Nitrooxide Spin-Label Quenching of Fluorophore’s Triplet State as a Tool for Studying Diffusion Mediated Reactions in Lipid Membranes

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In this work, we introduce an approach to study bimolecular interactions in model lipid bilayers and biological membranes, which exploits the influence of membrane-associated Electron Spin Resonance (ESR) labels on the fluorescence signal of likewise membrane-bound fluorophore markers. It is shown how one can exploit the high detection sensitivity of the fluorescence signal without losing the ability to follow low-frequency molecular interactions, taking place on a time scale well beyond that of the fluorescence lifetimes. The approach utilizes triplet state monitoring by Fluorescence Correlation Spectroscopy (FCS), whereby the fluctuations in a strong fluorescence signal is used to characterize transition rates to and from the lowest triplet state of the fluorophores, which take place on a time scale 3 to 6 orders of magnitude slower than the fluorescence lifetimes of the fluorophores. FCS measurements were performed on the dye Lissamine Rhodamine B (LRB) in aqueous solutions and bound to a lipid in a liposome, and in the presence of different local concentrations of the ESR label TEMPO. Both in the aqueous solution and in the lipid membrane measurements, the measured relative changes in the singlet-triplet transitions rates were found to well reflect the collisional frequencies between the LRB and TEMPO molecules. The proposed approach, allowing low-frequency interactions to be monitored with a bright fluorescence signal offers a broad applicability, both in terms of read-out means, types of molecular interactions that can be followed, and in what environment these interactions can be measured. From this point of view, it can prove useful for a broad category of molecular interaction studies.

INTRODUCTION

Many cellular processes are based on bimolecular, diffusion-mediated reactions occurring in biological membranes1. Such processes include oxidative phosphorylation and electron transport, enzymatic processes utilizing membrane-bound substrates as well as sensory and regulatory processes at membrane interfaces.

Several techniques can be used to study diffusion behavior in model bilayers or in biological membranes, such as NMR, fluorescence correlation spectroscopy (FCS) fluorescence recovery after photobleaching (FRAP) and single-particle tracking (SPT) (see 2-4 for recent reviews). However, many of these methods, primarily address self-diffusion and are less suited to directly monitor molecular encounters in membranes. Spin exchange detected in ESR experiments can provide this information, but the detection sensitivity is relatively low. ESR spectroscopy for membrane dynamics studies thus require high concentrations of spin-labels, which increases the risk of perturbation, and reduces the applicability. Bimolecular reactions in membranes have therefore to a large part been studied by quenching of luminescence from electronically excited probe molecules, labeled to one or several of the interaction partners.

In this work, we introduce an approach to study bimolecular interactions in model lipid bilayers and biological membranes, which exploits the influence of membrane-associated ESR labels on the fluorescence signal of a likewise membrane-bound fluorophore marker. It is well established that some molecular species with electron spin multiplicities >0 are good quenchers of excited electronic states of a wide range of fluorescent molecules6-8. Quenching of luminescence from fluorescent and phosphorescent probes by nitrooxide spin labels, based on a long-range electron transfer (LRET) mechanism, has also been demonstrated as a tool to monitor association/clustering of cell surface proteins9. While useful for monitoring physical association between membrane-associated reactants or conformational changes of membrane proteins, direct measurements of collisional encounters in membranes with sufficient sensitivity have proven difficult, both by fluorescence and phosphorescence quenching.

Using fluorescence emission as readout yields an excellent detection sensitivity. On the other hand, the excited state lifetimes of fluorescent probes are often too short (~ns) to allow for diffusion and collisional encounters of quenchers to significantly affect the emission of the fluorophores. A large fraction of the bimolecular diffusion-mediated processes between components in biological membranes take place in a time range of µs to ms. Fluorescent probes emitting in this relatively long time-scale are rare, in particular if one also adds as criteria that they should have a high fluorescence brightness and be efficiently quenched by contact.10

In contrast to fluorescence emission, photo-excited triplet state probes are much more long-lived and thus open for membrane dynamic studies at considerably longer time scales (µs-ms). Diffusion-mediated triplet-triplet annihilation in membranes has been successfully monitored by transient state absorption spectroscopy11,12. However, the instrumentation is relatively complicated and offers a limited sensitivity. This approach is therefore not widely applicable for diffusion-mediated reaction studies in membranes, in particular not for studies on cellular membranes. Alternatively, the extent of triplet state quenching can be followed via the phosphorescence signal of triplet state probes. Phosphorescence labels are far more
long-lived than fluorophores, and in this respect more suitable for bimolecular reaction studies in membranes. On the other hand, coupled to the long-lived emission is also the susceptibility of the triplet state to dynamic quenching by oxygen and trace impurities, which can be circumvented only after elaborate and careful sample preparation, or by creating oxygen diffusion barrier shields around the phosphorescent probes. This quenching not only shortens the triplet lifetime but also makes the luminescence practically undetectable. Molecular encounter studies in membranes by this readout is thus largely restricted to deoxygenated, carefully prepared samples, or to larger, shielded, and therefore less environment sensitive probes, which restricts the applicability.

In this work, we introduce FCS to measure the quenching of triplet states of fluorophores by nitrooxide labels, monitored via their fluorescence signal rather than via their several orders of magnitude weaker luminescence emission. Thereby, a high detection sensitivity can be maintained, without sacrificing the benefit of the long lifetime and environmental sensitivity following from their long lifetimes can be united with the signal strength of the fluorescence signal. FCS measurements have proven useful for monitoring several different photo-induced transient states, including triplet states, isomerised states, and states generated by photo-induced charge transfer.

Here, we monitor the quenching of the long-lived first excited triplet state of a fluorophore marker (Lissamine Rhodamine B, LRB) by a nitrooxide ESR label (TEMPOL), recorded via FCS and the strong fluorescence signal of the same fluorophore. The quenching mechanisms were first investigated in aqueous solution. Then the TEMPO-induced quenching of LRB were analyzed in unilamellar liposomes, with the labels covalently linked to lipid headgroups. A two-dimensional model for diffusion-controlled, bi-molecular quenching, based on and found to well predict the diffusion behavior and the collisional encounter frequencies between the LRB- and TEMPO-labeled lipids. The proposed approach can be applied to a wide range of molecular interaction studies in membranes.

MATERIALS AND METHODS

The fluorophore Lissamine Rhodamine B (LRB) and spin-label TEMPO choline were purchased from Invitrogen Inc., Carlsbad, USA. LRB was dissolved from powder into DMSO and then further diluted to nanomolar concentrations by adding ultra pure water.

Liposome preparation. SUVs were prepared by mixing 385 μl of a 10 mg/ml chlorof orm solution of the 18:1 (Δ9-Cis) PC (DOPC) lipid (1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine, Avanti Polar Lipids, Inc., Alabaster, AL, USA) with 7.1 μl of a 10 μg/ml chloroform solution of 14:0 LRB PE ((1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine Rhodamine B sulfonyl) (ammonium salt), Avanti Polar Lipids). Similarly, 18:1 TEMPO PC (1,2-dioleoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids) in concentrations ranging from 0% to 8% of the total lipid content were mixed into the same chloroform solution. Following evaporation under nitrogen flow for an hour, the lipids were dissolved in a solution containing 4 ml of 0.15 M NaCl and 4 μl of 0.3 M NaOH solution.

The lipid mixture was shaken for 30 minutes, using a vortex mixer, to form multilamellar liposomes, and then sonicated, using a tip sonicator, until the solution became transparent (about 1 hour). The liposome solution was then centrifuged for 30 minutes (10000g) to remove residual multilamellar liposomes and metal particles from the sonicator tip. The sonicated liposomes were calculated to have a hydrodynamic radius of 20 ± 6 nm, based on the determined diffusion coefficients from the FCS measurements. A radius of 20 nm corresponds to ~7200 lipids per monolayer, if the area per lipid is 72.2 Å². The ratio of LRB PE to non-fluorescent lipids was 1:120000 to ensure that each liposome contained at the most one fluorophore.

FCS measurements. FCS measurements were performed on a home-built confocal setup, largely arranged as described in. In brief, a 568 nm laser beam from a linearly polarized Kr/Ar-ion laser (Melles Griot 643-RYB-A02, Carlsbad, USA) was focused by a 63×, NA 1.2 objective (Zeiss, Plan-Neofluar, 160mm tube length), down to a 1/e² radius of ~0.6 μm. Emitted fluorescence was collected by the same objective, passed through a diacoric mirror (Semrock FF 576/661) focused onto a 30 μm in diameter pinhole in the image plane, split by a 50/50 non-polarizing beam-splitter cube (BS010, Thorlabs, USA), and finally detected via a band-pass filter (HQ601/75, Chroma Technology Corporation, Rockingham, USA) by two avalanche photodiodes (APDs) (SPCM AQR-14/16, Perkin-Elmer Optoelectronics, Wellesley, MA, USA). The detection volume was assumed to be a three-dimensional Gaussian with an axial 1/e²-extension approximately 5 times the transverse 1/e²-radius. The APD signals were processed by an ALV-5000/E correlator (model ALV-5000-E, ALV, Langen, Germany, with an ALV 5000/FAST Tau Extension board). The excitation power of the laser was varied between 0.1 and 1 mW, corresponding to mean irradiance values of 25 and 250 kW/cm² in the focal plane of the detection volume.

Deoxygenation was obtained by placing the samples in a sealed chamber. Argon gas was then flushed through a water chamber and then flushed through the chamber for at least 40 min prior to measurements.

Fluorescence lifetime measurements. Time-correlated single photon counting (TCSPC) measurements were performed on a spectrofluorometer with a TCSPC option (FluoroMax3, Horiba Jobin Yvon, Longjumeau, France). To avoid re-absorption and re-emission effects, the fluorophore concentrations were kept strictly below 1 μM. In the TCSPC measurements the samples were excited by a NanoLED source emitting at 495 nm with a repetition rate of 1 MHz and pulse duration of 1.4 ns. Typically 10000 photon counts were collected in the maximum channel using 2048 channels. The decay parameters were determined by least squares deconvolution using a mono-exponential model, and their quality was judged by the reduced χ² values and the randomness of the weighted residuals.

THEORY

Electronic state model. The electronic states of LRB involved in the processes of fluorescence at 568 nm excitation wavelength can be modeled as shown in Fig. 1. In the model, S₀ denotes the ground singlet state, T₁ is the excited singlet state and T₃ is the lowest triplet state. kᵣ, kₓ, kₛ, and kₜ are the rate constants for excitation from S₀ to T₁, relaxation of S₀ to Sₓ intersystem crossing from Sₓ to T₃, and relaxation of T₃ back to S₀ respectively. kₓ can be written as σₓ T₃ I / J, where σₓ is the excitation cross section for transitions from Sₓ to S₀, and J is the excitation intensity. As reasonable approximations for the triplet state population kinetics of LRB in water at 568 nm excitation, effects of excitation to higher singlet and triplet states can be neglected, excitation and emission dipole moments
can be considered isotropic and the triplet state completely non-luminescent. Typically, $k_{ISC}$ is in the order of $10^8 \text{s}^{-1}$ and $k_{ISC}$ is in the order of $10^5 \text{s}^{-1}$. Hence, transition to the triplet state only takes place in about one out of one thousand excitation-emission cycles. However, once populated, the triplet state is quite long-lived ($\mu$s-ns), with $k_{ISC}$ in the order of $10^{-3}$-$10^6 \text{s}^{-1}$. As a consequence, the steady state triplet-state population can accumulate strongly. This can in particular take place at excitation intensities, at which $k_{ISC}$ is comparable to $k_{ISC}$ and in the absence of triplet state quenchers, such as molecular oxygen, yielding very low $k_{ISC}$ rates.

Presence of paramagnetic compounds like oxygen or TEMPO can strongly influence the rates of singlet-triplet transitions. Molecular oxygen $^{16}$ is in its ground state in a triplet state, which makes it to an efficient quencher of triplet states fluorophores by triplet-triplet energy transfer. Due to the paramagnetic properties of molecular oxygen, significant changes in the triplet state lifetimes as well as in the intersystem crossing rates can be observed in solutions upon variation of the oxygen concentration $^{17,22}$.

Similarly, TEMPO has been observed to affect the triplet state of Rhodamine dyes. In comparison to oxygen however, the triplet state quenching constant was reported to be about 10 times lower.

**Fluorescence Correlation Spectroscopy.** In FCS, fluorescence intensity fluctuations from fluorescent molecules excited by a focused laser beam are exploited to monitor their dynamic processes. The emitted fluorescence, $F_{c}(\tau,t)$, from a fluorescent molecule observed in an FCS experiment, located at position $\tau$ at time $t$ is proportional to the probability that the excited singlet state, $S_1$ of the fluorophore is occupied at time $t$:  

$$F_{c}(\tau,t) = \Phi_s \int_{V} CEF(\tau) c_{s}(\tau,t)F_{c}(\tau,t) dV$$

Here, $\Phi_s$ is the fluorescence quantum yield and $S_1(\tau,t)$ denote the occupation probability of $S_1$ at time $t$ and at location $\tau$. Typically, the FCS measurements are performed in a confocal arrangement. The total detected fluorescence from the detection volume can then be expressed as:

$$F(t) = \Phi_{d} \int_{V} \int_{V} CEF(\tau)c_{s}(\tau,t)F_{c}(\tau,t)d\tau dV$$

Here, $\Phi_{d}$ is the detection quantum yield of the instrument, $CEF(\tau)$ is the collection efficiency function, and $c_{s}(\tau,t)$ is the concentration of fluorescent molecules. For fluorophores undergoing transitions between the fluorescent singlet entity ($S_0$ and $S_1$) and a non-fluorescent transient state, kinetic information about these transitions can be extracted from the fluorescence intensity fluctuations, analyzed in terms of a normalized auto-covariance function, $G(\tau)$. For molecules undergoing electronic state transitions between their two lowest singlet states ($S_0$,$S_1$) and a dark triplet state ($T_1$), as well as diffusion into and out of the FCS detection volume, $G(\tau)$ can be expressed as $^{17}$:

$$G(\tau) = \frac{< F(t)F(t+\tau) >}{< F(t)^2 >} =$$

$$= \frac{1}{N(1-T)} \left( \frac{1}{\tau_0} \frac{1}{1+\sigma_{exc}} \tau \tau_0 \right)^{\frac{1}{2}} \times$$

$$\left( \left[ 1-T + T \exp(-\tau/\tau_0) \right] + 1 \right)$$

$$= \frac{1}{N(1-T)} \left( \left[ 1-T + T \exp(-\tau/\tau_0) \right] + 1 \right)$$

Here, $N$ is the average number of fluorescent molecules in the detection volume. $G(\tau)$ represents the diffusion dependent part of $G(\tau)$ normalized to unity amplitude. $G(\tau)$ decays with a time $\tau_0$ corresponding to the average dwell times of the fluorescent molecules in the detection volume. $\bar{T}$ denotes the average steady-state probability for the fluorescent molecules within the detection volume to be in their triplet states. The triplet relaxation time $\tau_T$ represents the equilibration time between the two singlet states and the triplet state. In an air-equilibrated aqueous solution this equilibration typically occurs in the microsecond time range. For a fluorescent molecule, with electronic states as depicted in Fig. 1, located at $\bar{T}$ and experiencing an excitation intensity, $I(\tau)$:

$$\tau_T(\bar{T}) = \frac{k_{ISC} + \sigma_{exc} I(\bar{T})}{k_T k_{ISC} + \sigma_{exc} I(\bar{T}) (k_T + k_{ISC})}$$

$$\bar{T}(\tau_T) = \frac{\sigma_{exc} I(\bar{T})}{\sigma_{exc} I(\bar{T}) (k_T + k_{ISC}) + k_{ISC} k_T \tau_T}$$

Experimentally, by fitting Eq. 3 to the recorded FCS curves, the parameters $\bar{T}$ and $\tau_T$ represent average values within the detection volume, and can be estimated as described in $^{17}$.

A simple relation between $\bar{T}$ and $\tau_T$ can be expressed if $\sigma_{exc} I(\bar{T})$ is extracted from Eq. 5, i.e.:

$$\sigma_{exc} I(\bar{T}) = \frac{k_{ISC} k_T \bar{T}(\tau_T)}{k_{ISC} - \bar{T}(\tau_T) (k_T + k_{ISC})}$$

and substituted into Eq. 4, whereby $\tau_T$ can be expressed as a linear function of $\bar{T}$:

$$\tau_T = \frac{k_{ISC} (k_T - \bar{T}(\tau_T) (k_T + k_{ISC})) + k_T k_{ISC} \bar{T}(\tau_T)}{k_T k_{ISC} (k_T - \bar{T}(\tau_T) (k_T + k_{ISC})) + k_{ISC} k_T \bar{T}(\tau_T) (k_T + k_{ISC})}$$

$$= \frac{1 - \bar{T}(\tau_T)}{k_T}$$

**Diffusion controlled reactions.** In bimolecular diffusion-con-
trolled reactions, expressions relating the rate of reactions to parameters such as the diffusion coefficients, the reaction radii and concentrations of the two substrates have been derived for two or three dimensions assuming that the reactions take place in an infinite area or volume, respectively. In three dimensions, the number of reactions per second between molecules, B, and a single molecule, A, is given by:

$$\varphi = 4\pi(D_A + D_B)P \frac{P^4}{1.1} \ln \left( \frac{1.2}{4\pi \langle B \rangle^3} \right)$$

(8)

Here, $D_A$ and $D_B$ are the diffusion coefficients of A and B, respectively, and $P$ is the probability of a reaction to occur when the reaction partners are within the reaction distance, $s$, from each other. Eq 8 is based on the assumption that the concentration of B is constant in time at an infinite distance from A. When the reactions take place in a spherical shell, i.e. for reactions in a membrane, Berg and Purcell provided an approximate expression for the number of reactions per second between molecules B and a single molecule A:

$$\varphi = \frac{4\pi(D_A + D_B)P}{1.1} \ln \left( \frac{1.2}{4\pi \langle B \rangle^3} \right)$$

(9)

This expression is based on the understanding that the reaction distance, $s$, is much smaller than the shell radius. In the special case when all molecules occupy the same average area, $A$, then $\varphi$ may be written as:

$$\varphi = \frac{4\pi(D_A + D_B)P}{1.1} \frac{L}{A} \ln \left( \frac{1.2A}{4\pi Ls^3} \right)$$

(10)

Here, $L$ is the fraction of B molecules relative the total number of molecules in the membrane and $P$ is the probability that a reaction occur at a distance $s$.

RESULTS AND DISCUSSION

Solution measurements. Time-correlated single photon counting (TCSPC) measurements of LRB in aqueous solution show that upon addition of TEMPO in mM concentrations a small decrease in the fluorescence lifetime of LRB can be observed. The enhancement of $k_Q$ by the addition of TEMPO follows to a first approximation a linear relationship $k_Q = (0.70 \pm 0.01) \times 10^7 + (8.8 \pm 0.8) \times 10^6 \cdot [\text{TEMPO}] \cdot M^{-1} \cdot s^{-1}$. The presence of 1 mM TEMPO thus yields a relative change of $k_Q$ of LRB of about 1 percent. A similar relative change in the fluorescence intensity can be expected when recorded under conventional excitation conditions (non-saturating excitation intensities). Given the paramagnetic properties of TEMPO, a considerably stronger relative effect can be expected on the transition rates to and from the triplet state of LRB.

To investigate the quenching properties of TEMPO on the triplet states of LRB, a series of FCS measurements were performed in aqueous solution, both under air-saturated and deoxygenated conditions. Under air-saturated conditions, FCS curves were recorded from LRB sample solutions with varying concentrations of TEMPO (0 mM to 2.5 mM), and for each TEMPO concentration, the excitation power was varied from 100 to 1000 μW (corresponding to an average excitation irradiances in the detection volume of 25 to 250 kW/cm²). Upon addition of TEMPO an increase in the triplet amplitude, $\bar{T}$, and a drastic shortening of the triplet relaxation time, $\tau_T$, could be observed in the correlation curves (Fig. 2A). The correlation curves could be well fitted to Eq. 3 without the need to add any additional exponential relaxation terms in the fitting process. At TEMPO concentrations higher than 10 mM the triplet relaxation times become so fast that the full relaxation process cannot be adequately analyzed within the time resolution accessible by our correlator (first time channel is 12.5 ns). By plotting the measured parameters $\tau_T$ versus $\bar{T}$, the triplet deactivation rate, $k_T$, was directly determined by a linear fit according to Eq. 7. For each concentration of TEMPO, the intersystem crossing rate, $k_{ISC}$, could then be obtained as a global fitting parameter from the excitation irradiance dependence of $\tau_T$ and $\bar{T}$ using Eq. 4 and Eq. 5. Here, the determined $k_T$ values were used as fixed parameters. Also, the Gaussian-Lorentzian distribution of excitation irradiances experienced in the detection volume were taken into consideration (see Ref. 17 for details), and the minor relative quenching of $k_Q$ by TEMPO was also taken into consideration (see above). The resulting $k_Q$ and $k_{ISC}$ are plotted versus TEMPO concentration in Fig. 2B. From Fig. 2B it can be noted that both $k_Q$ and $k_{ISC}$ are influenced by the concentration of added TEMPO, [TEMPO], displaying a close to linear dependence. Considering the influence of TEMPO on $k_Q$ and $k_{ISC}$ to be a bimolecular reaction, it can be described by the equations:

$$k_{ISC} = k_{ISC}^{0} + k_{ISC} \cdot [\text{TEMPO}]$$

(11)

$$k_T = k_{T}^{0} + k_{Q} \cdot [\text{TEMPO}]$$

(12)

Fitting Eq. 11 and Eq. 12 to the data in Fig. 2B then yields the molar quenching rate coefficients $k_{Q}^{0} = 1.6 \times 10^6 M^{-1} s^{-1}$ and $k_T^{0} = 4.0 \times 10^9$ M⁻¹s⁻¹, respectively.

The FCS measurements were repeated on the same samples under deoxygenized conditions (see methods and materials). In Fig. 2C, the corresponding correlation curves are shown for a fixed excitation irradiance. Similar to the observation in air-saturated measurements, the triplet relaxation times were found to decrease upon addition of TEMPO. However, in contrast to the air-saturated measurements, the triplet state amplitudes, $\bar{T}$, decreased with increasing [TEMPO]. In air-saturated aqueous solutions most of the deactivation of $T$ is due to quenching by molecular oxygen. In the absence of oxygen, $k_T$ is on the order $10^7$ s⁻¹. Addition of TEMPO then leads to a much stronger relative increase of $k_T$ compared to the relative increase it would generate in an air-saturated solution. With this in mind, and with reference to Eq. 4 and Eq. 5, it is clear that the decrease of $\bar{T}$ with increasing [TEMPO] found in deoxygenated solutions reflects the same influence of TEMPO on the $k_T$ and $k_{ISC}$ rates of LRB, as found under air-saturated conditions. For the deoxygenated measurements, the kinetic rates were determined in the same manner as for the air-saturated measurements, using Eqs. 7, 4 and 5. The determined rate parameters are plotted versus TEMPO concentration in Fig. 2D. By a linear fit, according to Eq. 11 and Eq. 12, the molar quenching rate coefficients $k_{Q}^{0} = 2.3 \times 10^6 M^{-1} s^{-1}$ and $k_T^{0} = 5.2 \times 10^8 M^{-1} s^{-1}$. These values are slightly higher than the corresponding quenching rates determined in the air-saturated measurements (Fig. 2B). A possible explanation to this small difference is that TEMPO may react with molecular oxygen. A possible annihilation between TEMPO and $O_2$, which both are in their triplet states and which both can enhance $k_Q$ and $k_T$ will be present in the air-saturated measurements but can be negligible in the deoxygenized measurements.

The expected collision frequency between a single LRB molecule and TEMPO molecules was calculated from Eq. 8 to be $8 \times 10^{10}$ m⁻³s⁻¹. In these calculations, the diffusion coefficients of LRB and TEMPO were specified to be $4 \times 10^{-10}$ m²/s and $6 \times 10^{-10}$ m²/s.
s\textsuperscript{26}, respectively. From the diffusion coefficients, their corresponding hydrodynamic radii were determined from the Stokes-Einstein relationship to 0.4 and 0.6 nm, respectively. From the sum of the hydrodynamic radii, we then estimate \( s = 0.4 \text{nm} + 0.6 \text{nm} = 1.0 \text{nm} \).

Based on the determined \( k_{ISC} \) and \( k_T \) and by use of Eqs. 11 and 12, the probability, \( P \), that a collision results in a reaction affecting \( k_{ISC} \) or \( k_T \), was then determined to be 7% and 26%, respectively. The quenching of \( T_1 \) accounted for by \( k_{QT} \) is presumably mediated by triplet-triplet annihilation. For this quenching mechanism, and according to the spin statistical factor, only every ninth collision should affect the \( k_T \) rate. From that perspective, the 7% probability is very close to that limit. The other hand, the quenching of \( S_1 \) into \( T_1 \), accounted for by \( k_{QISC} \), is likely to be caused by spin-orbit coupling, which does not have a similar limitation by a spin-statistical factor. For the \( k_{QISC} \), we note that only every fourth collision results in a reaction affecting this rate.

From the quenching coefficients one can see that the effect of TEMPO is about 4 times stronger on the \( k_{ISC} \) rate, than on the \( k_T \), which results in the observed increase of the triplet state fraction under air-saturated conditions. The opposite effect was observed in a previous study\textsuperscript{28} where the effect of TEMPO on the triplet state parameters of Rhodamine 6G (Rh6G) was investigated. However, Rh6G has a lower maximum absorption wavelength than LRB, i.e. a higher lying first excited singlet state. Since the energy levels of

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**Figure 2:**

A. Set of FCS curves of LRB, measured in an air-saturated aqueous solution with different concentrations of TEMPO added (0 – 2.5 mM). Excitation irradiance 100 kW/cm\(^2\). Fits and residuals obtained according to Eq. 3. The amplitudes of the triplet relaxation term \( T \) and the triplet relaxation time \( \tau_T \) for the different curves were determined to: 0.15 / 3.0 \( \mu \)s (0 mM), 0.19 / 2.6 \( \mu \)s (0.1 mM), 0.23 / 1.8 \( \mu \)s (0.25 mM), 0.29 / 1.1 \( \mu \)s (1 mM), 0.30 / 0.57 \( \mu \)s (2.5 mM). The characteristic diffusion time \( \tau_D \) was for the curves determined to 95 \( \mu \)s ± 10%.

B. The [TEMPO] dependence of \( k_{ISC} \) and \( k_T \) as obtained from the FCS parameters \( T_{eq} \) and \( \tau_T \) (Eqs. 4 and 5), measured at different excitation irradiances (25 kW/cm\(^2\) – 250 kW/cm\(^2\)) in an air-saturated aqueous solution. Lines represent linear regression fits to Eqs. 11 and 12, yielding the following intrinsic and quenching rates: \( k_{ISC} = 4.2 \times 10^5 \text{s}^{-1} \) and \( k_{QISC} = 1.6 \times 10^9 \text{M}^{-1}\text{s}^{-1} \) for the intersystem crossing to \( T_1 \), and \( k_I = 4.0 \times 10^8 \text{M}^{-1}\text{s}^{-1} \) for the \( T_1 \) deactivation.

C. Set of FCS curves of LRB, measured in a deoxygenized aqueous solution with different concentrations of TEMPO added (0 – 2.5 mM). Excitation irradiance 250 kW/cm\(^2\). Fits and residuals obtained according to Eq. 3. The amplitudes of the triplet relaxation term \( T \) and the triplet relaxation time \( \tau_T \) for the different curves were determined to: 0.87 / 18 \( \mu \)s (0 mM), 0.71 / 5.6 \( \mu \)s (0.1 mM), 0.60 / 2.0 \( \mu \)s (0.25 mM), 0.57 / 0.89 \( \mu \)s (1 mM), 0.51 / 0.35 \( \mu \)s (2.5 mM). The characteristic diffusion time \( \tau_D \) was for the curves determined to 80 \( \mu \)s ± 10%. (The longer \( \tau_D \) compared to that obtained in Fig 2A, can be attributed to saturation broadening of the fluorescence emission profile in the detection volume due to a higher triplet state build-up).

D. The [TEMPO] dependence of \( k_{ISC} \) and \( k_T \) as obtained from the FCS parameters \( T_{eq} \) and \( \tau_T \) (Eqs. 4 and 5), measured at different excitation irradiances (25 kW/cm\(^2\) – 250 kW/cm\(^2\)) in a deoxygenized aqueous solution. Lines represent linear regression fits to Eqs. 11 and 12, yielding: \( k_{ISC} = 3.4 \times 10^5 \text{s}^{-1} \) and \( k_{QISC} = 2.3 \times 10^9 \text{M}^{-1}\text{s}^{-1} \) for the intersystem crossing to \( T_1 \), and \( k_I = 5.4 \times 10^3 \text{s}^{-1} \) and \( k_{Q_T} = 5.2 \times 10^8 \text{M}^{-1}\text{s}^{-1} \) for the \( T_1 \) deactivation.
S1 and T1 are typically closely related, also the T1 state of Rh6G can be expected to lie higher in energy than that of LRB. Because of this, charge transfer reactions to the ground triplet state of TEMPO are more likely to occur from the T1 state of Rh6G than from the T1 state of LRB. This additional de-activation channel of the T1 state should then be reflected in the FCS measurements as a decreased steady-state triplet populations and a shortening of the triplet relaxation times. Indeed, FCS measurements on Rh6G and TEMPO showed not only a decrease of the triplet state fraction (in agreement with results reported in 28), but also the presence of a second relaxation component in the correlation curves. This additional term can be attributed to formation of Rh6G radicals via their T1 states. 29 However, for LRB the FCS measurements give no evidence of a strong charge-transfer-mediated quenching of T1. This effect was therefore not included in the analysis, and the effects of TEMPO on other fluorophores fall outside the scope of this investigation and were not further investigated in this work.

Liposome measurements. FCS measurements were performed on LRB-labeled liposomes in aqueous solution, with varying fractions of TEMPO labeled lipids included. Measurements were recorded for LRB-labeled liposomes in an air-saturated aqueous solution, with different fractions, L, of TEMPO-labeled lipids included (see Fig 3A). For each L, a series of FCS curves were recorded at different excitation intensities (from 25 kW/cm² to 175 kW/cm²), yielding the different \( \tau_T \) and \( |T| \) values in the graph. Solid lines: Fittings of the measured \( \tau_T \) versus \( |T| \) parameters for each L, according to Eq. 7. From these fits, the following \( k_T \) values were obtained (given in \( 10^6 s^{-1} \)): 0.34 (0%), 0.42 (0.75%), 0.50 (2%), 0.67 (5%), 0.95 (8%).

![Graph A](image1.png)  
**Figure 3: A.** Set of FCS curves of LRB-labeled liposomes (maximum one LRB-labeled lipid per liposome), measured in an air-saturated aqueous solution. In the liposomes, the fraction \( L \) of the lipids labeled with TEMPO varied from 0 to 8%. Excitation irradiance 100 kW/cm². Fits and residuals obtained according to Eq. 3. The amplitudes of the triplet relaxation term \( |T| \) and the triplet relaxation time \( \tau_T \) for the different curves were determined to: 0.26/2.2 µs (0%), 0.30 / 1.8 µs (0.75%), 0.43 / 1.1 µs (2%), and 0.50/ 0.5 µs (8%). The characteristic diffusion time \( \tau_D \) was for the curves determined to 1.45ms±10%. B. Relaxation times, \( \tau_T \), and amplitudes, \( |T| \), obtained by fitting FCS curves to eq. 3. The FCS curves were recorded for LRB labeled liposomes in an air-saturated aqueous solution, and with different fractions, L, of TEMPO-labeled lipids included (see Fig 3A). For each L, a series of FCS curves were recorded at different excitation intensities (from 25 kW/cm² to 175 kW/cm²), yielding the different \( \tau_T \) and \( |T| \) values in the graph. Solid lines: Fittings of the measured \( \tau_T \) versus \( |T| \) parameters for each L, according to Eq. 7. From these fits, the following \( k_T \) values were obtained (given in \( 10^6 s^{-1} \)): 0.34 (0%), 0.42 (0.75%), 0.50 (2%), 0.67 (5%), 0.95 (8%).

![Graph B](image2.png)  
![Graph C](image3.png)  
![Graph D](image4.png)
performed both under air-saturated and under de-oxygenized conditions. For the air-saturated measurements, the fraction of TEMPO-labeled lipids in the liposomes, \( L \), was varied from 0 to 8%. For each fraction, FCS curves were recorded at different excitation irradiances, from 25kW/cm² up to 175kW/cm². In Fig. 3A, a series of correlation curves are shown, recorded at the same excitation irradiance, but where the SUVs contained different fractions of TEMPO labeled lipids. Apart from a distinct decay of the curves \( t = 11\% \). \( P \) dependence was fitted to Eq. 10, yielding a reaction probability per molecular encounter \( L \) fusion of the SUVs. When increasing \( L \), a strong increase in the triplet amplitude, \( T \), and the triplet deactivation rate, \( k_T \), for each local concentration of TEMPO-labeled lipids, \( L \), could be directly determined from a linear fit according to Eq. 7. From the obtained \( k_T \) rates, the corresponding intersystem crossing rates for each \( L \) were then determined from Eq. 4 and Eq. 5. Additional FCS measurements on SUVs were performed under de-oxygenized conditions, as described in Methods and Materials. SUVs were investigated, with \( L \) varying from 0 to 2%. For each \( L \), FCS measurements were performed under excitation irradiances ranging from 25 kW/cm² to 175 kW/cm². With increasing \( L \) the triplet state parameters showed the same principal behavior as found for [TEMPO] in the de-oxygenized solution measurements (Fig. 3C). Higher concentrations of TEMPO-labeled lipids in the SUVs lead to reduced triplet relaxation times and, in contrast to the air-saturated SUV measurements but in correspondence with the de-oxygenated solution measurements, decreased triplet state

Figure 4: A. Average times between two quenching reactions leading to deactivation of the \( T \) state of LRB in liposomes in a deoxygenized solution. The average times were determined for each fraction \( L \) from the difference of the inverse \( k_T \) rates, in the presence and absence of TEMPO-labeled lipids (Eq. 13). The average times versus \( L \) dependence was fitted to Eq. 10, yielding a reaction probability per molecular encounter \( P=11\% \). B. Average times between two quenching reactions leading to deactivation of the \( T \) state of LRB in liposomes in an air-saturated solution. Fitting the average times versus \( L \) dependence to Eq. 10, yielded \( P=8\% \). C. Average times between two quenching reactions leading to intersystem crossing from the \( S \) to the \( T \) state of LRB for liposomes in a deoxygenized solution. The average times were determined for each fraction \( L \) from the difference of the inverse \( k_{ISC} \) rates, in the presence and absence of TEMPO-labeled lipids (Eq. 14). The average times versus \( L \) dependence was fitted to Eq. 10, yielding a reaction probability per molecular encounter \( P=59\% \). D. Average times between two quenching reactions leading to intersystem crossing from \( S \) to \( T \) in LRB for liposomes in an air-saturated solution. Fitting the average times versus \( L \) dependence to Eq. 10, yielded \( P=36\% \). Analogous to the solution measurements, the measured parameters were plotted \( \tau_T \) versus \( T \) (Fig. 3B), and the triplet deactivation rate, \( k_T \), for each local concentration of TEMPO-labeled lipids, \( L \), could be directly determined from a linear fit according to Eq. 7. From the obtained \( k_T \) rates, the corresponding intersystem crossing rates for each \( L \) were then determined from Eq. 4 and Eq. 5.
amplitudes. The measured parameters were plotted, $\tau_T$ versus $T$ (Fig. 3D) and the $k_L$ rates for each $L$ were obtained by fitting the $\tau_T$ versus T plot to Eq. 7. The corresponding $k_{ISC}$ rates were then determined for each $L$ from the excitation intensity dependence of $\tau_T$ and $T$ and by use of Eq. 4 and Eq. 5, in the same manner as for the previous FCS measurement series.

From the $k_L$ and $k_{ISC}$ rate parameters, as determined above (Figs. 3B and 3D), the average time between two quenching reactions could be determined for each fraction $L$ from the difference of the inverse rates, in the presence and absence of TEMPO-labeled lipids:

$$\frac{1}{\varphi_T} = \frac{1}{k_L(L) - k_L(0)}$$  
$$\varphi_{ISC} = \frac{1}{k_{ISC}(L) - k_{ISC}(0)}$$  

Here, $\varphi_T$ and $\varphi_{ISC}$ denote the molecular encounter flow rates as given by Eqs. 9 and 10, for triplet deactivation from $T$ and intersystem crossing to $T$, respectively. $k_L(L)$ and $k_{ISC}(L)$ denote the rates of $k_L$ and $k_{ISC}$ at a lipid fraction of $L$, and $k_L(0)$ and $k_{ISC}(0)$ are the corresponding rates in the absence of TEMPO-labeled lipids ($L=0$).

The average times between two quenching reactions, for the two different reactions, $1/\varphi_T$ and $1/\varphi_{ISC}$, were calculated from the $k_L$ and $k_{ISC}$ rates determined from the liposome measurements (under air-saturated and deoxygenized conditions) and are plotted in Fig. 4A to Fig. 4D. The average times $1/\varphi_T$ and $1/\varphi_{ISC}$ were fitted to the inverse of Eq. 10. In this fitting procedure, the typical lipid area, $A$, was specified to be 72.2 $Å^2$, and the diffusion coefficient of the DOPC lipids specified to $9 \mu m^2/s$. The determined value of the reaction probability, $P$, assuming the same reaction distance, $s$, as that estimated for TEMPO and LRB in the solution measurements (1 nm) was for the air saturated liposome measurements, 8% for a reaction affecting $k_L$ and 36% for a reaction affecting $k_{ISC}$. For the deoxygenated measurements, the corresponding probabilities were determined to 11% and 59%, respectively. It can be noted that these reaction probabilities are slightly larger than those determined in the solution measurements. One contributing reason to this difference can be that the LRB and TEMPO molecules may be constrained in their mutual orientation with respect to each other when labeled to lipids in a membrane, which may favor a reaction to take place upon a collisional encounter. Secondly, the environmental conditions experienced by LRB and TEMPO are likely to differ slightly between the liposome measurements and those in aqueous solution. The reaction probabilities determined for the air-saturated liposome measurements were found to be slightly lower than those determined under deoxygenized conditions. This is in analogy to the solution measurements, where higher quenching rates $k_{air}$ and $k_{ISC}$ were found in the absence of oxygen. Similar to the solution measurements, we expect TEMPO and molecular oxygen to react with each other, leading to slightly lower reaction probabilities in the air-saturated liposome measurements. From the liposome measurements under deoxygenized conditions, it be noted that the average times between reactions are much more precisely determined for $T$ deactivation (Fig. 4A) than for $S$ to $T$ intersystem crossing (Fig. 4C). In general, in our measurements, the $k_L$ parameters could be determined directly from the $T$ versus $\tau_T$ plots (Figs. 3B and 3D) by use of Eq. 7, without the need to include assumptions about the excitation irradiances applied. Moreover, for the deoxygenized measurements, it should be noted that for triplet state measurements by FCS, the intersystem crossing rates are more difficult to determine when the relative difference between the $k_{ISC}$ and $k_L$ rates is large. This circumstance is likely to make the determination of the reaction times for $k_{ISC}$ enhancement more precise when performed under air-saturated (Fig. 4D) than under deoxygenized conditions (Fig. 4C). However, taken together it can also be noted that the combination of the determined $T$ and $\tau_T$ parameters, as plotted in Figs. 3B and 3D, well reflects quite small differences in molecular encounter frequencies / local TEMPO concentrations in the investigated lipid vesicles.

CONCLUDING REMARKS

In this work, it is shown how one can exploit the detection sensitivity of the fluorescence signal without losing the ability to follow low frequency molecular interactions, taking place beyond the time scale of the fluorescence lifetimes. By FCS, transitions to and from long-lived transient states can be monitored via the fluctuations they generate in the fluorescence signal. Similarly, we show that presence of the electron spin label TEMPO leads to an increase of the transition rates to and from the lowest triplet state of LRB, both in aqueous solution, as well as in lipid membranes. The triplet state transition rates were shown to be sensitive to molecular encounter frequencies between TEMPO and LRB far lower than those recordable by traditional fluorescence parameters, such as by fluorescence intensity or fluorescence lifetime measurements. In the absence of oxygen, the triplet state lifetimes get significantly longer and even slower molecular interaction frequencies can be followed. In lipid membrane studies, the concentrations of both fluorophore and quencher molecules can be kept quite low, which minimizes the risk to influence the membrane by high label concentrations. The approach can be extended to other fluorophores and electron spin labels than those used in this study. Consequently, similar interaction studies can also be performed on e.g. membrane proteins, which can be readily labeled with both fluorescence and electron spin probes. FCS is not the only method that combines the possibility to follow the influence of an electron-spin label on the triplet state parameters of a fluorophore, via a strong fluorescence signal. Recently, it was shown that by use of modulated excitation, triplet state population and kinetics of fluorophores can be monitored in a highly parallel fashion, with far less instrumental and sample constraints. The presented principal approach, allowing low-frequency interactions to be monitored with a bright fluorescence signal thus offers a broad applicability, both in terms of read-out means, types of molecular interactions that can be followed, and in what environment these interactions can be measured.

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