Fluorescent Dyes

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Geminate Recombination as a Photoprotection Mechanism for Fluorescent Dyes**

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Abstract: *Despite common presumption due to fast photodestruction pathways through higher excited states, we show that further improvement of photostability is still achievable with diffusion-limited photoprotection formulas. Single-molecule fluorescence spectroscopy reveals that thiolate ions effectively quench triplet states of dyes by photoinduced electron transfer. Interestingly, this reaction rarely yields a radical anion of the dye, but direct return to the ground state is promoted by an almost instantaneous back electron transfer (geminate recombination). This type of mechanism is not detected for commonly used reductants such as ascorbic acid and trolox. The mechanism avoids the formation of radical cations and improves the photostability of single fluorophores. We find that a combination of β-mercaptoethanol and classical reducing and oxidizing systems yields the best results for several dyes including Atto532 and Alexa568.*

In recent years, we have witnessed a significant advancement in the understanding of single-molecule photoblinking and -bleaching so that a group of recipes has evolved as the golden standard for biomolecular single-molecule measurements as well as for superresolution microscopy.[1] Strategies to stabilize the fluorescence and photostability of fluorescent dyes commonly involve oxygen depletion by one of several enzymatic oxygen-scavenging systems[2] and the depopulation of various reactive intermediate states such as triplet states and radical-ion states. Triplet states are, for example, depopulated by cyclooctatetraene or by electron transfer reactions with oxidants such as methyl viologen (MV), nitrobenzyl alcohol, and trolox quinone (TXQ), and reductants including trolox (TX) and ascorbic acid (AA). According to the ROXS principle,[3] radical cations formed by photoionization or (triplet) oxidation are restored by reductants and radical anions formed by reductive photoprotectants are returned to the ground state by oxidants in solution (Figure 1).[3a,4]

While such photoprotection formulas improve the photostability up to 5000-fold for some fluorescent dyes and more than 10^8 photons can be emitted from single molecules under biologically relevant conditions,[5] the photostability is less improved for other dyes, especially for those that are excited at wavelengths below 600 nm.[3a,6] It was suggested that shorter wavelength excitation goes along with photodestruction pathways via higher excited states that cannot be blocked by diffusion-limited protection mechanisms involved in common photostabilizing formulas.[5,7] On the other hand, even if the first step towards photobleaching is absorption to higher excited singlet states, the cascade of events towards photobleaching might still involve longer-lived states that could be recovered by appropriate additives.[8]

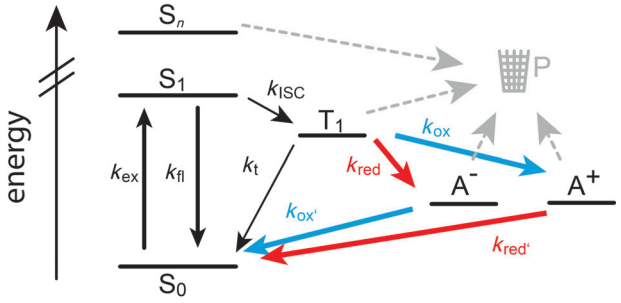

Figure 1. Photophysical model of organic fluorophores in the presence of reductants and oxidants. The dye is repeatedly excited (k_{ex}) from the ground state S_0 to the first excited singlet state S_1 and can return to S_0 by emission of a photon. Every now and then, intersystem crossing (k_{ISC}) causes the dye to enter a triplet state T_1 instead. From here, pathways to return to S_0 (k_t) compete with the reduction of the dye to a radical anion state A^- (k_{red}, red), which is re-oxidized ($k_{ox'}$, blue) to S_0. These electron-transfer reactions can also occur in reverse order. Photobleaching (gray arrows) predominantly occurs from states with long lifetimes and higher excited states (S_n).

Here we show a photoprotection mechanism, reduction with efficient back electron transfer (geminate recombination, GR), that significantly increases the photostability of dyes in the wavelength range below 600 nm. We present evidence that the increased propensity of photobleaching at these shorter wavelengths is related to photo-oxidized states that are avoided by GR. The approach requires only a single aliphatic thiol compound. We deduce the mechanism from single-molecule measurements of dyes attached to oligonucleotides immobilized on BSA/biotin/neutravidin surfaces.

Aliphatic thiols such as β-mercaptoethanol (ME) and β-mercaptoethylamine have been used in fluorescence microscopy for many years because they display photoprotecting properties without quenching fluorescence (see Ref. [9] and references therein). Many beneficial characteristics have been attributed to them including antioxidant properties, triplet quenching, oxygen removal, and scavenging of reactive

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oxygen species.[4,9b,10] Recently, they have also become popular for superresolution microscopy because single fluorescent dyes can be reversibly switched on and off in the presence of thiols using UV light and visible light, respectively.[11] Although thiols are common photostabilizing agents they have not been studied in the context of current photostabilizing strategies that focus on depopulation of reactive intermediates.

The shortcoming for dyes absorbing below 600 nm such as Atto532, Alexa532, and Alexa568 is exemplified by a fluorescence image and an intensity transient in Figure 2a. Alexa568 shows stable fluorescence emission without blinking in the presence of reducing and oxidizing compounds (2 mM mixture of TX/TXQ [3b]) but photobleaches quickly (oxygen is always removed enzymatically).

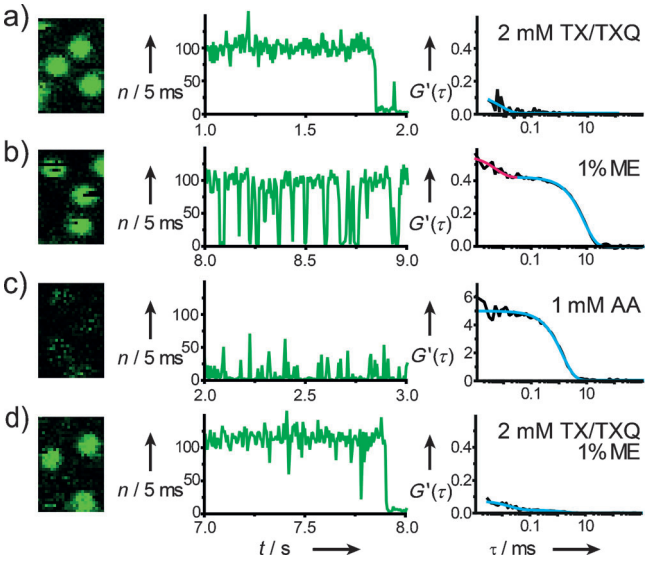

Figure 2. Confocal images (1.2×1.7 μm², 2 ms px⁻¹, 50 nm px⁻¹, color scale 0–150 counts px⁻¹), 1 s sections of fluorescence transients in the absence of oxygen and corresponding AC functions (right, offset subtracted $G'(\tau)=G(\tau)-1$) with biexponential fits (blue, red) for Alexa568 molecules. a) In ROXS buffer (TX/TXQ), the molecule emits stably but bleaches quickly. No significant AC amplitude is observed. b) The thiol ME induces blinking of the dye which is visible in the scan and in the transient. AC analysis reveals intensity fluctuations on two timescales: fast triplet dynamics and dynamics in the ms range (redox blinking). c) The reductant AA induces blinking as well, but the number of detected photons before blinking is strongly reduced compared to (b). d) Best performance is achieved with a mixture of ROXS and ME, where the fluorophore emits without significant blinking and survives longer than without ME.

Using ME instead of common ROXS reagents, we observe strongly increased photostability in combination with frequent blinking (Figure 2b and Figure S1b–d for fluorescence transients with increasing ME concentration). Blinking in the millisecond range is also observed when a classical reductant such as AA is used. However, the quantitative appearance of the transients with ME or with AA is drastically different (compare images and transients in Figure 2b,c) and photostability is also markedly higher with ME. As ME is a known reductant, we ascribe the fluorescence intermittencies (dark states) to radical anions formed by the photoinduced reduction of the triplet state.[12] This interpretation is supported by the disappearance of the off states in the presence of an oxidant (a mixture of TX/TXQ in Figure 2d).[3b] This mixture of the thiol compound with ROXS compounds appears to be the optimal solution for single-molecule measurements as blinking is minimized while high photostability is preserved (vide infra).

These data raise the question of how the mode of action of the thiol compound differs from that of the classical reductants AA and TX commonly used in ROXS formulas.[3a,b] We therefore analyze the intensity fluctuations in more detail. Autocorrelation (AC) analysis (Figure 2b, Figure S2, see the Supporting Information for details on AC analysis) reveals intensity fluctuations on two timescales in the presence of thiols. We assign the short component to the triplet state which is more quickly depopulated with increasing ME concentration (Figure S1b–d). Interestingly, the photostability thereby increases further but the long timescale blinking due to radical anion formation (long component in AC) appears similar. From a biexponential fit of the AC function we calculate the transition rates and the number of detected photons before the dye enters the triplet (N_T) or radical state (N_{on}). Triplet lifetimes t_T of different dyes including Atto532, Alexa568, and Atto647N decrease similarly (Figure 3a). The analogous effect of triplet reduction and photostabilization is achieved by increasing the pH instead of increasing ME concentration (Figure S3), indicat-

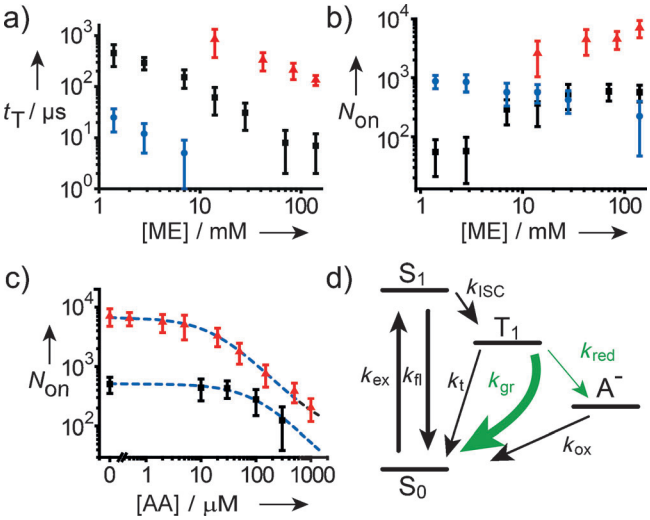

Figure 3. Analysis of thiol quenching mechanism. Error bars indicate standard deviations of Gaussian fits (Figure S2). a) The triplet lifetime t_T of Atto532 (blue circles), Alexa568 (black squares), and Atto647N (red triangles) are inversely proportional to the ME concentration. b) Corresponding N_{on} for the same dyes exhibit markedly different behavior. c) N_{on} in the presence of 1% ME decreases when AA competes with ME for triplet quenching. Dashed lines are fits ($N_{on}=N_T/(1-\Phi^0_{gr}/(1+\alpha[AA]t_T))$) to the experimental data, where N_T and t_T are fixed at the values in absence of AA. Statistical weighting yield Φ^0_{gr} of 99.0% (Atto647N) and 99.6% (Alexa568) and α of 4.0 μM⁻¹s⁻¹ (Atto647N) and 5.4 μM⁻¹s⁻¹ (Alexa568) (see text and the Supporting Information). d) Scheme of GR-ROXS, where GR successfully competes with the formation of radical cations.

ing that the thiolate anion is the active species in accordance with Ref. [13].

Commonly, N_{on} decreases with increasing reductant concentration because the reduction competes with direct triplet–singlet transitions (k_t) and at higher reductant concentration also singlet quenching sets in.[12] For Atto647N and Alexa568, N_{on} surprisingly increases with thiol concentration (Figure 3b). In the case of Atto532, N_{on} is slightly decreasing, but much less than that expected based on the ROXS concept and the efficient triplet quenching evident in Figure 3a. These contrasting effects of thiols and the common reductant AA on the photophysics is readily visualized with confocal scans of immobilized Alexa568 molecules (Figure S4). Because increasing AA concentration drastically accelerates the rate of dark-state formation (lower N_{on}), the molecules are barely detectable in the absence of oxygen. In contrast, increasing the ME concentration improves the visibility of the molecules because they enter dark states less frequently. We therefore quantify the effect of the AA concentration on N_{on} in presence of 1% ME for the dyes Atto647N and Alexa568. Figure 3c shows that N_{on} remains constant for low AA concentrations and is only affected when the concentration becomes high enough that AA can compete with the present thiolate for triplet quenching. The fact that the triplet lifetime of Alexa568 is shorter than that of Atto647N (Figure 3a) explains why the decrease in N_{on} for Atto647N starts already at lower concentrations. In addition, we find that N_T for Atto647N remains constant when N_{on} is reduced (Figure S5), which supports that the reduction occurs from the T_1 and not from the S_1 state.

Combining all this information, we conclude that thiolate ions are in fact triplet quenchers. However, they do not simply act as reductants like AA and TX. Instead, they are able to induce an additional triplet-quenching mechanism. Our findings are in accordance with a mechanism in which reduction and GR compete with reduction and escape from the solvent cage as was also found for alkyl sulfides (Figure 3d).[14] For this mechanism, the observed weak dependence of N_{on} on the thiolate concentration is expected. In first approximation, N_{on} should be constant because the yield of GR (Φ_{gr}), which is defined as the fraction of GR events with respect to all thiolate reductions including GR and radical escape, should be independent of the thiolate concentration. Considering additionally the intrinsic rate k_t that competes with the thiolate reaction, N_{on} should slightly decrease with increasing thiolate concentration. On the other hand, dyes exhibit some sort of blinking slower than triplet blinking already in the absence of redox-active agents due to photooxidation or reduction by redox-active groups in the local proximity of the dyes (e.g. guanosine in the DNA used for immobilization or interaction with the BSA-coated surface).[3a,4] Because such influences are subordinate at increasing thiolate concentration, N_{on} can also increase slightly.

Our data make it possible to extract the yield Φ_{gr} by comparing the number of detected photons before the dye enters the triplet and radical state ($\Phi_{gr} = 1 - N_T/N_{on}$) and we find that it is near 99% for all dyes studied. Because the formed thiyl radical is uncharged and the dyes are similar in size, this similarity in yields is reasonable. Interestingly, Φ_{gr} increases with increasing viscosity from 99.0 to almost 99.5% for Atto647N as expected for a GR mechanism (Figure S6). At higher viscosity the rate at which the products of the initial reduction can leave the solvent cage is reduced. The increase by 0.5% implies a doubling of the number of detected photons before the radical anion is formed. The fact that the off-times do not decrease with increasing thiolate concentration (Figure S7) is also in accordance with our interpretation of the geminate recombination (GR) ROXS mechanism rather than the two-component (tc) ROXS mechanism.[3b] The slight increase of off-times is in accordance with the reported oxygen depletion property of thiols.[10b,13a]

To elucidate the optimal photostabilizing system for blue/green dyes or even a general system for all dyes we studied the photobleaching of single molecules by total internal reflection microscopy. For Alexa568, we find that the photostability is increased in the presence of thiols and the best performance is achieved with a mixture of ME and classical tc-ROXS (Figure 4a, Figure S8 and Supporting Video 1) which also suppresses residual blinking. In contrast, Atto647N, which experiences an increase of the on-counts as well (Figure 3b), shows strikingly different behavior (Figure 4b). Whereas Atto647N is extremely photostable with tc-ROXS,[3a] apparent photobleaching is about 20-fold enhanced with thiols. Reactivation with UV (405 nm), however, restores fluorescence and shows that the dyes are not irreversibly bleached (Figure S9). It is known that thiols are not only antifading agents but that they also induce the formation of reversible long-lived dark states, likely by a photoaddition.[11a,d,13b] Thus, most dyes can be switched on and off by reactivating them with UV light as has been exploited for superresolution microscopy.[11b–d] This explains why the photostability of Atto647N in the presence of thiols appears to be reduced (Figure 4b). Further stabilization enhancement could therefore be achieved by UV reactivation. Similarly, we find for other dyes including Atto532, Alexa532, and Alexa488 (Figure S10) that apparent photostability can be higher with tc-

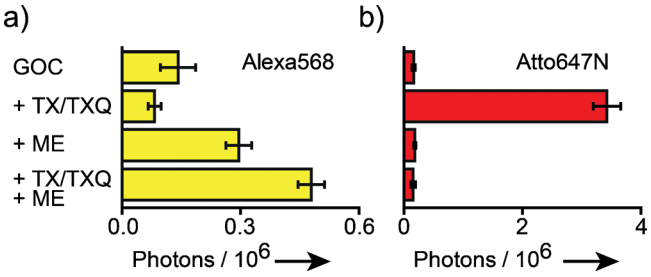

Figure 4. Total number of detected photons before photobleaching (mean+SD of at least three measurements) in different buffers. "GOC": enzymatic oxygen removal in Tris pH 8.6, "+TX/TXQ": GOC+2 mM TX/TXQ; "+ME": GOC+1% ME; "+TX/TXQ+ME": GOC+2 mM TX/TXQ and 1% ME. a) For Alexa568, the photon count is increased with ME with respect to conventional tc-ROXS and the best performance is achieved with both ME and TX/TXQ. b) In the case of Atto647N, addition of ME reduces the number of photons due to the formation of a long-lived dark state (Figure S9). Under "GOC" and "+ME" conditions, the molecules exhibit significant blinking (Figure 2).

ROXS or with GR-ROXS. In all cases, however, UV photoactivation can further enhance the overall photon count in the presence of thiols.[11c] Higher photostability with GR-ROXS is reasonable because the dyes reside for less time in potentially reactive radical anionic states and especially radical cationic states are avoided. The improvement compared to tc-ROXS also shows that the remaining photobleaching pathways for shorter-wavelength dyes were not only related to excitation to higher excited singlet states. While other explanations for the improved photostability including active involvement of redox compounds such as AA and MV in photobleaching cannot be excluded, it appears that especially semioxidized forms are prone to act as photobleaching intermediates (Figure S11 and discussion in the Supporting Information).

We have introduced geminate recombination as a photostabilizing mechanism in single-molecule biophysics. Our data indicate that thiols in contrast to classical reductants such as AA and TX exhibit a GR yield exceeding 99%, thus reducing triplet and redox blinking even in the absence of oxidants. As thiols also induce the switching of molecules to long-lived dark states, the number of detected photons before apparent photobleaching can decrease (Atto647N) or increase (Alexa568), depending on the individual gain from GR relative to the propensity of switching. This work represents a paradigm change in the sense that better photostabilization might still be feasible with diffusion-limited mechanisms and optimized antifade reagents because current protocols involve sensitive photo-oxidized states.[5,7] Our findings expand on the ROXS mechanism and (re-)launch the quest for photostabilizing agents that induce a high yield of GR without causing switching. The photostabilizing mechanism presented here resembles the mechanism of self-healing dyes except that no covalent binding is required and the protecting unit is steadily renewed.[15]

From a practical perspective, we find that generally a mixture of tc-ROXS and ME improves the performance of organic dyes, although the relative concentrations might need adjustment for individual cases. Triplet quenching by GR appears also promising for measurements on diffusing molecules because even a small number of GR cycles directly increases the photon count rate and the diffusion time is generally short enough that rare and long-lived radical dark states are negligible. Substances for the GR-ROXS mechanism are also biocompatible and frequently used in buffers (e.g. dithiothreitol (DTT), GR-ROXS with DTT in Figure S12) and occur in living cells (e.g. glutathione).

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