list to DPBF and ABDA. For comparing such probes one also needs to take into account their sensitivity towards $^1O_2$, especially for the investigation of processes which produce $^1O_2$ with only small yield. The high sensitivity of DPBF to $^1O_2$ is well documented [6], but it is more difficult to find direct information on the sensitivity of ABDA,

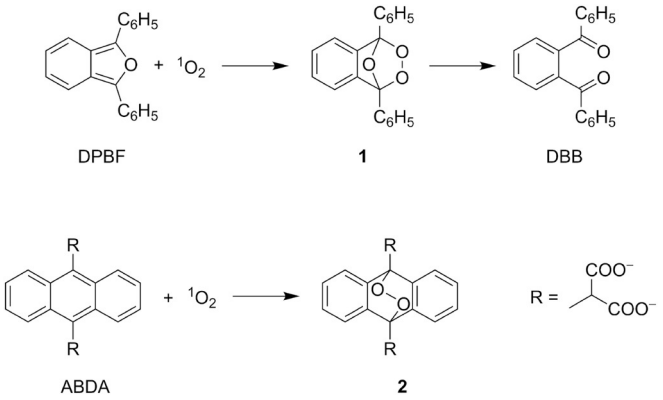

Fig. 1. Reaction of $^1O_2$ with DPBF (top) and ABDA (bottom).

which in principle is the more useful probe due to its good water solubility [29] and exclusive sensitivity to $^1O_2$ [17,27], and has been used increasingly in the last few years, particularly for investigating biological questions [9,33]. Recently, it was reported that the reactivity of ABDA in aqueous buffer solution is significantly lower than that of DPBF in organic solvents [34]. Thus, it seems possible that, in spite of its shortcomings, DPBF still might be the probe of choice for samples with low $^1O_2$ yield.

In the present work, we report a quantitative comparison of the $^1O_2$ sensitivity of DPBF and ABDA, where we define the $^1O_2$ sensitivity as the probability of $^1O_2$ to chemically react with the probe and thus cause its bleach before being deactivated. Since the intrinsic lifetime of $^1O_2$, and therefore the probability of deactivation before a reaction with the probe, is strongly dependent on the solvent [6,13], we investigated a range of solvents; in addition to H_2O, the probes were tested in D_2O which significantly increases the lifetime of $^1O_2$ and hence its chances of reacting with the probe; to allow for the poor solubility of DPBF in water, we used mixed water/ethanol solvents and micellar solutions for this probe. The results not only allow a comparative assessment of the usefulness of the two $^1O_2$ probes, but also provide a reference for an accurate absolute quantification of the amount of $^1O_2$ produced in an experiment using either of these probes in different aqueous environments.

2. Materials and Experimental Methods

2.1. Materials and Sample Preparation

ABDA, Rose Bengal (RB), sodium azide (NaN_3), sodium dodecyl sulfate (SDS) and D_2O (all from Sigma-Aldrich) and DPBF, ethanol (EtOH) and dimethylsulfoxide (DMSO) (all from Fisher Scientific) were used as received. Milli-Q water (> 18.2 MΩ cm) was prepared freshly before the experiment using a Barnstead Smart2Pure water purification system (Thermo Scientific).

Prior to use, all glassware, cuvettes and stirrer bars were left in aqua regia (1:3 HNO_3:HCl, from Sigma-Aldrich) for 15 min and rinsed several times with Milli-Q water. All solutions were prepared and kept stirring in the dark until used.

A stock solution of DPBF in EtOH (~0.1 mM) was used to prepare DPBF solutions in 50/50 (v/v) EtOH/H_2O or EtOH/D_2O immediately prior to use. SDS solutions (0.1 or 0.5 M) were prepared by dissolving SDS in milli-Q water and sonicating for 10 min; a small aliquot (1% of the total volume) of a ~4 mM stock solution of DPBF in EtOH was added immediately prior to use. Since it was found to be difficult to fully dissolve ABDA in neat water, a stock solution of ABDA in DMSO (~10 mM) was used to prepare solutions of ABDA in H_2O, D_2O or 50/50 (v/v) EtOH/D_2O with 1% (v/v) DMSO. The concentrations of DPBF (~0.05 mM) and ABDA (~0.1 mM) were chosen to give an absorbance near 1, which allows for the most accurate measurement of the

absorbance. For determining the extinction coefficients of DPBF and ABDA a minimum of 10 mg of each compound was weighed in accurately on a digital analytical balance to achieve sufficient accuracy. A stock solution of RB (~0.5 mM) was prepared in H_2O, a small aliquot of which was added to the ABDA or DPBF solutions to achieve the desired concentration of RB, which always was below 10^{-5} M to avoid aggregation [35]. For experiments in the presence of azide, a small aliquot of a 2 M sodium azide stock solution in H_2O or D_2O, as appropriate, was added to yield an azide concentration of 20 mM.

2.2. Irradiation Experiments

For irradiation experiments, the solutions were placed into a 10 mm path-length cuvette (Starna Special Optical Glass, SOG) equipped with a magnetic stirrer bar, and sealed with an airtight stopper and parafilm. The solution was kept stirring before and during irradiation. Solution stability in the dark was verified for a period of 30 min prior to irradiation using UV–Vis absorbance spectra (Genesys 10UV or Ocean Optics USB4000).

Irradiation at 532 nm was performed using a continuous-wave diode pumped solid state laser (Opus 532, Laser Quantum). The laser power of 200 mW, verified using a power meter (Ophir Optronics Nova, with a 30A-P-SH probe) was reduced to values of 0.14 mW or 2.4 mW using calibrated neutral density filters. These powers are further reduced by reflection losses of 4.6% on the front face of the cuvette. The rate of photon absorption during irradiation, N_{abs}, was calculated from the power incident on the sample itself and the sample absorbance at 532 nm, which results exclusively from the sensitizer (Rose Bengal, RB), see Fig. 2.

At regular intervals, irradiation was stopped and the absorbance spectrum recorded (Genesys 10UV or Ocean Optics USB4000). All irradiation experiments were repeated at least three times.

3. Determination of Singlet Oxygen Sensitivity

3.1. Singlet Oxygen Photogeneration

Rose Bengal is a well-known $^1O_2$ photosensitizer for the use with visible light (500–580 nm) which works *via* intersystem crossing of the photo-excited RB to its triplet state, ^{3}RB, and reaction with ground state O_2 [36]. Irradiation of RB results in photogeneration of $^1O_2$ with generally accepted quantum yields, Φ_{RB}, of 0.80 in methanol and ethanol [36,37] and 0.75–0.76 in H_2O or D_2O [16,36,38,39], which are

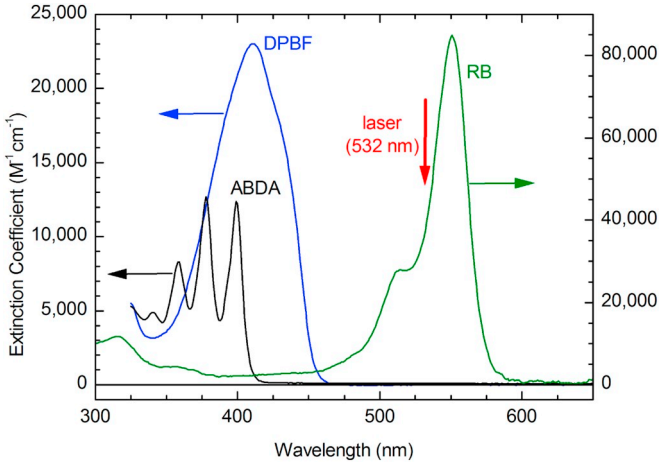

Fig. 2. UV–Vis spectra of RB in H_2O (green, right scale), ABDA in H_2O (black, left scale) and DPBF in 50/50 (v/v) EtOH/H_2O (blue, left scale). The red arrow indicates the wavelength of the laser used for photosensitization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

independent of the excitation wavelength. A value of $\Phi_{RB} = 0.75$ has also been reported for a 50/50 (v/v) mixture of methanol/H_2O [40] and for micellar solutions [41]. The $^1O_2$ quantum yield of RB was found to be independent of the concentration of RB [41], in line with the observation that RB in its ground state does not significantly quench $^1O_2$ [42]. Thus, it can be concluded that the value of Φ_{RB} should be quite similar for all samples and solvents used here (H_2O, D_2O, 50/50 (v/v) EtOH/H_2O or EtOH/D_2O, 0.1–0.5 M SDS solutions in H_2O) and a value of $\Phi_{RB} = 0.76$ will be assumed for calculating the rate of $^1O_2$ photogeneration from N_{abs}, the rate of photon absorption.

The $^1O_2$ quantum yield of RB is independent of the concentration of O_2 down to values well below those found in air-saturated solutions [43], due to the fact that the reaction of O_2 with triplet-RB is faster than the intrinsic triplet decay even at reduced oxygen concentrations [39]. Under atmospheric oxygen pressure, the concentrations of O_2 in aqueous solution and in 50/50 (v/v) EtOH/water mixtures are 0.3 and 0.7 mM, respectively [44], which is significantly higher than the probe concentrations of 0.05–0.1 mM used here. Thus, the yield or rate of $^1O_2$ photogeneration is not affected by the minor depletion of oxygen resulting from the reaction of the probes with singlet oxygen.

In parallel to the formation of $^1O_2$, photoexcitation of RB leads to the formation of superoxide (O_2^-) with a yield of approximately 0.20 in aqueous solution [39,45]. Whereas anthracene-based probes such as ABDA do not react with O_2^- [3,17,27], DPBF is known to do so [19]; however, as shown below, this has only a minor effect on our results.

3.2. Lifetime of Singlet Oxygen

The intrinsic lifetime of $^1O_2$ in solution depends strongly on the solvent since deactivation back to the triplet ground state is dominated by non-radiative energy transfer to solvent vibrations [6,13]. The reported values of the $^1O_2$ lifetime in neat H_2O cluster around 4 μs [6,13,46], whereas larger variations of the reported values are found for D_2O, ranging from 40 to 69 μs [6,13,27,39,46,47], caused by minor contamination by H_2O [6]. The strong variation of the $^1O_2$ lifetime with solvent and isotope content has been modelled using a model which assumes additive contributions from energy transfer to different types of bonds with bond-specific rate constants [13]; optimization of the latter reproduces the experimental results for a wide range of solvents with only a few model parameters.

This model predicts a reduction of the $^1O_2$ lifetime in D_2O from 68 μs to 52 μs upon the addition of only 2% H_2O, which is the typical H_2O content of our samples using D_2O as a solvent, as shown by FTIR spectroscopy, see section S1 of the *Supporting Information*, and we used this value for the analysis of data obtained in D_2O. For consistency, the $^1O_2$ lifetime in H_2O as calculated from the same model dataset, 4.2 μs, which is in good agreement with most experimental data, was used for the analysis of data obtained in H_2O. To the best of our knowledge, no experimental $^1O_2$ lifetimes have been reported for 50/50 (v/v) EtOH/H_2O or EtOH/D_2O, so again we used the model to estimate those lifetimes to be 6.6 μs and 24 μs, respectively.

The lifetime of $^1O_2$ in micellar solutions depends on the surfactant concentration; values near 5.5 μs have been reported for 0.5 M solutions of SDS in H_2O [5,48], which is in good agreement with predictions based on the relative solubility of oxygen in water and SDS micelles [49], using a lifetime of 4.2 μs for the aqueous phase and the experimentally observed lifetime within SDS micelles of 20 μs [48]. This model predicts a lifetime of 4.4 μs for a 0.1 M SDS solution in H_2O.

The rate constant for quenching of $^1O_2$ by RB has been reported to be less than 5×10^7 M^{-1} s^{-1} [42], thus having a negligible effect on the lifetime of $^1O_2$ at the concentration of RB used here.

3.3. Reaction of Probes with Singlet Oxygen

In the presence of a molecular $^1O_2$ probe, two additional pathways for the deactivation of $^1O_2$ back to the triplet ground state are present,

which can be described as chemical or physical quenching by the probe, with bimolecular rate constants k_r and k_q, respectively. The chemical quenching reactions of DPBF and ABDA involve [4 + 2]-cycloadditions, as shown in Fig. 1, whereas physical quenching refers to the fact that these molecules also could quench $^1O_2$ without undergoing a net chemical reaction. Detailed investigations of the temperature [12] and pressure [50] dependence of the reaction of DPBF with $^1O_2$ came to the conclusion that the first step of chemical and physical quenching is the reversible formation of an exciplex. Similarly, although the absolute rate constants for the [4+2]-cycloaddition of $^1O_2$ to 1,4-dimethylnaphthalene vary over several orders of magnitude in different solvents, the ratio of physical to chemical quenching is largely unaffected, suggesting a common exciplex intermediate [47]; the authors further proposed that anthracene- and furan-based probes react with $^1O_2$ by the same mechanism, albeit with different rate constants and different ratios of physical and chemical quenching, which is now widely accepted [32].

Physical quenching of $^1O_2$ by DPBF was found to be negligible, *i.e.* $k_q < 0.1\ k_r$, in a range of solvents, which included EtOH, methanol, mixtures of methanol with H_2O or D_2O [51] and aqueous SDS micellar solution [23], which are most relevant for the work presented here. More recent work also confirmed this conclusion for the organic solvents toluene and acetonitrile [34]. Similar observations have been reported for anthracene-based probes; thus, the rate constant for overall quenching of $^1O_2$ by ADPA, which is closely related to ABDA, in D_2O was found to be only 10% larger than that for chemical quenching [27,52]. Results for two other water soluble anthracene derivatives show that $k_q/k_r = 0.35$ for 9,10-bis(ethanesulfonate)anthracene in H_2O and D_2O [28] and $k_q/k_r = 0.3$ for bis-9,10-anthracene-(4-trimethylphenylammonium) [53]. The most direct and relevant observations in this context, however, is the recent observation that $k_q/k_r < 0.2$ for ABDA in phosphate buffer/D_2O [34].

3.4. Singlet Oxygen Sensitivity

Here, we define the *sensitivity* of a particular $^1O_2$ probe P, Φ_P, as the probability that photogenerated singlet oxygen reacts irreversibly with a probe molecule before it deactivates by solvent quenching under the given experimental conditions. Φ_P is related to the kinetics of the possible reaction pathways of $^1O_2$ outlined in the preceding sections as shown in Eq. (1), where k_r and k_q denote the bimolecular rate constants for a chemical reaction of $^1O_2$ with P and for physical quenching of $^1O_2$ by P, respectively, and k_0 the pseudo-first order rate constant for deactivation by the solvent, which is the inverse of the intrinsic lifetimes summarized in Section 3.2.

$$\Phi_P = \frac{k_r[P]}{(k_r + k_q)[P] + k_0} \tag{1}$$

The value of Φ_P depends on the solvent, which directly affects the intrinsic lifetime of $^1O_2$, *i.e.* k_0, and, *via* its viscosity and other solvent effects [47], the bimolecular rate constants for the reaction between $^1O_2$ and P. However, the value of Φ_P also depends on the concentration of the probe [P]. Hence, although of high interest for evaluating the usefulness of a particular $^1O_2$ probe under the given conditions, the value of Φ_P is not a universally applicable number. On the other hand, the rate constants k_q and k_r are independent of the probe concentration and allow the calculation of Φ_P for any given conditions, provided that the intrinsic life time of $^1O_2$ is known, Eq. (1). Therefore, in the following, we determined values for these rate constants from our data for different probes and solvents and then used these values for comparisons of the $^1O_2$ sensitivity under typical experimental conditions.

The sensitivity of a probe, Φ_P, under the given conditions can be determined experimentally by calculating the number of probe molecules which bleach during irradiation for a given time interval, Δt, and relating it to the amount of $^1O_2$ generated in this time interval. The former is given by the measured absorbance change ΔA, the extinction coefficient ε at the probe wavelength, and the sample volume V, whereas the latter can be calculated from the rate of photon absorption, N_{abs}, and the quantum yield of $^1O_2$ photogeneration by RB (d denotes the optical path-length and N_A is Avogadro's constant):

$$\Phi_P = \frac{\Delta A\ \ V N_A}{\varepsilon d\ \ N_{abs}\Phi_{RB}\Delta t} \tag{2}$$

However, due to the reaction of the probe with $^1O_2$, its concentration and hence also its sensitivity change over the course of the irradiation. Therefore, Eq. (2) is only valid for small changes or short time intervals, but it provides an absolute value of Φ_P, independent of any assumptions regarding the relative contributions of physical and chemical quenching (*vide infra*). A more thorough approach, which avoids the approximation of constant Φ_P over the experimental irradiation time intervals by explicitly accounting for the change of the probe concentration and allows the analysis of the full data set, has to take into account the kinetics of the reaction.

This approach is based on the observation that, since the lifetime of $^1O_2$ is very short compared to the experimental time scale (minutes), the steady-state concentration of $^1O_2$ at any given time t can be calculated by equating the rates of its photogeneration and its decay *via* solvent quenching or reaction with a probe molecule:

$$\frac{N_{abs}\Phi_{RB}}{VN_A} = [k_0 + (k_r + k_q)[P]]\ \ [^1O_2]_{ss} \tag{3}$$

Using this steady-state concentration of $^1O_2$ yields the rate of bleach of probe P due to the reaction with $^1O_2$:

$$\frac{d[P]}{dt} = -k_r[P][^1O_2] = -k_r[P]\frac{N_{abs}\Phi_{RB}}{VN_A[k_0 + (k_r + k_q)[P]]} \tag{4}$$

As shown explicitly in section S2 of the *Supporting Information*, this equation can be integrated, yielding the following relationship between the measured time-dependent absorbance during irradiation, $A(t)$, and time t (A_0 is the initial absorbance at $t = 0$):

$$t = C_1\left(C_2 \ln\frac{A_0}{A(t)} + C_3[A_0 - A(t)]\right) \tag{5}$$

where $C_1 = \frac{VN_A}{\varepsilon d N_{abs}\Phi_{RB}}$, $C_2 = \frac{\varepsilon d k_0}{k_r}$, and $C_3 = \frac{k_r + k_q}{k_r}$

The constant C_1 is given by parameters related to the $^1O_2$ sensitizer (Φ_{RB}), the probe investigated (ε) and the experimental conditions (V, d, N_{abs}), all of which are known. Parameter C_2 relates the rate constant for the chemical quenching of $^1O_2$ by the probe to the intrinsic $^1O_2$ lifetime ($1/k_0$), and C_3 provides the relationship of the rate constants for physical and chemical quenching of $^1O_2$ by the probe.

Eq. (5) was used to fit the experimental data, using a non-linear least-squares (Levenberg-Marquardt) fitting routine (Microcal Origin). Unfortunately, the time dependence of the two terms inside the bracket in Eq. (5) is not sufficiently different to allow for an independent determination of parameters C_2 and C_3 from the data. Therefore, parameter C_3, which quantifies the relative contributions of physical and chemical quenching of $^1O_2$ by the probe, was set to fixed values based on the literature results discussed in the preceding section; it will be shown below that the exact value chosen for C_3 does not significantly affect the singlet oxygen sensitivities reported here and has only a minor effect on the rate constant k_r. Thus, the fits were performed with only two free fit parameters, C_2 and A_0, and k_r was then determined from the value of C_2, using the solvent-dependent intrinsic lifetimes of $^1O_2$, $1/k_0$, given in Section 3.2.

4. Results

4.1. Extinction Coefficients

UV–Vis spectra of the $^1O_2$ sensitizer, RB, and the $^1O_2$ probes, DPBF

Table 1
Extinction coefficients of ABDA and DPBF in different solvents.

$^1O_2$ probe	Solvent	Wavelength[a] (nm)	Extinction coefficient ε (M^{-1} cm^{-1})[b]
ABDA[c]	H_2O	398-400	11,990 ± 60
	D_2O		11,770 ± 90
	EtOH/H_2O[d]		13,170 ± 40
	EtOH/D_2O[d]		13,310 ± 60
DPBF	EtOH/H_2O[d]	410–412	23,000 ± 250
	EtOH/D_2O[d]		22,710 ± 140
	0.1 M SDS/H_2O	414–416	22,730 ± 270
	0.5 M SDS/H_2O		22,690 ± 230

[a] Wavelength range over which results were averaged, here and in the analysis of the photobleaching experiments.
[b] Errors determined from the standard deviation of several repeat experiments.
[c] ABDA samples contained 1% (v/v) DMSO.
[d] 50/50 (v/v).

and ABDA, are shown in Fig. 2. These show that the probes do not have any absorbance at the excitation wavelength used in our experiments, 532 nm, whereas the sensitizer has almost no absorbance at the wavelengths where the probes have strong absorbance, 410 nm for DPBF and 380 or 400 nm for ABDA. It should be noted that the extinction coefficient of DPBF is almost twice that of ABDA, which contributes to its usefulness as $^1O_2$ probe.

Quantitative analysis of the probe bleaching due to $^1O_2$, *i.e.* the determination of the singlet oxygen sensitivity, requires knowledge of the probe extinction coefficient. We accurately determined these for the different solvents used here, see Table 1. The results show some variation of the extinction coefficient in different solvents, in particular for ABDA, whereas within the uncertainty of the measurement no difference was found when replacing H_2O by D_2O, as expected. There is also no effect of the concentration of SDS on the extinction coefficient of DPBF, since at the concentrations used here there is only one DPBF molecule per micelle. The extinction coefficients reported here are in good agreement with literature values reported for DPBF in organic solvents [22,31,34] and surfactant solutions [25] and for ABDA in aqueous buffer solution [29,34].

4.2. Probe Bleaching upon Photoexcitation of Rose Bengal

The $^1O_2$ sensitizer, RB, and both $^1O_2$ probes, ABDA or DPBF, when used separately, are stable in the dark as well as under the irradiation conditions used here, see Fig. S2 in the *Supporting Information*. In the presence of RB both probes undergo significant photobleaching, see Fig. 3, as is expected due to their reaction with singlet oxygen, *i.e.* the cycloaddition reactions shown in Fig. 1 leading to endoperoxide products, which result in the loss of absorbance in the near UV or visible spectral region. It should be noted that upon direct photoexcitation 9,10-substituted anthracenes and DPBF photosensitize $^1O_2$ with significant and solvent-dependent yield [54,55] and thus extreme care has to be taken if the process under investigation requires excitation at wavelengths below 450 nm, and neither DPBF nor ABDA may be suitable as $^1O_2$ probe under those conditions.

It should be noted that RB does not bleach at all under our irradiation conditions, which means that the experiments are conducted under conditions of constant $^1O_2$ formation rates. It is also obvious from the results presented in Fig. 3 that the rate of DPBF photobleaching is significantly higher than that of the ABDA photobleaching, in spite of the lower rate of $^1O_2$ formation resulting from the lower concentration of RB used with DPBF in these particular sets of experiments. This is a first indication of the significantly higher sensitivity of DPBF for $^1O_2$, which will be quantified explicitly below.

The probe bleaching efficiency upon photosensitization by RB was quantified from spectra as those shown in Fig. 3 by measuring the probe

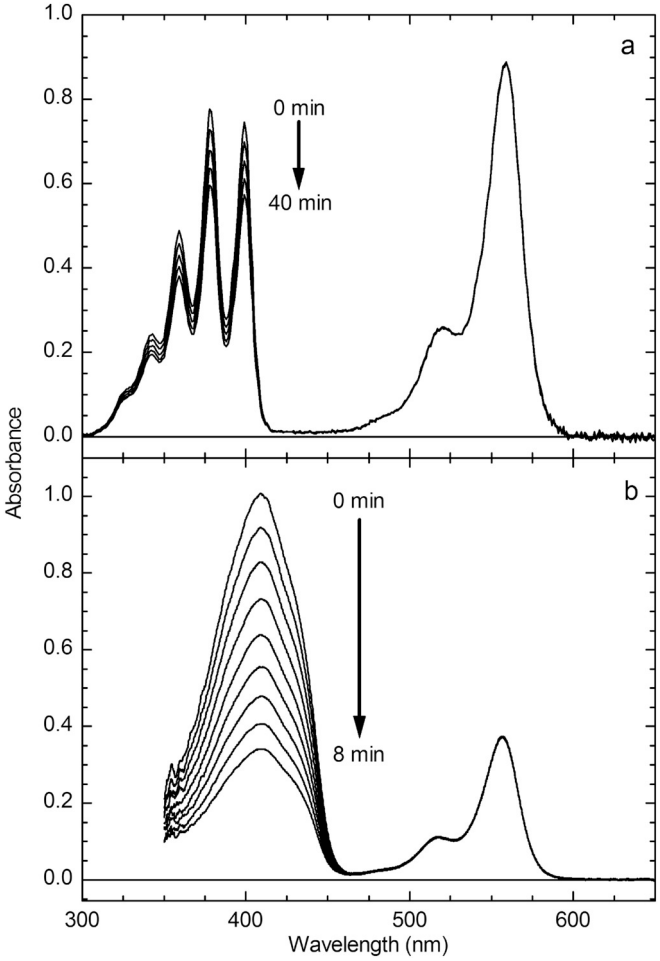

Fig. 3. Photobleaching of ABDA (a) and DPBF (b) in 50/50 (v/v) EtOH/D_2O upon irradiation with 0.14 mW cw laser light at 532 nm in the presence of photosensitizer RB; the spectra were taken every 10 min over a time interval of 40 min (ABDA, a) and every minute over a time interval of 8 min (DPBF, b).

absorbance at the maximum of the near-UV absorbance band, averaged over a limited wavelength range to improve the signal-to-noise (ABDA: 398–400 nm, DPBF: 410–412 nm) and subtracting the small residual absorbance of RB at those wavelengths. For a first comparison, the probe absorbance was normalized to the absorbance at the start of the irradiation. Fig. 4 shows some typical results, although it should be emphasized that the concentration of RB, and hence the amount of photogenerated $^1O_2$, varied to some extent between the different curves, so that they are not fully quantitatively comparable. Nevertheless, several trends are clearly shown in these results: (i) the solvent has a significant effect on the probe bleaching, with particularly pronounced bleaching found in D_2O, whereas the slowest bleaching occurs in H_2O, in good agreement with the solvent-dependent $^1O_2$ lifetimes summarized in Section 3.2; (ii) as already shown in Fig. 3, when comparing results in the same solvent system, ABDA bleaches significantly more slowly than DPBF, indicating that it has a significantly lower sensitivity for $^1O_2$ than DPBF; (iii) the addition of the $^1O_2$ quencher NaN_3 suppresses the photobleaching almost completely, which proves that photobleaching is largely due to $^1O_2$, as discussed in more detail in the next section.

4.3. Probe Bleaching is due to Singlet Oxygen

Two alternative mechanisms for the bleaching of ABDA or DPBF after photoexcitation of RB which do not involve the formation of $^1O_2$ need to be considered, based on reports in the literature: (i) direct

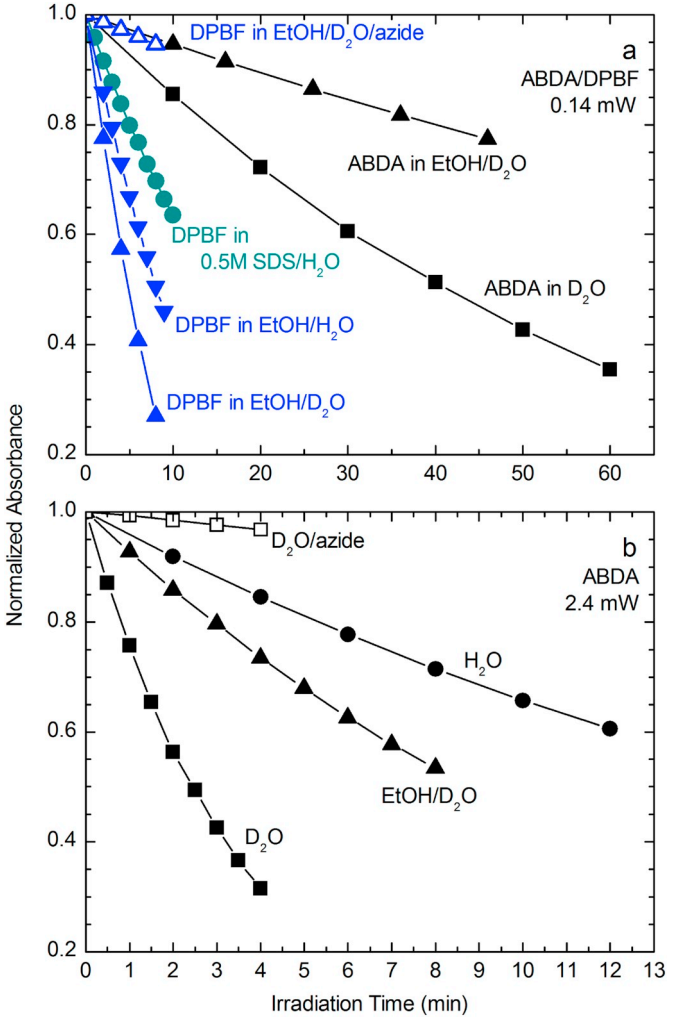

Fig. 4. Photobleaching of ABDA (black) and DPBF (blue, dark cyan) in different solvents upon irradiation with 0.14 mW (a) or 2.4 mW (b) cw laser light at 532 nm in the presence of photosensitizer RB; shown here is the probe absorbance at 398–400 nm (ABDA) or 410–412 nm (DPBF), normalized to the absorbance at the start of the irradiation, averaged over several repeat experiments at a similar concentration of RB, resulting in standard deviations for the individual data points which are smaller than the size of the symbols; however, the concentration of RB (~2–4 μM), and hence the amount of photogenerated $^1O_2$, varied between the different curves, so that they are quantitatively not fully comparable; solid and open symbols refer to measurements in the absence and presence of 20 mM NaN_3, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

triplet energy transfer from RB resulting in bleaching, and (ii) the reaction with superoxide, O_2^-, which is formed upon photoexcitation of RB.

The triplet energies of 9,10-substituted anthracene (~41 kcal mol^{-1}) [56] and DPBF (~34 kcal mol^{-1}) [57] in organic solvents suggest the possibility of triplet-triplet energy transfer from ^{3}RB (40–42 kcal mol^{-1} [37]). In experiments using ADPA, a water-soluble anthracene derivative similar to ABDA, triplet energy transfer from ^{3}RB, followed by efficient endoperoxide formation by the reaction of ^{3}ADPA with ground state oxygen, was proposed to explain the observation that the well-known $^1O_2$ quencher NaN_3 was not able to quench all of the ADPA bleach upon RB photosensitization even at saturating concentrations [38]. Although 23% of the experimentally observed bleaching of the ADPA absorbance was associated with this pathway, it was shown that quenching of ^{3}RB by ADPA is only a minor decay path for ^{3}RB and does not lead to a significant decrease of its $^1O_2$ yield; the reason why this minor ^{3}RB quenching path can make a measurable contribution to the bleaching of ADPA is the fact that anthracene-based probes have only low $^1O_2$ sensitivity (*vide infra*), so that only a small fraction of the generated $^1O_2$ results in bleaching of a probe molecule. These conclusions were confirmed in experiments which directly measured the rate constant for quenching of ^{3}RB by ADPA and O_2, respectively [39]. It seems highly likely that triplet energy transfer from the RB dianion to the tetraanion ABDA, which also has bulkier substituents, is significantly slower than that to the dianion ADPA, and indeed virtually complete quenching of ABDA photobleaching upon RB photosensitization has been reported in the presence of 20 mM NaN_3 [29], suggesting that the triplet energy transfer pathway can be ignored for ABDA. In similar experiments, the rate of DPBF bleaching upon RB photosensitization in pyridine has been shown to drop to approximately 10% of the quencher-free rate in the presence of the $^1O_2$ quencher tetramethylethylene at saturating concentrations [58], suggesting that the contribution of DPBF photobleaching due to triplet energy transfer from ^{3}RB to DPBF also is small.

The formation of O_2^- upon photoexcitation of RB, with a yield of approximately 0.20 in aqueous solution [39,45], provides another possible mechanism for the observed photobleaching of DPBF [19,20], whereas anthracene-based probes such as ABDA do not react with O_2^- [17,27].

We investigated the extent to which these two alternative mechanisms contribute to the observed photobleaching of ABDA or DPBF under our experimental conditions by repeating experiments in the presence of sodium azide, NaN_3, which is a well-known quencher of $^1O_2$, but is not expected to interfere with triplet energy transfer from RB to ABDA or DPBF, and does not quench O_2^- [19]. However, care has to be taken since azide may quench the triplet state of RB (^{3}RB), *i.e.* the $^1O_2$ precursor [59]; although the reaction of azide with ^{3}RB is much slower than its reaction with O_2 (rate constants of 0.07×10^9 M^{-1} s^{-1} *vs.* 1.6×10^9 M^{-1} s^{-1} in H_2O [39,59]), this is partly compensated by the higher concentration of azide. A concentration of 20 mM NaN_3 was chosen here, which is well above the concentration at which saturation of $^1O_2$ quenching has been observed in similar experiments [38], due to the well-characterized fast reaction of azide with $^1O_2$, which occurs with a rate constant of $\sim 5 \times 10^8$ M^{-1} s^{-1} [7], *i.e.* is much faster than the oxidation of ABDA or even DPBF, given their concentrations and rate constants for the reaction with $^1O_2$, see Table 2.

These experiments showed a significant reduction of the probe absorbance bleach in the presence of NaN_3, see Fig. 4. In the case of ABDA in D_2O, the rate of absorbance bleach in the presence of NaN_3 was found to be only 4% of that in the absence of a $^1O_2$ quencher under otherwise identical conditions; for DPBF, the addition of NaN_3 reduced the rate of bleaching to 5% in EtOH/D_2O and 10% in EtOH/H_2O, respectively, compared to that found in the absence of a $^1O_2$ quencher. This significant difference between the (relative) effect of azide in H_2O and D_2O containing solvents shows that it is mostly due to the quenching of $^1O_2$ and not ^{3}RB, since the $^1O_2$ lifetime depends strongly on the isotopic solvent composition (see Table 2), whereas no such effect is expected for ^{3}RB. Overall, these results show that, as expected from the discussion above, neither triplet energy transfer from ^{3}RB nor the formation of O_2^- upon photoexcitation of RB greatly affect the outcome of the experiments presented in the previous section. For ABDA and for DPBF in EtOH/D_2O, the effect from these two alternative mechanisms is at most a few percent, essentially within the uncertainty of our results, whereas for DPBF in EtOH/H_2O, the $^1O_2$ sensitivity might be overestimated by ~10% when neglecting these effects. For DPBF in SDS/H_2O solution, neither of these effects is expected to make any significant contribution since the negative surfactant molecules surrounding each DPBF molecule will prevent the RB dianion and superoxide O_2^- from reacting with DPBF with any significant yield. Therefore, we conclude that the photobleach shown in Fig. 3, and in similar experiments in different solvents, directly reflects the reaction of the probe with the $^1O_2$ generated by RB photosensitization.

Table 2
Rate constant k_r for deactivation of $^1O_2$ by a chemical reaction with ABDA or DPBF, respectively, and resulting $^1O_2$ sensitivity Φ_P at probe concentrations corresponding to an absorbance of 1 in the band near 400 nm (ABDA) or 410 nm (DPBF), in different solvents; also given is the life time $\tau = 1/k_0$ of $^1O_2$.

$^1O_2$ probe	Solvent	$\tau = 1/k_0$ (μs)[a]	k_r (10^7 M^{-1} s^{-1})[b]	Φ_P[b]
ABDA[c,d]	H_2O	4.2	5.61 ± 0.12	0.0192 ± 0.0004
	D_2O	68	3.92 ± 0.18	0.178[f] ± 0.005
	EtOH/D_2O[g]	24	2.78 ± 0.14	0.050[f] ± 0.002
DPBF[e]	EtOH/H_2O[g]	6.6	283 ± 11	0.449 ± 0.010
	EtOH/D_2O[g]	24	231 ± 9	0.722[f] ± 0.008
	0.1 M SDS/H_2O	4.4	230 ± 9	0.308 ± 0.008
	0.5 M SDS/H_2O	5.5	177 ± 29	0.299 ± 0.035

[a] From the literature, see Section 3.2.
[b] Errors determined from the standard deviation of several repeat experiments.
[c] ABDA samples contained 1% (v/v) DMSO.
[d] Data analyzed with $C_3 = 1.2$, *i.e.* assuming $k_q = 0.2\ k_r$, see text.
[e] Data analyzed with $C_3 = 1$, *i.e.* assuming $k_q = 0$, see text.
[f] Assuming neat solvents, *i.e.* no contamination by H_2O.
[g] 50/50 (v/v).

4.4. Singlet Oxygen Sensitivity

The sensitivity of a $^1O_2$ probe, Φ_P, as defined in Eq. (1), can be determined experimentally from the amount of $^1O_2$ generated during irradiation for a given time interval and the number of probe molecules which bleach in this time interval, determined by the bleach of the probe absorbance, Eq. (2). This analysis yields values for Φ_P of approximately 0.018, 0.05 and 0.12 for ABDA in H_2O, 50/50 EtOH/D_2O and D_2O, respectively, and 0.4 and 0.65 for DPBF in 50/50 EtOH/H_2O and 50/50 EtOH/D_2O, respectively, at the start of the irradiation from the data presented in Fig. 4. It should be noted that these values do not depend on the concentration of RB, which is accounted for in Eq. (2), but depend on the concentration of the probe, which here was chosen so that the initial absorbance was in the vicinity of 1. Since the sensitivity decreases with probe concentration, its value decreases during the irradiation experiment; for example, the $^1O_2$ sensitivity of ABDA in D_2O decreases from 0.12 to 0.05 over the course of the experiments summarized in Fig. 4b.

In any application where a particular probe is used for detecting $^1O_2$, the value of Φ_P is important for quantifying the amount of $^1O_2$ produced; similarly, it is also important for comparing the usefulness of different $^1O_2$ probes. However, due to its dependence on the concentration and its variation during the experiment, the value of Φ_P is not a universally applicable number. On the other hand, Φ_P can be calculated for any given conditions from the rate constants k_q and k_r, which are independent of the probe concentration, Eq. (1). Therefore, here we determined the values for these rate constants for ABDA and DPBF in different solvents and then used them for comparing their $^1O_2$ sensitivity at concentrations corresponding to a probe absorbance of 1 in a 1 cm pathlength cell, which is a typical experimental condition, since it allows for high signal-to-noise absorbance measurements.

The determination of k_q and k_r is based on the theoretical treatment described in Section 3.4; in particular, Eq. (5) was used to fit the time-dependent absorbance data. As described above, the fits were performed with only two free fit parameters, C_2 and A_0. Parameter C_1 was calculated from the experimental parameters, the extinction coefficient (Table 1) and the $^1O_2$ quantum yield of RB. Parameter C_3, which quantifies the relative contributions of physical and chemical quenching of $^1O_2$ by the probe, was fixed to a range of values based on the literature results discussed in Section 3.3. The rate constant k_r was then determined from the value of C_2, using the solvent-dependent intrinsic lifetimes of $^1O_2$, $\tau = 1/k_0$, given in Section 3.2. Some example sets of experimental data and the resulting fits are shown in Fig. S3 in the *Supporting Information*, showing that Eq. (5) allowed a good fit of all of our data. Table S1 in the *Supporting Information* summarizes the fit results obtained for a wide range of values of C_3; these data show that the results for k_r reported here, and even more so those for the $^1O_2$ sensitivity Φ_P, do not greatly depend on the exact values of C_3 assumed in the fits, if these are kept within a reasonable range from those suggested by the literature results.

Thus, the value of k_q for DPBF has been reported to be less than 10% of that of k_r, *i.e.* $C_3 < 1.1$ and there is no significant difference between the $^1O_2$ sensitivities Φ_P obtained using values of 1 or 1.1 for C_3. In fact, our data directly rule out values of $C_3 \geq 1.6$ for DPBF, since they lead to poor fits and unphysical results for k_r, compare Fig. S4 and Table S1 in the *Supporting Information*. Furthermore, Table S1 shows that the ratio of the values of k_r obtained in EtOH/D_2O and EtOH/H_2O increases with increasing C_3, with the reaction becoming faster in EtOH/D_2O than in EtOH/H_2O for $C_3 \geq 1.2$. Since the viscosity of D_2O is higher than that of H_2O, this is unlikely to be correct, which further supports the literature evidence of negligible physical quenching of $^1O_2$ by DPBF. In the following, we will therefore use the fit results obtained for $C_3 = 1$. As discussed above, for ABDA it was reported that $C_3 \leq 1.2$ [34] and in general, anthracene-based probes have values of C_3 which vary between 1 and 1.5. Table S1 shows that C_3 can be varied over this range without significantly affecting the $^1O_2$ sensitivity Φ_P and without greatly affecting the values of k_r, which is due to the fact that for ABDA with its low reactivity towards $^1O_2$, $(k_r + k_q)$ [P] $< < k_0$, so that Eq. (1) can be approximated by $\Phi_P \approx k_r$ [P] / k_0, which is independent of k_q. Here, we will use the fit results obtained for $C_3 = 1.2$.

Experiments were undertaken at different laser powers and with different concentrations of the $^1O_2$ sensitizer, RB. Although both of these factors directly affect the rate of $^1O_2$ production and hence the rate of probe bleaching, see Fig. 4, there was no effect of either of these factors on the resulting values of the rate constants k_r or the $^1O_2$ sensitivities Φ_P within the error of the measurements.

Table 2 summarizes the results of these fits. It is obvious that the bimolecular rate constant k_r changes for both probes when replacing H_2O by D_2O, as expected from the viscosity of D_2O, which is ~25% higher than that of H_2O [60], resulting in correspondingly slower diffusion of O_2 [61]. Similarly, the higher viscosity of a 50/50 (v/v) mixture of EtOH and water, compared to neat water [62], slows down the reaction. Most importantly, however, the reactivity of DPBF with $^1O_2$ is shown to be significantly higher than that of ABDA, with a rate constant which is almost two orders of magnitude larger when comparing the same solvent.

The resulting $^1O_2$ sensitivities, Φ_P, at probe concentrations corresponding to a maximum absorbance of 1 for ABDA and DPBF in different solvents reflect these viscosity-induced variations of the reactivity, but are further modified by the significant variation of the lifetime of $^1O_2$, as discussed above. The short lifetime of only 4 μs in H_2O, *i.e.* the rapid deactivation by the solvent, makes it much more difficult to detect $^1O_2$, compared to the situation in D_2O, where $^1O_2$ has a lifetime of 68 μs.

5. Discussion

5.1. Reactivity of $^1O_2$ with DPBF and ABDA

The reactivity of DPBF with $^1O_2$ has been studied widely in organic solvents. Values for the bimolecular rate constant in the range 40–160 × 10^7 M^{-1} s^{-1} were reported in a range of neat organic solvents, including ethanol [5,6,12,22,24,34]. To the best of our knowledge, no values have been reported for the rate constant k_r in the solvent conditions used here, 50/50 (v/v) EtOH/H_2O and 50/50 (v/v) EtOH/D_2O; however, the highest value for k_r reported in the literature, 510 × 10^7 M^{-1} s^{-1}, is that for 50/50 (v/v) methanol/H_2O [22], which is in reasonably good agreement with our result for 50/50 (v/v) EtOH/H_2O (280 × 10^7 M^{-1} s^{-1}, Table 2), given the relative viscosity of methanol/H_2O (1.57 cP [63]) and EtOH/H_2O mixtures (2.4 cP [62]).

The observation that k_r scales with the inverse of the value of the viscosity when comparing its value in EtOH/H_2O with that in methanol/H_2O, but also with that in EtOH/D_2O (see Table 2, noting that the viscosity of D_2O is ~25% higher than that of H_2O [60]) suggests that in mixed alcolhol/water solutions the rate of this reaction is controlled by diffusion. This is further supported by an estimate of the diffusion-limited bimolecular rate constant k_d using the well-known expression [64]:

$$k_d = 4\,\pi\,(R_1 + R_2)\,(D_1 + D_2)\,N_A \quad (6)$$

here, R_1 and R_2 denote the effective radii and D_1 and D_2 the diffusion constants of the two reactants. The effective radii were approximated by the van-der-Waals radii of O_2 (2.1 Å) and benzene (~3 Å), since collisions of $^1O_2$ with the phenyl rings are not expected to contribute to the reaction. The main contribution to diffusion was assumed to arise from $^1O_2$ with a diffusion constant based on that of O_2 in H_2O (2 × 10^{-5} cm^2 s^{-1} [61]), but scaled by the inverse of the viscosity. This estimate suggests a diffusion-limited rate constant for the reaction of $^1O_2$ with DPBF in 50/50 (v/v) EtOH/H_2O of ~290 × 10^7 M^{-1} s^{-1} at room temperature, which is very close to the experimental value reported here, Table 2; a similar agreement is obtained for the result in EtOH/D_2O and the literature value in methanol/H_2O. Thus, it appears that the reaction is indeed close to the diffusion limit in these mixed solvent systems. This is different to the situation in organic solvents, all of which have significantly lower viscosity than the alcohol/water mixtures and thus would be expected to result in higher rate constants k_r; in contrast, k_r in all of these solvents is significantly smaller than those in the mixed solvents, see above. In fact, it has been shown explicitly that in toluene the reaction between $^1O_2$ and DPBF occurs under diffusion control only at low temperatures (below -30 °C), whereas at room temperature the pre-equilibrium mechanism prevails, resulting in a negative activation energy [12]. It appears that in alcohol/water mixtures with their unusual molecular structure the transition between diffusion-limited and pre-equilibrium reaction conditions is shifted to significantly higher temperatures than in organic solvents. Although this is of high interest in the context of fundamental reaction dynamics, a more detail investigation or explanation of this observation is beyond the scope of the current report.

Strictly speaking, the simple reaction scheme underlying our data analysis is not valid for the microheterogeneous environment of SDS solutions, where photogeneration of $^1O_2$ by the water-soluble sensitizer RB and quenching by the water-insoluble DPBF take place in separate phases. However, the exchange of molecular oxygen between the aqueous phase and the micelle interior occurs on the time scale of 10–100 ns [26], much faster than the intrinsic decay of $^1O_2$ or its reaction with DPBF. Therefore, the concentrations of $^1O_2$ in the two phases rapidly equilibrate and the values for k_r in Table 2 can be considered as "effective" rate constants [65,66]. Values in the range of (70–130) × 10^7 M^{-1} s^{-1} have been reported for the reaction of DPBF with $^1O_2$ in 0.1 M SDS solution in D_2O [6,25,26]. Even when accounting for the higher viscosity of D_2O compared to H_2O [60], these values are not in good agreement with our experimental results in H_2O, suggesting a more complex effect of the exact nature of the solvent on the reaction in micellar solution. On the other hand, a value of (450 ± 20) × 10^7 M^{-1} s^{-1} was reported for 0.1 M SDS solution in H_2O [24], but this value was based on a $^1O_2$ lifetime of 2 µs, and hence is too large by a factor of ~2; after correction for this inaccuracy, the result is in good agreement with our results. Similarly, a value of 9.7 × 10^{-5} M has been reported for the reactivity index $\beta = k_0/k_r$ for a 0.1 M SDS solution in H_2O [23], which also is identical to our result when accounting for a $^1O_2$ lifetime, $1/k_0$, of 4.4 µs (*vide supra*).

Lee and Rodgers developed a two-pseudophase model for describing the reaction of $^1O_2$ with a quencher in a microheterogeneous environment [48,65], which is based on the fast equilibration of molecular oxygen between the aqueous and micellar phases and different intrinsic $^1O_2$ lifetimes in the two phases [26], see section S5 of the *Supporting Information*. It was shown that in equilibrium the concentration of $^1O_2$ inside the micelle is larger than in the aqueous phase by a factor $K = 2.9$ and the intrinsic lifetime of $^1O_2$ inside micelles in the absence of an additional quencher was found to be 20 µs [48]. This model explains the decrease of the effective rate constant k_r with increasing SDS concentration (Table 2), since at higher SDS concentrations an increasing amount of $^1O_2$ is sequestered away into non-DPBF containing micelles. Moreover, it can be shown that the effective rate constant k_r relates to the microscopic parameters as shown in Eq. (7), see section S5 of the *Supporting Information* [65,66]:

$$k_r = k_{r,m}\frac{K}{K\ f + (1 - f)} \quad (7)$$

here, f denotes the total volume fraction of the micellar phase and $k_{r,m}$ denotes the microscopic rate constant for the reaction of $^1O_2$ with DPBF in the micellar environment. With $K = 2.9$, the observed effective rate constants k_r for our experiments in 0.1 and 0.5 M SDS/H_2O solution (Table 2) yield values of $k_{r,m}$ of (83 ± 3) × 10^7 M^{-1} s^{-1} and (75 ± 16) × 10^7 M^{-1} s^{-1}, respectively. Not only are these values in good agreement with each other, they also compare well with the rate constants observed in 50/50 EtOH/H_2O or EtOH/D_2O, given that the microviscosity of the interior of SDS micelles has been measured to be 9 cP [67], whereas the viscosity of 50/50 EtOH/H_2O has a value of 2.4 cP [62]. Thus, our results confirm the hindered diffusion in the micelle interior which is expected due to the presence of long hydrocarbon chains.

It is well known that the reactivity of anthracene-based $^1O_2$ probes is smaller than that of DPBF [6], with bimolecular rate constants in organic solvents in the range (0.1–7) × 10^7 M^{-1} s^{-1} for various 9,10-substituted anthracenes [6], where the variation is due to changing substituents and solvents. In particular, various water soluble anthracene derivatives have quenching rate constants in the range of (2–5) × 10^7 M^{-1} s^{-1} in H_2O [16,28,30,53], and ABDA has been shown to have a chemical quenching rate constant k_r = 5.5 × 10^7 M^{-1} s^{-1} in phosphate buffer/D_2O [34,68]. The latter value is close to the value found here for neat H_2O, and only slightly larger than the value for D_2O, suggesting at most a small effect of the presence of buffer.[1] It is interesting to note that for ADPA, which has two carboxylate substituent end groups compared to four for ABDA, slightly higher values of (6–8) × 10^7 M^{-1} s^{-1} (calculated from the reported rate constants of overall quenching assuming $k_q/k_r = 0.2$, see Section 3.3) have been reported in H_2O or D_2O [27,47,52], potentially reflecting increased

[1] We would like to note that values of k_r for ABDA in phosphate buffer solution have been reported which are smaller than the values determined here in H_2O or D_2O by a factor of 6–10 [29,69]; one of these reports also suggested that physical quenching of $^1O_2$ by ABDA becomes dominant in buffer solution [69]. However, the latter observation contradicts all that is known about the dominance of chemical quenching by anthracene-based probes, see Section 3.3, and these results could not be reproduced in a series of careful experiments [34].

steric hindrance by the additional carboxylate groups of ABDA.

In D_2O, the reaction of $^1O_2$ with ABDA is slower than in H_2O, and in 50/50 (v/v) EtOH/D_2O the rate is further reduced compared to neat aqueous solution, see Table 2, as expected from the higher viscosity of D_2O than H_2O [60] and the even higher viscosity of EtOH/water mixtures [62]. A comparison with the DPBF rate constants and the above estimates of a diffusion-limited rate constant makes is obvious that the reaction of $^1O_2$ with ABDA is not close to the diffusion-limit in any of the solvent systems used here, which also explains why the ratio of the rate constants for the different solvents are not in quantitative but only qualitative agreement with the ratio of the viscosities.

5.2. Singlet Oxygen Sensitivity

The sensitivity of a particular $^1O_2$ probe P, Φ_P, *i.e.* the probability that singlet oxygen will react with a probe molecule and thus contribute to the irreversible bleach of this probe before it deactivates by solvent quenching, is an important parameter for planning and analyzing any experiment investigating $^1O_2$ formation. In particular in situations where it is expected that only small amounts of $^1O_2$ will be released, one has to choose a probe with high sensitivity. Furthermore, the quantitative determination of the quantum yield of $^1O_2$ formation requires calibration of the sensitivity of the probe. The precise value of Φ_P allows one to calculate the amount of $^1O_2$ formed during the experiment from the observed absorbance bleach. The situation is complicated by the fact that Φ_P does not only depend on the probe, but also on the experimental conditions. The solvent determines the rate of quenching of $^1O_2$, whereas the probe concentration affects the rate of the competing bimolecular reaction of $^1O_2$ with the probe. Here, we focus on two $^1O_2$ probes, DPBF and ABDA, which can be used in combination with UV–vis spectroscopy, are soluble in aqueous (biological) environments and are commercially available at a reasonable price. For these two probes, the absolute numeric value of Φ_P in the range of solvents investigated here can be calculated from the kinetic parameters summarized in Table 2 for any given experimental conditions using Eq. (2). For comparative purposes, we also present some values of Φ_P in Table 2 for probe concentrations yielding an absorbance of 1 in the band near 400 nm (ABDA) or 410 nm (DPBF) in a 1 cm pathlength cuvette; this is motivated by the fact that an absorbance of 1 is close to the ideal absorbance which permits most accurate absorbance measurements in standard UV–vis spectrometers. It should be noted that the probe concentration, and hence Φ_P, changes during the course of the experiment as the probe is bleached due to its reaction with $^1O_2$.

The values for Φ_P in Table 2 confirm that DPBF is much more sensitive for $^1O_2$ than ABDA. In EtOH/D_2O more than 70% of the $^1O_2$ formed will irreversibly react with a DPBF molecule in a sample with a DPBF maximum absorbance of 1, whereas only 5% will do so with ABDA. In fact, the difference in the resulting absorbance change is even larger than suggested by these numbers, since DPBF has an extinction coefficient which is almost twice as large as that of ABDA, see Table 1, resulting in a DPBF absorbance change which is ~25 times larger than that of ABDA for the same $^1O_2$ formation rate. This advantage of high sensitivity is offset by the fact that DPBF is not soluble in neat water and is not specific for the detection of $^1O_2$, but readily reacts with other ROS [18–20]. The solubility issue can in principle be addressed by using mixed alcohol/water solvents or micellar solutions, provided that the experiment to be undertaken is compatible with these approaches, since DPBF retains its high reactivity and extinction coefficient in these solutions. The lack of specificity of DPBF for $^1O_2$, on the other hand, makes ABDA, which is specific for $^1O_2$ [3,17,27] and is soluble in neat water, a preferred option for any investigation where sufficient amounts of $^1O_2$ are produced so that they are detectable by this less sensitive probe.

Another factor which one needs to consider in optimizing the sensitivity of $^1O_2$ detection for a particular experiment is the solvent. For aqueous environments, it is best to use D_2O where possible, since the much slower intrinsic decay of $^1O_2$ in D_2O significantly enhances the chances of a reaction with a probe molecule. This is best seen by comparing the values of Φ_P for ABDA in D_2O and H_2O, which differ by a factor of almost 10. In neat D_2O the sensitivity of ABDA is approaching that of DPBF in micellar H_2O solutions, although the overall absorbance change, which also is affected by the different extinction coefficients, still is smaller than that of DPBF by factor of ~4. However, when designing such an experiment, one not only has to consider if the system under investigation is compatible with D_2O, which can be a problem for live cell experiments, or to what extent other differences, such as the viscosity, may affect the outcome, but one also has to very carefully assess any contamination of the solvent, either at the source or during final sample preparation, since even a small contamination with H_2O (of the order of 1%) measurably reduces the $^1O_2$ lifetime and hence the value of Φ_P, compare Section 3.2.

As noted above, the reaction of $^1O_2$ with DPBF in mixed alcohol/water solutions is close to diffusion-limited, whereas in organic solvents it follows the pre-equilibrium mechanism and therefore is somewhat slower. Therefore, alcohol/water solutions not only allow the use of DPBF in an at least partially aqueous environment, they also provide for the highest singlet oxygen sensitivity. It also should be noted that any estimate of the sensitivity in mixed alcohol/water solutions which might previously have been attempted on the basis of the reactivity of DPBF in organic solvents [10], would have underestimated the sensitivity, and hence overestimated the amount of $^1O_2$ detected.

6. Conclusions

The sensitivity for singlet oxygen ($^1O_2$) of two convenient $^1O_2$ probes, DPBF and ABDA, has been investigated in different aqueous environments. Both probes are commercially available at reasonable cost and can be used with standard UV–Vis spectrometers. Under the right conditions, both ABDA and DPBF have the capability of detecting $^1O_2$ accurately. However, DPBF is not soluble in neat water and thus requires the use of a mixed alcohol/water solvent or needs to be solubilized in micelles; furthermore, DPBF is not specific to $^1O_2$, but readily reacts with other ROS. On the other hand, DPBF reacts with $^1O_2$ in an essentially diffusion-limited reaction and thus has very high sensitivity, trapping up to 50% of all $^1O_2$ in alcohol/water or micellar solution, and even more when replacing H_2O by D_2O, which makes it the probe of choice for any application where only small amounts of $^1O_2$ are produced. ABDA has a much lower reactivity, reacting with only 2% of the $^1O_2$ in H_2O, and a smaller extinction coefficient, resulting in a much smaller spectroscopic response, but is soluble in neat water and is specific for $^1O_2$, allowing for discrimination from other reactive oxygen species. The results presented here also provide a reference for an accurate absolute quantification of the amount of $^1O_2$ generated in an experiment from the observed probe absorbance bleach.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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