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On the Mechanism of Trolox as Antiblinking and Antibleaching Reagent

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Currently, fluorescence microscopy is witnessing some revolutionary developments with respect to sensitivity and resolution that is, however, slowed down by the imperfections of fluorescent probes. Fundamental limitations are photobleaching, which reduces the absolute number of photons obtained from a dye molecule, and intermittent or fluctuating emission, i.e., blinking, that is observed when the fluorophore randomly enters transient, nonfluorescent states. Recently, two papers have shown strategies for strong improvement of the performance of fluorescent dyes.[1,2] Rasnik et al. showed that removal of oxygen together with millimolar concentrations of the vitamin E analogue Trolox (TX) could drastically reduce photobleaching. In contrast to essentially all other commonly applied antifading agents, TX also efficiently eliminated blinking related to triplet states as well as blinking occurring on longer time scales,[1] which has already been helpful in a number of single-molecule applications (see, e.g., refs 3−5). While an understanding of the working principle of TX is yet missing, a combination of an oxidizing and reducing system (ROXS) has similarly proven its applicability in single-molecule as well as super-resolution applications.[2,6,7] Using reducing and oxidizing agents simultaneously leads to rapid depopulation of triplet states via electron transfer and quickly recovers the formed radical ions through the complementary redox reaction.[2]

In this communication we investigated the fluorescence protecting mechanism of TX and conclude that its antiblinking effect is due to quinoid derivatives of TX that form in buffer through (photo-) reactions with molecular oxygen. The combination of TX and its oxidized form (TX-qinone, TQ) then acts according to the ROXS scheme.[2]

Since it is known that TX is a reductant that quenches triplet states via electron transfer,[8] the following possibilities regarding the antiblinking mechanism of TX were taken into account and were evaluated in the following: (i) oxidizing impurities in the used as received TX batches cause reoxidation of the reduced triplet states; (ii) oxidizing impurities form upon dissolving TX in buffer; (iii) TX has intrinsic yet unknown oxidizing capabilities; and (iv) TX reduces triplet states, and the formed radical ions quickly recombine in a geminate recombination process before they can escape the solvent cage.

We used ensemble and single-molecule fluorescence spectroscopy in combination with a recently established single-molecule redox sensor (SMRS) to find experimental evidence for the hypotheses above.[7] The oxazine SMRS (Figure 1) allows us to sensitively and independently detect reducing and oxidizing agents through a change of the blinking kinetics.[7] Oxazine dyes such as ATTO655 exhibit a low energy of their first reduced state and a high energy of their first oxidized state (see scheme in Figure 1).[7] This asymmetry of redox potentials excludes the pathway through radical cations and reduces the efficiency of reoxidation of radical anions in the presence of reductants and oxidants so that blinking is observed under ROXS conditions in contrast to most other

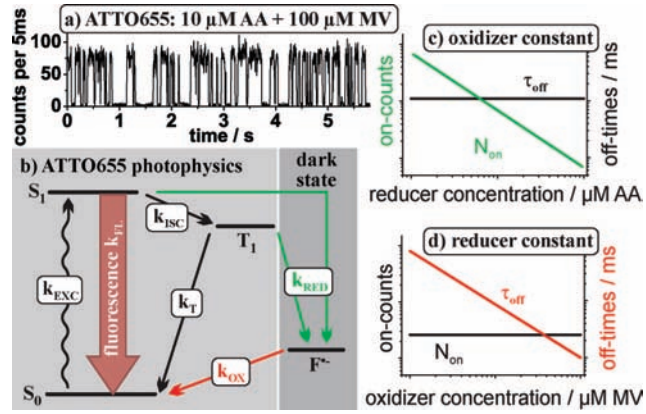

Figure 1. Schematic illustration of the single-molecule redox sensor (SMRS) using oxazine dyes. The left panels show a fluorescence transient of ATTO655 with pronounced blinking (a) and the energy levels and photophysical pathways (b) according to ref 7: the molecule cycles between S_0, S_1, and T_1 emitting N_{on} photons before entering a long-lived radical ion state $F^{\bullet-}$ via reaction with a reductant. In the presence of oxygen and a reductant, triplet states are so short-lived that they are not considered as off-states in single-molecule transients. Increasing concentrations of reductant (here ascorbic acid, AA) decrease the average on-counts N_{on}. Oxidizing agents (here *N*,*N*-methylviologen, MV) can depopulate the radical anion state and hence influence the off-times τ_{off}. (c) and (d) illustrate the sensing abilities: the on-counts are selectively modulated by the reductant (c), while the off-times are only influenced by the concentration of oxidizing agents (d).

dyes.[2,7] Accordingly, stable reduced states are formed when the first excited singlet (S_1) or triplet (T_1) state reacts with a reductant.[7] Such events are manifested as long-lived dark states in single-molecule fluorescence transients. The lifetime of the dark state is determined by the concentration of oxidant that recovers the oxazine back to its fluorescent singlet manifold. Hence, we recorded single-molecule fluorescence transients of immobilized double-stranded DNA labeled with ATTO655 in phosphate buffered saline (PBS) (as shown in Figure 1a). The number of photons emitted per on-time, N_{on}, and the off-state lifetime, τ_{off}, are the parameters that quantitatively report on the presence of reductants and oxidants (Figure 1c,d).

First we investigated whether an oxidizing effect of TX can be detected by measuring N_{on} and τ_{off} at different TX concentrations. As expected, the on-counts are reduced with increasing TX concentration (Figure 2a, black data points) representing the reducing properties of TX. The off-times are interestingly reduced as well, indicating that this TX sample has considerable oxidizing properties. Replacing TX by ascorbic acid, for example, yields a constant τ_{off} independent of concentration.[7] The indication of oxidative processes with gradually decreasing off-times implies that the geminate recombination mechanism (iv) is not efficiently active. Geminate recombination is a fast process that would quickly restore the fluorescent state without noticeable off-states in the single-molecule transient. This would involve a reduced number of off-

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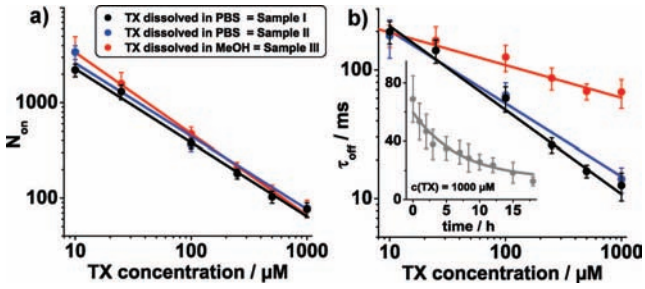

Figure 2. Dependency of the on-counts N_{on} (a) and off-state lifetime τ_{off} (b) on the concentration of different TX samples. The data were fitted using an allometric function of the form $f(x) = a \cdot x^b$ to guide the eye. The inset in panel (b) shows the temporal evolution of the off-times of sample III after dissolved in PBS (1 mM TX concentration).

times, whereas the off-time duration should remain constant. While our data cannot completely exclude that geminate recombination occurs to some small degree, it definitely can not account for the observed blinking reduction by TX, which would imply close to 100% yield of geminate recombination.

Next, we studied whether oxidizing impurities in the TX batch are the origin of its antiblinking capability by dissolving two different grades of TX (sample II: 97% purity as received from Sigma Aldrich; sample I: >98% purity with HPLC purification) in PBS (1 mM). Since TX is not very soluble in PBS, dissolving was carried out over ~18 h. The black data points in Figure 2 were obtained from sample I, and the blue data points, from sample II, yielding the same results within experimental error. If an impurity in the TX batch was the origin of the oxidizing properties, we had expected a systematic difference in the off-time dependency since it is unlikely that the oxidizing impurity had the same concentration in two different purity grades. We therefore exclude possibility (i).

To distinguish between mechanisms (ii) and (iii) we compared the properties of TX sample I to a TX sample that was prepared very freshly as a 100 mM solution in methanol (sample III). Methanol was used to circumvent the slow dissolving process in PBS. Control measurements were carried out to exclude the effect of methanol. Small amounts of this stock solution were added to PBS, and the concentration dependent on-counts and off-times were examined. For the very fresh TX sample, the on-counts show the same dependency on the concentration as observed for the TX dissolved in PBS over 18 h (Figure 2a, sample III, red data points). This proves that indeed the same TX concentrations were present and TX did not precipitate during the dilution of the methanol solution in PBS. The off-times, however, show that the oxidizing ability is drastically decreased for the fresh TX solution (Figure 2b) and only a weak reduction of off-times at higher TX concentration is observed. We conclude that a fraction of TX in sample I is oxidized during the slow 18 h dissolving process in PBS most likely due to reaction with oxygen, i.e., possibility (ii). This idea is further elaborated by recording fluorescence transients at a concentration of 1 mM TX (sample III) as a function of time after dissolving: the off-times gradually decrease as depicted in the inset of Figure 2b indicating the increasing oxidizing potential. Thus, TX essentially works according to the ROXS concept, and it is not required to assign unusual intrinsic oxidizing properties to TX (possibility (iii)). The difference between freshly prepared TX and partially oxidized TX samples was also found for the fluorescent dye ATTO647N. For this dye the close to complete removal of blinking by TX had already been demonstrated, evidencing the general importance of slow TX oxidation (see Supporting Information, Figure S3).[2]

These results are explained by the fact that, upon (photo-) oxidation, TX tends to form quinoid structures such as TX-quinone (TQ, Figure S4).[9] Comparing TQ with similar quinone derivatives in ref 10 suggests that the redox potential of TQ is on the order of −0.08 to −0.50 V, i.e., values that explain the observed reaction with the ATTO655 radical anion (E_{red} (ATTO655 vs SCE) = −0.42 V). With the known extinction coefficient of TQ,[9c] we estimate a concentration of ~25 μM of TQ after dissolving 18 h using UV−vis spectroscopy (Figure S4). The formation of TQ can also be induced by UV radiation.[9b] This was used to correlate TQ concentration with off-time reduction of SMRS transients as further proof of the concept elaborated here (Figure S4/5).

In summary, we used a single-molecule redox sensor that independently reports on reducing and oxidizing agents to investigate the mechanism by which the unusual antifading agent TX reduces blinking and photobleaching of single fluorescent dyes. Our data demonstrate that the antiblinking and antibleaching effect of TX is due to a combination of its reducing properties and a TX-quinone formed during the dissolving process in aerated PBS. In agreement with the ROXS scheme,[2] this oxidizing fraction depopulates reduced states and thereby minimizes blinking. The obtained unifying picture substantiates the potential of quenching triplet states via electron transfer reactions and recovering the singlet manifold via the complementary redox reaction. Very importantly, this work sheds light on many of the reports in the field that presented contradicting results concerning the efficiency and recommended concentrations of antifading agents (see ref 11 and references therein). Finally, a recipe for the reliable application of TX is provided that shows how to circumvent problems with fresh TX by determining the actual TX and TQ concentrations (see Supporting Information). For dye stabilization based on ROXS, this combination might be advantageous due to its lower toxicity.

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Supporting Information Available: Experimental methods and additional data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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