Understanding delayed fluorescence and triplet decays of Protoporphyrin IX under hypoxic conditions

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Abstract

Photosensitizers of singlet oxygen exhibit three main types of reverse intersystem-crossing (RISC): thermally activated, triplet-triplet annihilation, and singlet oxygen feedback. RISC can be followed by delayed fluorescence (DF) emission, which can provide important information about the excited state dynamics in the studied system. An excellent model example is a widely used clinical photosensitizer Protoporphyrin IX, which manifests all three mentioned types of RISC and DF. Here, we estimated rate constants of individual RISC and DF processes in Protoporphyrin IX in dimethylformamide, and we showed how these affect triplet decays and DF signals under diverse experimental conditions, such as a varying oxygen concentration or excitation intensity. This provided a basis for a general discussion on guidelines for a more precise analysis of long-lived signals. Furthermore, it has been found that PpIX photoproducts and potential transient excited complexes introduce a new overlapping delayed luminescence spectral band with a distinct lifetime. These findings are important for design of more accurate biological oxygen sensors and assays based on DF and triplet lifetime.

1 Introduction

The dynamics of a photophysical system containing a chromophore (photosensitizer) and oxygen in a solvent exposed to light is usually described by the following reactions: [1–3] The photosensitizer absorbs a photon and is excited to an S_n state. This very quickly relaxes to the first singlet state (S_1), which usually has a lifetime of several nanoseconds or less. From S_1 , it can either de-excite to the ground state (S_0) by fluorescence emission (rate constant k_f) or non-radiatively (k_{nr}), or undergo intersystem-crossing

(k_{ISC}) to a metastable first triplet state (T_1), which can live for many microseconds up to milliseconds. T_1 lives long enough to interact efficiently with surrounding ground state oxygen molecules (3O_2) and generate a very reactive singlet oxygen (1O_2) in an energy transfer reaction (k_q):

Quenching by oxygen in this energy transfer reaction is usually the most important deactivation channel of T_1 states in oxygen-rich samples [4, 5]. The rest of T_1 states are deactivated non-radiatively due to interactions with solvent, or they can also emit phosphorescence with a low probability—these together are represented by deactivation rate k_T .

The generated 1O_2 is quite efficiently quenched by electronic-to-vibronic interactions with the solvent/medium molecules, or it can enter in a chemical reaction if it encounters a suitable molecular target in its surroundings [5, 6]. With a very small probability, it can also emit a phosphorescence photon around 1270 nm at rates $\sim 1\text{ s}^{-1}$ [5]. These processes together will be represented by a deactivation rate k_Δ . This traditional description of a photophysical system can be schematically represented by the following reactions and their rate constants [2]:

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The reaction rates can be determined experimentally for the particular studied system based on measurement of excited state lifetimes and quantum yields (typical values of reaction rates for PpIX in dimethylformamide are listed in Table 1 in Discussion). When the sample is excited with a short light pulse, this set of reactions results in a one-exponential decay kinetics of triplet states:

$$[T_1](t) = [T_1](0) \exp(-t/\tau_T), \tag{3}$$

where the triplet lifetime $\tau_T = 1/(k_T + k_q[O_2])$, and a two-exponential rise-decay kinetics of singlet oxygen:

$$[{}^1O_2](t) = k_q[O_2][T_1](0) \frac{\tau_\Delta \tau_T}{\tau_T - \tau_\Delta} (e^{-t/\tau_\Delta} - e^{-t/\tau_T}), \tag{4}$$

where $\tau_\Delta = 1/k_\Delta$ is the lifetime of singlet oxygen. In abundance of oxygen, this expression can be slightly simplified, because $k_q[O_2] \approx 1/\tau_T$.

However, it is often overlooked that even in the simplest photophysical system that contains a solvent, photosensitizer, and oxygen, other excited state reactions can take place, namely reversed intersystem-crossing (RISC) processes [7]:

- (i) thermal fluctuations giving rise to thermally activated RISC (k_{therm}) [8, 9]

- (ii) triplet-triplet annihilation (TTA, k_{TTA}) [10–15]

- (iii) singlet-oxygen-feedback (SOF, k_{SOF}) [16–20]

All of these RISC mechanisms involve transition of T_1 back to S_1 , which can be followed by emission of a delayed fluorescence (DF) photon. Therefore, DF has a spectrum of prompt fluorescence (PF), but it happens at time-scales typical for phosphorescence. Time-resolved detection of DF can be used to investigate dynamics of the excited states in the system. Although TTA and SOF are referred to as RISC reactions, it should be noted that they are classified as spin-allowed reactions [5, 13].

Three main types of DF are recognized based on the underlying RISC mechanism: thermDF, TTADF, and SOFDF. The corresponding DF decay kinetics are different, and their analysis provides somewhat different information about the studied system [7]. Moreover, various types of DF can occur simultaneously, which makes interpretation of DF signals more complex. The thermDF is first order in the concentration of excited states, while the TTADF and SOFDF are second order, which makes their individual contributions dependent on the concentration of triplet states, which, itself, depends on the photosensitizer concentration and excitation intensity.¹

A great example for examination of the described phenomena is Protoporphyrin IX (PpIX), which has been reported to exhibit all three of the above-mentioned RISC reactions, as manifested by DF emission [21–24]. PpIX is one of the most common photosensitizers that is used in a range of clinical applications, such as fluorescence-guided tumor resection, or photodynamic therapy (PDT) of skin diseases and cancer [25–27]. A precursor of PpIX, delta-aminolevulinic acid (5-ALA) or one of its derivatives, is usually administered to the patient and then naturally metabolised into PpIX in mitochondria of most of the cells, with a preferential accumulation in lesions as compared to healthy tissue [26, 28]. Light excitation of PpIX in the presence of oxygen ultimately leads to formation of the cytotoxic singlet oxygen with quantum yields near 0.7 [29], which can damage the target tissue. Measuring of thermDF kinetics can be used to assess the oxygenation of the tissue which is an important clinical parameter [22, 30].

The aim of this work was to explore different mechanisms of RISC and DF in PpIX and determine their rate constants. This would make it possible to estimate the magnitude of individual RISC mechanisms (and their influence on excited state dynamics) in specific situations, and provide guidance for optimal design of assays based on analysis of triplet, DF, or 1O_2 signals.

2 Mathematical model

2.1 Triplet state kinetics

Dynamics of excited states of PpIX after a short excitation pulse (nanoseconds) in the presence of oxygen in a solvent that does not interact with 1O_2 can be approximated by the following set of differential equations:

¹ The term “intensity” can refer to the excitation pulse fluence in the case of short laser pulses, as in this work, or to excitation fluence rate in the case of cw irradiation.

$$\frac{\partial[S_1]}{\partial t} = (k_{\text{therm}} + k_{\text{TTA}}[T_1] + k_{\text{SOF}}[{}^1\text{O}_2])[T_1] - (k_f + k_{\text{nr}} + k_{\text{ISC}})[S_1] \quad (8)$$

$$\frac{\partial[T_1]}{\partial t} = k_{\text{ISC}}[S_1] - (k_T + k_{\text{therm}} + 2k_{\text{TTA}}[T_1] + k_q[{}^3\text{O}_2] + k_{\text{SOF}}[{}^1\text{O}_2])[T_1] \quad (9)$$

$$\frac{\partial[{}^1\text{O}_2]}{\partial t} = k_q[{}^3\text{O}_2][T_1] - k_{\Delta}[{}^1\text{O}_2] - k_{\text{SOF}}[{}^1\text{O}_2][T_1] \quad (10)$$

and a mass-balance equation

$$[{}^3\text{O}_2] = [{}^3\text{O}_2](0) - [{}^1\text{O}_2]. \quad (11)$$

Mathematical calculations can be done in the limit of a very short excitation laser pulse, with initial conditions $[S_1](0) = [S_1]_0$, $[T_1](0) = 0$, and $[{}^1\text{O}_2](0) = 0$. In practise, the laser will have a certain finite pulse duration (in our case 3 ns) and the acquisition of relevant time-resolved data will start after the end of the excitation pulse. In any case, we assume that there is no direct light excitation of S_1 states during the investigated time interval.

Here, we do not take into account chemical consumption of singlet oxygen, which is a justifiable approximation in pure solvents when the sample is stirred. However, the chemical consumption of oxygen during the course of data acquisition is often not negligible in biological samples, particularly when strong excitation is used, which poses a whole new set of challenges for analysis of time-resolved luminescence [31]. Chemical consumption of ${}^1\text{O}_2$ could be accounted for by adding terms $-k_{\text{ch}}[{}^1\text{O}_2]$ into the equation (10) and $-\int k_{\text{ch}}[{}^1\text{O}_2](t')dt'$ into the mass-balance equation (11), where k_{ch} can decrease in time as the molecular targets get depleted during irradiation.

The reaction rates listed in (2) are generally known or can be derived from available experimental data (see Table 1 in Discussion), whereas the RISC rates k_{therm} , k_{TTA} , and k_{SOF} are unknown and need to be experimentally determined. It has to be stressed out here that for further analysis, we assume that all the rates in the differential equation [Eq. (8)] are independent of the way the S_1 state was formed. If not stated otherwise, we will also assume that $[{}^3\text{O}_2](0) \gg [{}^1\text{O}_2]$, *i.e.* the concentration of ground state oxygen can be treated as approximately constant in the differential equations [Eqs. (9) and (10)]. This approximation basically leads to elimination of the mass-balance equation from the calculations.

Our interest is focused on microsecond time-scales $t > 1 \mu\text{s}$, *i.e.*, relatively long times after the excitation pulse. Practically, all S_1 states directly excited by the excitation laser pulse had been deactivated long before that, and all existing S_1 states have been populated by RISC from T_1 states at relatively small rates. The S_1 state acts as a short-lived intermediate, whose deactivation rate constants are much larger than those of formation, which

means that the newly formed S_1 states are cleared within nanoseconds. Therefore, there is no appreciable accumulation of S_1 states and their concentration remains very low during the investigated time interval. This allows us to use the steady-state approximation $\partial[S_1]/\partial t \approx 0$. Equation (8) then leads to

$$[S_1] = [T_1](k_{\text{therm}} + k_{\text{TTA}}[T_1] + k_{\text{SOF}}[{}^1\text{O}_2]) / (k_f + k_{\text{nr}} + k_{\text{ISC}}). \quad (12)$$

When this is plugged into Eq. (9), we can write

$$\frac{\partial[T_1]}{\partial t} = -[T_1](k_T + k'_{\text{therm}} + k'_{\text{TTA}}[T_1] + k_q[{}^3\text{O}_2] + k'_{\text{SOF}}[{}^1\text{O}_2]), \quad (13)$$

where $k'_{\text{therm}} = (1 - \Phi_T)k_{\text{therm}}$, $k'_{\text{SOF}} = (1 - \Phi_T)k_{\text{SOF}}$, and $k'_{\text{TTA}} = (2 - \Phi_T)k_{\text{TTA}}$, with $\Phi_T = k_{\text{ISC}} / (k_f + k_{\text{nr}} + k_{\text{ISC}}) \approx 0.7$ being the triplet quantum yield of PpIX in organic solvents [29]. This way, Eq. (8) can be eliminated. Let us define

$$k_1 = k_T + k'_{\text{therm}} + k_q[{}^3\text{O}_2] \quad (14)$$

as a sum of rate constants that do not involve second-order RISC processes.

Several important special cases can be distinguished.

a) Anoxic conditions: In absence of oxygen ($[{}^3\text{O}_2] = 0$) the set of differential equations is reduced to a single equation

$$\frac{\partial[T_1]}{\partial t} = -[T_1](k_1 + k'_{\text{TTA}}[T_1]), \quad (15)$$

where $k_1 = k_T + k'_{\text{therm}}$ is the overall rate of first-order deactivation. This is a special case of a Bernoulli differential equation with solution

$$[T_1](t) = \frac{k_1}{e^{k_1 t} \left(\frac{k_1}{[T_1](0)} + k'_{\text{TTA}} \right) - k'_{\text{TTA}}} =: \mathcal{T}(t). \quad (16)$$

Under conditions when the triplet-triplet annihilation is negligible as a deactivation pathway ($k'_{\text{TTA}}[T_1]^2 \ll k_1[T_1]$), *e.g.* at a small concentration of triplet states, the solution can be approximated as a simple mono-exponential function (a first-order decay)

$$[T_1](t) = [T_1](0) \exp(-k_1 t) =: \mathcal{T}_1(t). \quad (17)$$

On the other hand, when the triplet-triplet annihilation is dominant ($k'_{\text{TTA}}[T_1] \gg k_1$), *e.g.*, at a large concentration of triplet states, the solution at short times can be approximated by a second-order decay [32]

$$[T_1](t) = \frac{[T_1](0)}{1 + k'_{\text{TTA}}[T_1](0)t} =: \mathcal{T}_2(t). \quad (18)$$

When the decay function (16) cannot be reduced to one of these two special cases, a mixed-order decay takes place [24].

b) Normoxic: In abundance of ground state oxygen and/or low triplet concentration ($[^3\text{O}_2] \gg [\text{T}_1]$, $\partial[\text{T}_1]/\partial t \approx k_q[^3\text{O}_2][\text{T}_1]$, $\partial[^3\text{O}_2]/\partial t \approx 0$), the triplet decay can be approximated by a single exponential as in Eq. (17). This is because ground-state oxygen concentration remains practically constant and it is by far the most important quencher of the triplet state. Under assumption that $k_\Delta \gg k_{\text{SOF}}[\text{T}_1]$, *i.e.*, at relatively small concentrations of triplet states, the solution for the singlet oxygen kinetics is the well-known biexponential rise-decay (4). The concentration of oxygen in air-saturated dimethylformamide (DMF) is ≈ 1.1 mM [33]

$$I_{\text{TTADF}}(t) = A\mathcal{T}^2(t) + d = A_2 \left(\frac{k_1}{e^{k_1 t} \left(\frac{k_1}{[\text{T}_1](0)} + k'_{\text{TTA}} \right) - k'_{\text{TTA}}} \right)^2 + d \quad (22)$$

and the triplet lifetime $\tau_{\text{T,air}} \approx 0.41$ μs [23] which results in a quenching constant of $k_q \approx 2.2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$. This value is similar to k_q in water ($\tau_{\text{T,air}} \approx 2.0$ μs , $[^3\text{O}_2]_{\text{air}} \approx 0.27$ mM) [20].

c) Hypoxic: In intermediate cases, *i.e.*, relatively low oxygen and/or relatively high triplet concentration, the differential equations cannot be simplified as in the previous two cases. The SOF mechanism interconnects the individual differential equations, and the triplet decay and singlet oxygen kinetics become more complex. This situation can occur in biological samples during PDT, and such scenarios are investigated further in this report.

2.2 DF kinetics

Triplet state kinetics can be measured by means of delayed fluorescence. DF mechanism that is the dominant source of the detected luminescence signal is referred to as a *measurement pathway*. Such mechanism can be but does not have to be a *deactivation pathway*, *i.e.*, a pathway that appreciably contributes to triplet state deactivation [24].

In the absence of oxygen, thermDF and TTADF can take place. In the case of thermDF (and also phosphorescence or transient absorption), the emission rate is directly proportional to the triplet concentration

$$I_{\text{thermDF}}(t) \propto k_{\text{thermDF}}[\text{T}_1](t), \quad (19)$$

and thermDF kinetics has identical lifetime to triplet kinetics.

In the case of TTADF, the DF intensity is proportional to the square of the triplet concentration

$$I_{\text{TTADF}}(t) \propto k_{\text{TTADF}}[\text{T}_1]^2(t). \quad (20)$$

If TTADF is a measurement pathway but not a deactivation pathway, *i.e.*, the second-order term k'_{TTA} is much smaller than $k_1/[\text{T}_1](0)$ in (16), then the triplet decay is approximately an exponential in the form $a \exp(-k_1 t)$. Thus

$$I_{\text{TTADF}}(t) \propto \exp(-2k_1 t) \propto \exp\left(\frac{-t}{\tau_{\text{T}}/2}\right), \quad (21)$$

i.e., the TTADF lifetime is half of the triplet lifetime τ_{T} . Otherwise, when TTADF is a measurement pathway and also a deactivation pathway alongside the first-order deactivation, the kinetics of TTADF emission can be described by a model function

where the parameter d accounts for a constant background signal. Often, the emitted DF can be a mixture of the second-order TTADF and first-order thermDF. Then, the model function requires addition of one amplitude fit parameter

$$I_{\text{DF}}(t) = A_1\mathcal{T}(t) + A_2\mathcal{T}^2(t) + d \quad (23)$$

In presence of oxygen, SOFDF can take place in addition [20, 23]:

$$I_{\text{SOFDF}}(t) \propto k_{\text{SOFDF}}[\text{T}_1](t)[^1\text{O}_2](t). \quad (24)$$

In the previously described normoxic scenario b), *i.e.*, in abundance of oxygen when $^3\text{O}_2$ is the main triplet state quencher, I_{SOFDF} is proportional to the product of a one-exponential T_1 decay (3) and a biexponential rise-decay of $^1\text{O}_2$ (4), which yields another biexponential rise-decay function

$$I_{\text{SOFDF}}(t) = k_{\text{SOFDF}}[\text{T}_1]^2(0)k_q[^3\text{O}_2] \frac{\tau_{\text{T}}\tau_{\Delta}}{\tau_{\text{T}} - \tau_{\Delta}} \left(e^{-t/(\tau_{\text{T}}/2)} - e^{-t(\frac{1}{\tau_{\text{T}}} - \frac{1}{\tau_{\Delta}})} \right). \quad (25)$$

Naturally, SOFDF, thermDF, and TTADF can all take place at the same time, which can happen particularly at lowered but non-zero oxygen concentrations, *i.e.*, under hypoxic conditions, leading to complex DF decay kinetics. This is especially relevant to photodynamic therapy.

Fig. 1 **Left:** Transient absorption kinetics of PpIX dissolved in N₂-saturated DMF at two different excitation laser pulse energies (setup D). The sample was excited at 532 nm (absorbance of 0.3) and triplet–triplet absorption was detected at its maximum at 450 nm. The orange curve clearly shows a presence of triplet–triplet annihilation, a second-order decay process. **Inset:** Triplet-minus-singlet transient

absorption spectrum of a nitrogen-purged PpIX at $t = 1 \mu\text{s}$ (setup C). **Right:** DF kinetics at 635 nm of 10 μM PpIX in DMF at low oxygen concentration, excited with two different intensities 1.8 mJ/cm²/pulse and 0.18 mJ/cm²/pulse (setup A). The kinetics were fitted with the TTADF model function (22) (black dashed line) and alternatively with the model function (23) (red line).

3 Materials and methods

Protoporphyrin IX (PpIX, Frontier Scientific, catalog number P562-9, purity > 97%) was dissolved in dimethylformamide (DMF, Lab-Scan, catalog number C33C11X, purity > 99.8%), usually at 10 μM concentration. Experiments were conducted at 22°C.

Setup A: To measure delayed fluorescence kinetics, a setup built around a microscope was used. It was described in detail elsewhere [34]. A tunable pulsed laser (EKSPLA NT242) at 420 nm, 500 Hz repetition rate, and 3 ns pulse duration was used for excitation and was attenuated by a set of ND filters to obtain the desired excitation intensity. The laser was reflected by a 600 nm longpass dichroic mirror and focused on the back aperture of the objective to produce a collimated beam with 200 μm diameter above the objective. The luminescence collected by the objective passed through the above-mentioned dichroic filter, and then, it was split into VIS and NIR path by a 1000 nm shortpass filter. In the VIS branch, the emission passed through a spectrograph (Princeton Instruments ARC-SP-2358) which allowed us to choose the desired detection wavelength. The DF kinetics were recorded by a gated photomultiplier (Hamamatsu, H11526-20-NF) and DF time-resolved spectra were collected by a gated intensified camera (Princeton Instruments, PI-MAX 4). In the NIR path, singlet oxygen emission around 1275 nm was isolated by a bandpass filter (Sigma Optical, 1270/40 nm) and spectrograph (Princeton Instruments ARC-SP-2358), and detected by a gated photomultiplier (Hamamatsu H12694-45).

Setup B: This setup was built at a later stage to replace the setup A and avoid the unnecessary use of a microscope. The laser was brought to the sample cuvette by an optical fiber and was collimated to yield a beam diameter of 1 mm. The luminescence was collected by a lens in the right-angle geometry, passed through a bandpass filter, and was coupled into an optical fiber to bring the signal to the photomultiplier.

Setup C: Time-resolved spectra of transient absorption were measured by a pump-probe setup using a 420 nm laser at 10 Hz repetition rate for pumping and a xenon lamp for probing. The probe light was dispersed into spectrum by a spectrograph and then detected by a gated intensified camera which enabled us to set a precise delay after the excitation pulse. When the probe light was switched off, this setup also could be used to measure time-resolved spectra of delayed fluorescence. The setup was described in detail elsewhere [20].

Setup D: Alternatively, transient absorption kinetics were measured by a setup using a 532 nm laser at 10 Hz for pumping, a xenon lamp for probing, and a photomultiplier for detection of light transmitted at a selected wavelength. This setup was described in detail elsewhere [35].

4 Results

4.1 Transient absorption kinetics

One of the commonly used methods to measure triplet kinetics is transient absorption in a pump-probe experiment, where the decay of an absorption band corresponding to

a T_1 - T_n transition is monitored. Absorption measurements are usually not as sensitive as luminescence measurements and hence usually strong pumping laser pulses have to be used to induce a detectable concentration of triplet states. Figure 1-left shows a nitrogen-purged sample of PpIX in DMF excited with 532 nm laser pulses with absorbance of 0.3 at the excitation wavelength (setup D). Transient absorption kinetics at 450 nm were recorded at two different excitation intensities: 0.3 mJ/cm² and 7.5 mJ/cm² per pulse. With the lower excitation intensity, the triplet kinetics was a single exponential (17). With the large excitation power, second-order deactivation processes took place and the kinetics became non-exponential. The curve was fitted by the model function (16). This is in line with similar experiments conducted at a fixed excitation power and varying PpIX concentration [24]. It should be noted that the latter kinetics could be easily misinterpreted as a biexponential or multiexponential function.

4.2 Triplet–triplet annihilation delayed fluorescence

Triplet state kinetics can be examined indirectly also by measuring DF. In the absence of oxygen, PpIX can emit thermDF or TTADF, whereas SOFDF is not possible.

First, it was investigated which of these two DF mechanisms was dominant in a nitrogen-purged sample of 10 μ M PpIX in DMF excited with pulse energies $\gtrsim 0.1$ mJ/cm² (setup A). To remove a potential interference of SOFDF due to a small residual amount of oxygen, 10 mM NaN₃ was added to quench singlet oxygen. It was found that the ratio of DF and PF integral intensities (I_{DF}/I_{PF}) grew proportionally to I_{PF} for pulse energies $\gtrsim 0.1$ mJ/cm², i.e., I_{DF} was proportional to the second power of the concentration of excited states (data not shown). This clearly points to a dominant role of TTADF.

DF kinetics at two different excitation pulse energies (0.18 mJ/cm² and 1.8 mJ/cm² per pulse) are shown in Fig. 1-right. At the higher excitation pulse energy, the initial decay was steeper due to the more pronounced TTA. The kinetics were fitted with the TTADF model function (22) (black dashed line). To include the potential thermDF contribution, particularly at lower T_1 concentration, the model function (23) was also tested (red line). However, the difference between these two fits was quite small and it is questionable whether the contribution of thermDF can be reliably distinguished. Although the fitting with TTADF model (22) provides the value of k'_{TTA} , this fit parameter is strongly inter-related with the $[T_1](0)$ parameter, and for a more accurate determination of k'_{TTA} , a sample with a well-defined $[T_1](0)$ has to be prepared.

To this end, the sample was excited with a saturation intensity of 20 mJ/cm²/pulse, as shown later in Fig. 3-left. Under these conditions, most of PpIX molecules were

excited and we can assume $[T_1](0) = \Phi_T[S_1](0) \approx 7 \mu$ M, where $\Phi_T \approx 0.7$ is the triplet quantum yield of PpIX [29]. This value was plugged into the fit function (22) as a fixed parameter. The fit procedure then yielded $k'_{TTA} \approx 1.7 \times 10^9$ M⁻¹s⁻¹ (and $k_1 \approx 1.8 \times 10^3$ s⁻¹).

4.3 Singlet oxygen emission under hypoxic conditions

Figure 2A shows the DF emission and NIR singlet oxygen emission in a nitrogen bubbled hypoxic sample of 10 μ M PpIX in DMF excited with 0.18 mJ/cm²/pulse (setup A), this time without NaN₃. The tails of the DF and ¹O₂ kinetics were fitted each with one exponential with lifetimes 91 μ s and 180 μ s, respectively. The decay-time of singlet oxygen corresponded to the triplet lifetime in this case (so-called inversed lifetimes [2]). DF lifetime was twice shorter than the triplet lifetime which is consistent with the second-order DF mechanisms, namely TTADF and SOFDF (Eqs. (21) and (25)). The oxygen saturation of the sample was estimated at $\approx 0.05\%$, as derived from the fact that the triplet lifetime was $\sim 400\times$ longer than that in an air-saturated solution ($\tau_{T,air} \approx 0.4 \mu$ s, 21% oxygen saturation). This experiment shows that singlet oxygen was present in detectable amounts even at very low oxygen saturation.

4.4 SOFDF

In the previous sections, it is shown that TTADF was a dominant DF mechanism in the absence of oxygen under our experimental conditions. However, even a small amount of oxygen turned out to induce a non-negligible emission of SOFDF. Figure 2B shows DF kinetics in a nitrogen-purged 10 μ M PpIX in DMF using excitation pulses at 0.18 mJ/cm²/pulse. The long DF lifetimes indicate that the oxygen saturation was smaller than 0.1%. The addition of 10 mM NaN₃, a specific singlet oxygen quencher, caused a significant drop in the DF intensity and a loss of the characteristic concave curvature, proving that SOFDF was contributing to the DF emission.

Figure 2C displays DF kinetics in a nitrogen-purged sample that was left open to see the effect of gradual increase in oxygen saturation, causing a shortening of the triplet lifetime and conversely SOFDF decay-time. NIR singlet oxygen kinetics were measured simultaneously and are shown in Fig. 2D. The lifetime of the ¹O₂ NIR emission decay was 14 μ s in air-saturated DMF, which is in accordance with the previously measured values [36]. The DF kinetics were a mixture of TTADF and SOFDF, and besides that, the rise-parts of the ¹O₂ kinetics were overlaid with a background signal, which prevented us from conducting a more detailed quantitative analysis of DF lifetimes vs ¹O₂ lifetimes.

Fig. 2 10 μM PpIX in DMF at low oxygen concentration, excited with 0.18 $\text{mJ}/\text{cm}^2/\text{pulse}$. **A:** DF kinetics at 635 nm is compared to singlet oxygen kinetics at 1275 nm. The tails of the kinetics were fitted each with one exponential with lifetimes 91 μs and 180 μs . The decay-time of singlet oxygen corresponds to the triplet lifetime. **B:** DF kinetics

with and without 10 mM NaN_3 . **C:** DF kinetics in an open cuvette where oxygen concentration was gradually increasing over time. **D:** The corresponding $^1\text{O}_2$ kinetics. The oxygen saturations were roughly estimated at $\sim 1\%$, 2% , 2.5% , and 15% for these curves based on the DF decay-time

Fig. 3 Determination of RISC and DF rate constants. **Left:** 10 μM PpIX in DMF with 10 mM NaN_3 at low oxygen concentration, excited with a saturation intensity 20 $\text{mJ}/\text{cm}^2/\text{pulse}$. The black solid curve that overlaps with the data is a fit by the model function (22) yielding $k'_{\text{TTA}} = 1.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$. For comparison, the grey dashed curve is a fit into the initial part of the kinetics by a model

that assumes a purely second-order triplet deactivation according to (18). **Right:** Determination of TTADF rate constant from comparison of PF and DF emission intensities. 2 μM PpIX in DMF with 10 mM NaN_3 at low oxygen concentration, excited with a saturation intensity 4.4 $\text{mJ}/\text{cm}^2/\text{pulse}$. PF was collected in time interval 0–40 ns, whereas DF in time interval 1–3 μs

4.5 Determination of rate constants

It has been shown in the previous paragraphs that TTADF and SOFDF became a dominant source of PpIX DF emission at higher excitation pulse energies, whereas earlier reports showed that thermDF is dominant at low excitation energies [21, 24, 37, 38]. Here, we report on the rates of these individual RISC processes. Section 4.2 estimated the k'_{TTA} at $\approx 1.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ based on fitting of the DF decay.

In the following paragraphs, a different approach is used to estimate the rates of individual RISC processes: the ratio $R = I_{\text{DF}}/I_{\text{PF}}$ of DF integral intensity to PF integral intensity was experimentally determined in three different scenarios on the setup A using the gated camera for detection. In each scenario, the experimental conditions (PpIX concentration, oxygen concentration, pulse excitation intensity, and presence/absence of NaN_3) were set to favor only one specific RISC mechanism.

First, PF integral intensity was measured with 0–40 ns gate window (S_1 lifetime $\tau_{S_1} = 16 \text{ ns}$). During this time interval, the PF emission rate dropped more than 10×, and hence, more than 90% of PF photons were emitted. Therefore, we will be using $I_{\text{PF}} = \alpha \Phi_f [S_1](0)$ in the following discussion for simplicity, where the collection efficiency factor α is equal for PF and DF. Afterwards, the gate was set to collect DF, with gate settings dependent on the DF mechanism under investigation. Apart from the different gate widths, the conditions were identical for the PF and DF detection.

The measured quantity was the emission intensity and emission rate. To determine the rates of the underlying RISC mechanisms, it was assumed that all the rates in the differential equation [Eq. (8)] are independent of the way the S_1 state was formed. This includes the assumption of an invariable fluorescence rate k_f and fluorescence quantum yield $\Phi_f = \Phi_f^{\text{TTA}} = \Phi_f^{\text{SOF}} = k_f/(k_f + k_{\text{nr}} + k_{\text{ISC}})$.

4.5.1 Thermal activation rate

To assess the rate constant of thermDF, 2 μM PpIX in nitrogen-saturated DMF with 10 mM NaN_3 was used. Small excitation intensity of 15 $\mu\text{J}/\text{cm}^2$ was employed to suppress the second-order processes. The $R = I_{\text{DF}}/I_{\text{PF}}$ intensity ratio was found to be growing only slightly around this excitation intensity which confirmed that second-order processes did not play a major role. The DF gate was set to 1 μs – 800 μs , which basically embraced the full DF emission interval. Although the DF kinetics was weak, it did not show significant deviations from a single exponential, implying that the triplet decay kinetics was also a single exponential. Then

$$R = I_{\text{DF}}/I_{\text{PF}} = \frac{\alpha \int_{1\mu\text{s}}^{800\mu\text{s}} k_{\text{thermDF}} \Phi_T [S_1](0) \exp(-t/\tau_T) dt}{\alpha \Phi_f [S_1](0)} \quad (26)$$

The experimental values were $R = 1.8 \times 10^{-3}$, $\tau_T = 267 \mu\text{s}$, $\Phi_f \approx 0.1$, [29], and $\Phi_T \approx 0.7$ [29]. Given that the collection time interval was much longer than the triplet lifetime, this can be simplified to

$$k_{\text{thermDF}} \approx \frac{R \Phi_f}{\Phi_T \tau_T}, \quad (27)$$

which yields $k_{\text{thermDF}} = 1.0 \text{ s}^{-1}$ and $k_{\text{therm}} = R/(\Phi_T \tau_T) = 10 \text{ s}^{-1}$ at 22°C.

4.5.2 TTA rate

To determine the rate constant of TTADF (k_{TTADF}), 2 μM PpIX in nitrogen-saturated DMF with 10 mM NaN_3 was used. Large excitation intensity of 4.4 $\text{mJ}/\text{cm}^2/\text{pulse}$ was employed to saturate the sample. Under these conditions, most of PpIX molecules got excited.² The DF gate was set to 1 μs – 3 μs , *i.e.* $\Delta t = 2 \mu\text{s}$. Given the long DF lifetime, the triplet concentration in this short time interval was very close to the initial triplet concentration and the DF emission intensity was basically constant over the interval. Taking into account the mentioned approximation, we can write

$$R = \frac{I_{\text{DF}}}{I_{\text{PF}}} \approx \frac{(\Phi_f^{\text{TTA}} k_{\text{TTA}} [T_1](0) + \Phi_f k_{\text{therm}}) [T_1](0) \Delta t}{\Phi_f [S_1](0)} \quad (28)$$

After substituting $[T_1](0) = \Phi_T [S_1](0)$ and rearrangement, we get

$$k_{\text{TTA}} = \frac{R - k_{\text{therm}} \Phi_T \Delta t}{\Phi_T^2 [S_1](0) \Delta t} \cdot \frac{\Phi_f}{\Phi_f^{\text{TTA}}} \quad (29)$$

The experimental value of R was 4.4×10^{-4} , and hence, we can recognize that $R \gg k_{\text{therm}} \Phi_T \Delta t$, since the latter term amounts only to 1.4×10^{-5} . This allows us to neglect this term in further calculations. Using $k_{\text{TTADF}} = \Phi_f^{\text{TTA}} k_{\text{TTA}}$, we obtain

$$k_{\text{TTADF}} = \frac{R \Phi_f}{\Phi_T^2 [S_1](0) \Delta t} \quad (30)$$

$$q := \frac{k_{\text{TTADF}} [T_1]^2(0)}{k_f [S_1](0)} = R \frac{\tau_{S_1}}{\Delta t} \quad (31)$$

Experimental values $R = 4.4 \times 10^{-4}$, $[S_1](0) \approx 2 \mu\text{M}$, $\Delta t = 2 \mu\text{s}$, $\tau_{S_1} = 16 \text{ ns}$, $\Phi_T \approx 0.7$, [29] $\Phi_f \approx 0.1$, [29] yield

² PpIX was excited into S_2 state which undergoes rapid internal conversion into S_1 state within picoseconds [29], whereas the excitation pulse is much longer (3 ns). Hence, stimulated emission is expected to be negligible. On the other hand, the pulse duration was $\approx 5\times$ shorter than the S_1 lifetime which means that cyclic excitation was improbable.

Fig. 4 Kinetics of PpIX in DMF simulated on the basis of the system of differential equations (8)–(11) at two different initial oxygen concentrations. Left: air-saturated, right: oxygen lowered $\sim 500\times$

$k_{\text{TTADF}} \approx 2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $q = 3.5 \times 10^{-6}$. Under the assumption $\Phi_f^{\text{TTA}} = \Phi_f$, this yields $k_{\text{TTA}} = 2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $k'_{\text{TTA}} = (2 - \Phi_T)k_{\text{TTA}} = 2.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. Given the approximations taken along the way, this value can be considered a lower limit.

4.5.3 SOF rate

To determine the singlet-oxygen-feedback rate constant k_{SOF} , $5 \mu\text{M}$ PpIX in air-saturated DMF without NaN_3 was excited with saturation intensity of $4.4 \text{ mJ/cm}^2/\text{pulse}$. Therefore, we assume that $[S_1](0) = 5 \mu\text{M}$. The DF gate was set to $1 \mu\text{s} - 2 \mu\text{s}$. The triplets were decaying monoexponentially (due to dominant quenching by oxygen) from the initial concentration of $[T_1](0) = \Phi_T[S_1](0)$ with lifetime $\tau_T = 0.4 \mu\text{s}$ [23]. Triplets transferred energy to singlet oxygen that had lifetime $\tau_\Delta = 14 \mu\text{s}$. In the investigated gate window, the triplet concentration dropped more than $10\times$ from the initial value but meanwhile the singlet oxygen reached its maximal concentration. Therefore, SOFDF was strongly favored over TTADF. Under such conditions, the SOFDF emission rate is described by the Eq. (24). Due to abundance of oxygen, we can use $k_q[\text{O}_2] \approx 1/\tau_T$ and write

$$I_{\text{DF}} = \alpha[T_1]^2(0) \left(k_{\text{SOFDF}} \int_{1 \mu\text{s}}^{2 \mu\text{s}} \frac{\tau_\Delta}{\tau_T - \tau_\Delta} (e^{-t/(\tau_T/2)} - e^{-t(\frac{1}{\tau_T} - \frac{1}{\tau_\Delta})}) dt + P \right). \quad (32)$$

Here, the value of the integral is $0.2 \mu\text{s}$ and

$$P = k_{\text{TTADF}} \int_{1 \mu\text{s}}^{2 \mu\text{s}} e^{-t/\tau_T} dt = 0.663 \quad (33)$$

when using the value of k_{TTADF} derived in the previous section. Then, we get

$$R = \frac{I_{\text{DF}}}{I_{\text{PF}}} = \frac{\alpha[S_1]^2(0)\Phi_T^2(k_{\text{SOFDF}} \times 0.2 \mu\text{s} + P)}{\alpha\Phi_f[S_1](0)} \quad (34)$$

and

$$k_{\text{SOFDF}} = \left(\frac{R\Phi_f}{\Phi_T^2[S_1](0)} - P \right) \frac{1}{0.2 \mu\text{s}}. \quad (35)$$

The experimental values $R = 8.3 \times 10^{-5}$, $[S_1](0) = 5 \mu\text{M}$, $\Phi_f \approx 0.1$, and $\Phi_T \approx 0.7$ yield $k_{\text{SOFDF}} = 1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Under assumption $\Phi_f^{\text{SOF}} = \Phi_f$, we get $k_{\text{SOF}} = k_{\text{SOFDF}}/\Phi_f = 1.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. Similarly to the previous paragraph, this can be considered a lower limit.

4.6 Examples of applications of rate constants

Let us make two simple simulated examples to illustrate how the estimated values of rate constants can be used.

a) Fig. 4 left: An air-saturated solution of PpIX in DMF with a high initial concentration of triplets $[T_1](0) = 10 \mu\text{M}$. The triplet lifetime $0.4 \mu\text{s}$ corresponds to

decay rate $k_1 = k_T + k'_{\text{therm}} + k_q[{}^3\text{O}_2] = 2.5 \times 10^6 \text{ s}^{-1}$, where $k_T < 2 \times 10^3 \text{ s}^{-1}$ is practically negligible, and the rate constant k_1 is absolutely dominated by bimolecular quenching with ground-state oxygen (whose concentration is in an mM range and remains basically constant). Singlet oxygen lifetime is $\tau_\Delta = 14 \mu\text{s}$ ($k_\Delta = 1/\tau_\Delta = 7.1 \times 10^4 \text{ s}^{-1}$). Furthermore, let us assume that $k_{\text{TTA}} = k_{\text{SOF}} = 1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (i.e., larger values than the experimentally determined lower limits). In that case $k_{\text{TTA}}[T_1](0) = 1 \times 10^4 \text{ s}^{-1}$, which is

Fig. 5 Time-resolved PF and DF spectra of fresh 10 μM PpIX, photobleached 10 μM PpIX, and fresh 100 μM PpIX

two orders of magnitude weaker than the first-order decay constant k_1 and hence quite negligible. Similarly, $k_{\text{SOF}}[^1\text{O}_2]$ is always smaller than $1 \times 10^4 \text{ s}^{-1}$ and hence negligible too. Therefore, the triplet decay is a single exponential with a good accuracy. As to DF emission, let us assume $k_{\text{SOFDF}} = k_{\text{TTADF}} = 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, and $k_{\text{thermDF}} = 1 \text{ s}^{-1}$. As the triplets transfer energy to singlet oxygen, $[^1\text{O}_2]$ quickly becomes larger than $[T_1]$ and SOFDF becomes more intense than TTADF. The thermDF would become dominant over SOFDF when $k_{\text{thermDF}} > k_{\text{SOFDF}}[^1\text{O}_2]$, i.e., when $[^1\text{O}_2]$ drops below 50 nM. However, in our example, this would happen only after $[T_1]$ drops practically to zero level, leaving DF undetectable. Therefore, thermDF is negligible in this case.

b) Figure 4 right: On the other hand, let us have the same sample, but this time at very low oxygen, 500× decreased compared to the air-saturated one, resulting in $k_1 = k_T + k'_{\text{therm}} + k_q[^3\text{O}_2] = 6 \times 10^3 \text{ s}^{-1}$ ($\tau_T = 160 \mu\text{s}$ as determined at longer times). Now, the second-order decay rate $k_{\text{TTA}}[T_1](0) = 1 \times 10^4 \text{ s}^{-1}$ at time zero is larger than k_1 , and therefore, triplet-triplet annihilation is a major deactivation pathway at short times. When the triplet concentration drops down to $[T_1](t) \lesssim 1 \mu\text{M}$ after $\sim 250 \mu\text{s}$, the triplet-triplet annihilation again becomes negligible as a deactivation pathway and the triplet decay at later times will be governed by first-order processes and hence will be exponential. However, TTADF could still be a dominant DF emission pathway (i.e., the *measurement pathway*). This is illustrated in the Fig. 4 right. TTADF is a dominant source

of DF until thermDF becomes more intense at time $\sim 700 \mu\text{s}$ when $k_{\text{TTADF}}[T_1] = k_{\text{thermDF}}$, i.e. $[T_1] \approx 5 \times 10^{-8} \text{ M} = 50 \text{ nM}$. Naturally, if the initial triplet concentration is smaller than 10 μM , which will be the case in most experiments, this threshold concentration will be achieved earlier. It should be also noted that even at such a low oxygen concentration the SOFDF contribution is not negligible, which was confirmed in our experiments.

The situation in a biological sample is distinctive and more complex. It has been shown in our previous work that the PpIX triplet lifetime in mouse skin spans from $\sim 20 \mu\text{s}$ to $\sim 2000 \mu\text{s}$ depending on the degree of oxygenation, and it is also strongly spatially dependent [38]. The singlet oxygen lifetime in biological samples is usually shorter than that in DMF, roughly around $0.5 - 1 \mu\text{s}$ [31]. The rise-part of the SOFDF kinetics would become short ($< 1 \mu\text{s}$) and the lifetime of the decay-part will correspond to a half of the triplet lifetime [7]. The short $^1\text{O}_2$ lifetime would lead to a smaller concentrations of $^1\text{O}_2$ and less intense SOFDF emission rate relative to the SOFDF emission rates in the examples above. The rate of $^1\text{O}_2$ formation in normoxic tissue is much higher than that in the example b) and smaller than that in the example a). The mobility and distribution of oxygen and PpIX is very heterogeneous and depends on the subcellular localization, which makes it difficult to predict contributions of individual DF mechanisms.

4.7 The effect of photobleaching

PpIX is known to readily photobleach both in the presence and absence of oxygen. In cell samples, it was reported that a photoproduct with fluorescence peak around 675 nm was favored at low oxygen, whereas another photoproduct with a peak around 655 nm was mainly formed at well-oxygenated samples [39]. First, it was investigated how the formation of photoproducts influences the kinetics of delayed fluorescence in a sample of 10 μM PpIX. Figure 5 shows PF and DF spectra of a nitrogen-saturated fresh sample at various time-delays after the excitation pulse. The same is shown for a sample that was photobleached with the 420 nm laser (in this case, photobleaching was done in an air-saturated sample, and subsequently, this was purged with nitrogen before the DF measurement.)

The time-dependent spectra were decomposed into two spectral components corresponding to fresh PpIX and a photoproduct. The photoproduct fluorescence band around 670 nm clearly had a shorter PF lifetime than the native PpIX spectrum with maximum at 635 nm (5 ± 1 ns vs. 16 ± 3 ns), which is in line with previous reports [40, 41]. In contrary, the photoproduct band had a significantly longer DF lifetime than the standard PpIX bands (145 ± 10 μs vs. 99 ± 10 μs). Similar results were obtained also with a sample photobleached at absence of oxygen, but in that case, the new photoproduct fluorescence band was found around 660 nm. This is in contradiction with Dysart et al. [39], who reported that the band of a photoproduct formed at low oxygen has the longer wavelength.

Interestingly, the longer lived DF band in the 660–680 nm region was observed also in fresh samples of a more concentrated PpIX (50 or 100 μM), whereas this band was not present in the PF spectrum. At longer delays, this DF band became dominant over the standard 635 nm and 700 nm bands of PpIX, similarly to the photobleached sample. The shape and position of this new DF spectral band in the fresh 100 μM sample was practically identical to the photoproduct DF band in 10 μM samples. Spectral decomposition showed that the time-dependent intensity of the new DF band in the fresh 100 μM PpIX sample might have a rise-decay character. There was no distinct photobleaching detected in this sample during the course of the experiment. This is quite an intriguing result: It is possible that after the excitation pulse, there are transient phenomena and excited state reactions that involve excimer formation, [42] dimerization, photoproduct formation, or a not yet understood interplay among these. This deserves further investigation.

5 Discussion

To correctly interpret the DF signals, it is important (i) to know what are the contributions of individual DF mechanisms, and (ii) to understand whether and how the second-order RISC deactivation processes influence the triplet kinetics. To this end, we estimated the RISC and DF rate constants for the important case of PpIX.

Thermally activated delayed fluorescence of PpIX has been proposed as a novel tool to measure tissular oxygenation in clinics [21, 22, 30]. Lifetime of thermDF corresponds to the lifetime of the triplet states, which in turn is related to the oxygenation by Stern–Volmer equation $1/\tau_T = \tilde{k}_q p\text{O}_2 + k_0$ [22]. It is important to ensure that thermDF is indeed a dominant DF mechanism in the particular settings, because otherwise contributions of TTADF and SOFDF could make the analysis too complex.

The thermally activated RISC rate was determined here to be $k_{\text{therm}} \approx 10 \text{ s}^{-1}$ at 22 °C. With $\Phi_f \approx 0.1$, this yields the emission rate constant $k_{\text{thermDF}} \approx 1.0 \text{ s}^{-1}$ (this is quite comparable to the rate of NIR phosphorescence emission of singlet oxygen) [5]. These values are supposed to increase with temperature as they scale with the Boltzmann factor $\exp(-\Delta E/k_B T)$, where $\Delta E \approx 35 \text{ kJ/mol}$ is the T_1 – S_1 energy gap of PpIX [43, 44]. The value of k_{thermDF} in DMF obtained here is in line with our experiments conducted in a sacrificed mouse that was previously injected 5-ALA, where we found that the peak DF emission rate was about seven orders of magnitude smaller than the peak PF emission rate [37, 38].

Triplet–triplet annihilation reaction rate has been determined by two different methods. (a) Fitting of the non-exponential DF decay in Sect. 4.2 resulted in $k'_{\text{TTA}} \approx 1.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. b) Comparison of DF and PF emission rates in Sect. 4.5.2 resulted in $k_{\text{TTADF}} \approx 2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Under the assumption $\Phi_f^{\text{TTA}} = \Phi_f$, this experiment yielded $k_{\text{TTA}} = 2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and from here $k'_{\text{TTA}} = (2 - \Phi_T)k_{\text{TTA}} = 2.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. The difference between k'_{TTA} and k_{TTA} was explained in Sect. 2.1. There is a discrepancy between k'_{TTA} values obtained in experiments a) and b), which could be explained in several ways: (i) In the experiment b), the PF is linear in the concentration of excited states, whereas TTADF is quadratic, which means that PF could be effectively detected from a larger volume than DF due to a non-uniform distribution of excited states, which could lead to a significant underestimation of k_{TTA} . We made efforts to maximize the uniformity of excitation using a strong saturation pulses, relatively small PpIX concentration, a thin layer of the solution, and detection in the center of the illuminated spot, but still there could be a significant effect. (ii) Another possible source of the discrepancy could be that fluorescence emission after TTA RISC is less probable than that for an isolated molecule,

Fig. 6 Simulated data. **Left:** The ratio of TTADF and thermDF emission rates in 1 mm-thick sample-layer of PpIX in DMF at low oxygen [³O₂] = 2 μM under pulsed excitation as a function of excitation pulse intensity and PpIX concentration. The dashed vertical line points to the pulse energy used in our paper on DF in ALA-treated mice [38].

Right: The fraction of triplets that decay through TTA or SOF in PpIX in DMF at low oxygen [³O₂] = 2 μM under pulsed excitation. Only a thin layer incident to the light beam was taken into account. The rectangle shows the span of excitation intensities commonly used in PDT [45].

Table 1 Excited state reaction rates for PpIX in DMF

S₁ deactivation [29]		
k_f	k_{ISC}	
$\approx 6.3 \times 10^6 \text{ s}^{-1}$	$\approx 4.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	
T₁ deactivation		
k_T	k_q	$k_q[\text{}^3\text{O}_2]_{\text{air-sat}}$
$< 2 \times 10^3 \text{ s}^{-1}$	$\sim 2 \times 10^9 \text{ s}^{-1}$	$2.5 \times 10^6 \text{ s}^{-1}$
DF		
k_{thermDF}	k_{TTADF}	k_{SOFDF}
1.0 s^{-1}	$> 2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	$> 1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
RISC		
k_{therm}	k_{TTA}	k_{SOF}
10 s^{-1}	$> 2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$	$> 1.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$
¹O₂ deactivation		
k_{Δ}		
$7.1 \times 10^4 \text{ s}^{-1}$		

i.e., $\Phi_f^{\text{TTA}} < \Phi_f$. However, investigation of this is beyond the scope of this report.

The k'_{TTA} rate of $1.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ determined in Sect. 4.2 is comparable to k_q , the rate of triplet state quenching by oxygen, which is generally considered to be a very efficient process [5]. Therefore, when the concentration of T₁ states gets close to the concentration of ground state oxygen, the TTA can become a significant deactivation pathway for triplet states.

It has been shown that a broad distribution of pO₂ in live samples translates to convoluted DF decays with multiple lifetimes. To recover correctly the underlying pO₂ distribution from the DF kinetics, it is important to ensure that thermDF is a dominant DF emission pathway, because otherwise the presence of TTADF and SOFDF which have faster decays could lead to a bias in pO₂ determination. To this end, it is a good practice to check the linearity of DF emission intensity vs. laser pulse fluence [38]. It has been shown that the presence of second-order processes depends both on excitation intensity and PpIX concentration. When designing an experiment, the potential contribution of second-order processes (TTA and SOF) should be estimated based on the values and equations which we provided. As an example, Fig. 6 shows a numerical simulation of how the contributions of second-order deactivation processes depend on the excitation intensity and PpIX concentration in our experimental sample. This was a hypoxic solution of PpIX in DMF with ground state oxygen concentration of 2 μM, *i.e.*, $\sim 500\times$ lower than air-saturated (using $k_q = 2.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) [33]. It has to be remembered that the rate constants in DMF reported here cannot be directly applied in biological environment. Although DMF has a similar viscosity as water, the rate constant of SOF and TTA in the intracellular environment would be smaller due to larger viscosity and smaller diffusion coefficients. However, the general approach as shown here can facilitate understanding the data even in biological samples.

The SOFDF emission rate constant was determined to be $k_{\text{SOFDF}} > 1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ in DMF (using $\Phi_f \approx 0.1$). Under the assumption that k_f , k_{nr} , k_{ISC} are independent of the way S₁ state was formed, this yields the RISC rate

$k_{\text{SOF}} = k_{\text{DF}}/\Phi_f > 1.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. In our older paper, the SOF rate of water-soluble porphyrins was estimated at $\sim 1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ [20]. SOFDF can be a dominant source of DF in samples with a sufficient concentration of oxygen. We might ask if SOF mechanism can be an appreciable pathway of singlet oxygen deactivation. In biologically relevant samples, the $^1\text{O}_2$ deactivation rate is likely to fall into the range $1 \times 10^5 \text{ s}^{-1} < k_{\Delta} < 1 \times 10^7 \text{ s}^{-1}$ due to interactions with the medium and water [31]. If we assume $k_{\text{SOF}} \sim 1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, then a very large $[\text{T}_1]$ concentration of 100 μM would be needed to reach $k_{\text{SOF}}[\text{T}_1] \geq 1 \times 10^5 \text{ s}^{-1}$, so that SOF would compete with other pathways of $^1\text{O}_2$ deactivation. Therefore, SOF is unlikely to become an appreciable deactivation pathway for $^1\text{O}_2$ in biological systems.

It is often possible to assume that relative changes of $[\text{}^3\text{O}_2]$ during the triplet decay are only small, *i.e.* $[\text{}^1\text{O}_2] \ll [\text{}^3\text{O}_2](0)$. This means that $[\text{}^3\text{O}_2]$ can be treated as a constant in the differential equations (9) and (10), which allows us to simplify them and provide analytical solution. This assumption can become problematic under certain conditions in hypoxic samples with a large concentration of triplet states (strong illumination) and long $^1\text{O}_2$ lifetime. This is illustrated in simulated curves in Fig. 4, which did not use this approximation. The figure on the left is normoxic and the graph shows that $[\text{}^3\text{O}_2]$ remains practically constant during the triplet decay. The figure on the right corresponds to severely hypoxic conditions with a high initial triplet state concentration and quite long $^1\text{O}_2$ lifetime as is characteristic for pure organic solvents. We can see that there are certain changes in the $[\text{}^3\text{O}_2]$ at short times, on the level of 20%.

We also have to be aware that oxygen particularly in biological samples can be depleted very quickly due to chemical reactions of the generated singlet oxygen with surrounding molecules. This will dramatically change the measured triplet lifetime. As an example, we examined how the measured DF lifetime was influenced by stirring of the sample. In a stirred hypoxic sample, the DF lifetime was around 50 μs , whereas when the stirring was stopped, the DF lifetime increased to 300 μs . The possible light-induced oxygen depletion always has to be taken into account especially in biological samples, where stirring is not possible and oxygen diffusion is slower.

Finally, it has been found that (apart from thermDF, TTADF, and SOFDF of PpIX) the photoproducts and/or transient complexes also contribute to the delayed emission with their own long-lived luminescence of different spectrum and lifetimes. This could further complicate the analysis of DF kinetics for determination of pO_2 . Therefore, it is advisable in oxygen sensor applications to detect selectively the main fluorescence peak with maximum around 635 nm.

6 Conclusions

The rate constants of different types of reverse intersystem-crossing and delayed fluorescence have been estimated for Protoporphyrin IX in dimethylformamide (Table 1). It has been discussed when and how the presence of RISC can lead to distortion of triplet state kinetics. Furthermore, the knowledge of rate constants will make it possible to estimate contributions of individual delayed fluorescence mechanisms to the overall delayed emission, which will enable a more accurate analysis and interpretation of long-lived luminescence signals under diverse conditions.

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Declarations

Conflict of Interests On behalf of all authors, the corresponding author states that there is no conflict of interest.

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