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Cy3 Photoprotection Mediated by Ni^{2+} for Extended Single-Molecule Imaging: Old Tricks for New Techniques

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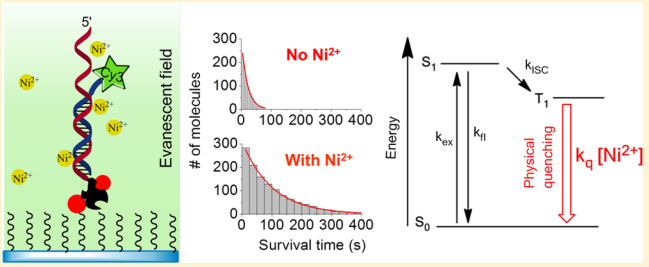
Supporting Information

ABSTRACT: The photostability of reporter fluorophores in single-molecule fluorescence imaging is of paramount importance, as it dictates the amount of relevant information that may be acquired before photobleaching occurs. Quenchers of triplet excited states are thus required to minimize blinking and sensitization of singlet oxygen. Through a combination of single-molecule studies and ensemble mechanistic studies including laser flash photolysis and time-resolved fluorescence, we demonstrate herein that Ni^{2+} provides a much desired physical route (chemically inert) to quench the triplet excited state of Cy3, the most ubiquitous green emissive dye utilized in single-molecule studies.

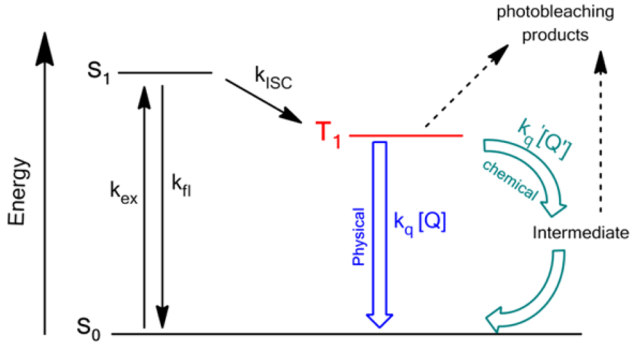


INTRODUCTION

Extended imaging times are of paramount importance in single-molecule fluorescence experiments in order to acquire relevant structural and dynamic information before photobleaching of a reporter fluorophore occurs.[1] A large number of strategies have thus been reported in order to increase the photostability of the different fluorophores employed.[2−5] These protocols mostly target the triplet excited state of a fluorophore.[6−12] Triplet states are undesirable, as they are long-lived and non-emissive, reducing the duty cycle of a fluorophore and leading to blinking under the rapid continuous excitation conditions prevalent in single-molecule fluorescence experiments.[1,4] Although triplet states are efficiently quenched by oxygen, this happens at the expense of sensitizing singlet oxygen, a strongly oxidizing species that may rapidly react with its sensitizer, leading to permanent photobleaching of the fluorophore.[13−15] Current strategies to enhance fluorophore photostability in single-molecule studies thus work via a two-pronged approach: reducing oxygen concentration, e.g., with an enzymatic oxygen scavenger preparation to prevent singlet oxygen sensitization, and introducing a triplet quencher to rapidly regenerate the fluorophore in its ground state, thus minimizing the undesired blinking and off-times characteristic of triplet states. The reduced triplet excited-state lifetime in the presence of a quencher further prevents singlet oxygen sensitization.

Importantly, most water-soluble triplet quenchers for single-molecule studies operate through chemical rather than physical routes (Scheme 1). A plethora of protocols have been reported relying on redox-active compounds that undergo photoinduced electron transfer with the triplet excited state of the fluorophores of interest, acting either as electron acceptors or electron donors and yielding, respectively, the fluorophore radical cation or radical anion. The resulting radical ions are long-lived transient species that are potentially very reactive and may lead to the photodegradation of the dyes. A cocktail combining both an oxidizing agent and a reducing agent (ROXS) has thus been shown to be optimal to scavenge the long-lived radical intermediates, regenerating the fluorophore in its ground state.[11] β-Mercaptoethanol (ME), a most popular "first-generation" single-molecule photostabilizer, has recently been shown to work also via photoinduced electron transfer from the thiolate form.[9] In the case of ME, rapid geminate recombination of counterions precludes the use of an oxidative counteragent and in turn minimizes the photooxidation of semioxidized intermediates. Thiols are thus optimal for green emissive fluorophores where photoionization of a radical anion might otherwise occur, yet they undergo photoaddition reactions with the highly extended conjugated vinyl linkers of

Scheme 1. Jablonski Diagram Illustrating a Physical Route and a Chemical Route To Quench a Triplet Excited State



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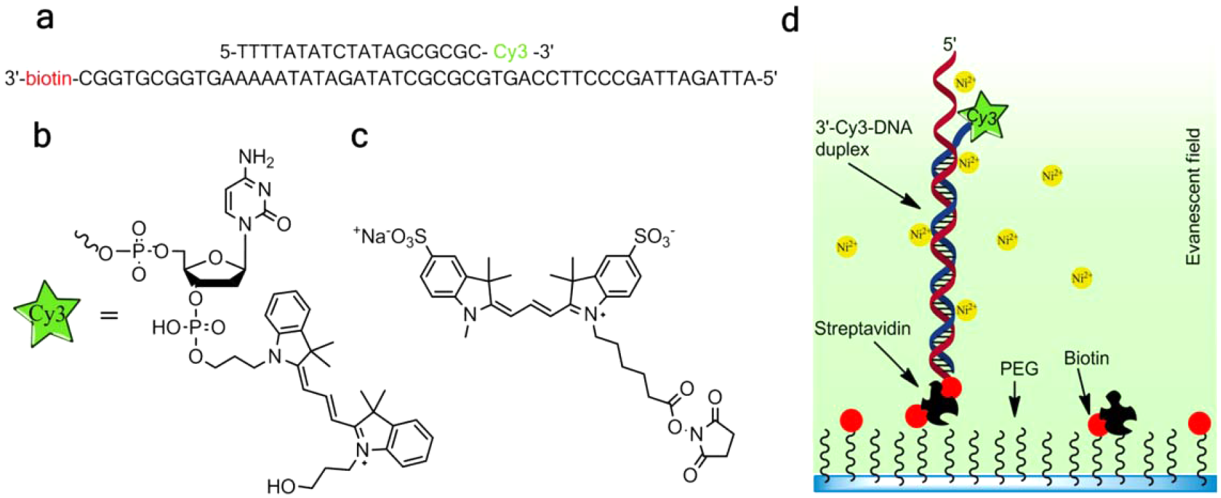

Figure 1. (a) Construct of Cy3-labeled DNA duplex used in our single-molecule and ensemble fluorescence studies. (b) Chemical structure of Cy3 attached to the 3′-terminal cytosine through phosphoramidite chemistry. (c) Structure of sulfo-Cy3 NHS ester used for mechanistic laser flash photolysis studies. (d) Cartoon illustrating the setup of the single-molecule experiment. The Cy3-DNA duplex is immobilized on a PEG-coated glass coverslip via biotin−streptavidin interactions, and different concentrations of Ni^{2+} are flowed with the imaging buffer (50 mM Tris pH 8, 40 mM NaCl); an evanescent field is utilized to excite the sample.

red-emissive cyanine dyes such as Cy5.[16] While advantageous toward super-resolution imaging, this is an undesired reaction in single-molecule imaging studies that rely on the steady signal from the fluorophore, e.g., single-molecule resonance energy transfer.

To date there has been little success implementing triplet quenchers that operate via a physical mechanism in single-molecule studies in aqueous media. For example, triplet−triplet (T-T) energy transfer is a convenient physical process to quench triplet excited states, as it does not involve a chemical reaction. Yet the relatively low energy of the first triplet excited state of most organic fluorophores that emit in the visible limits the choices of good triplet energy acceptors to highly conjugated organic molecules, which are in turn insoluble in aqueous media.[13] Recently, Blanchard et al. circumvented the solubility limitation by covalently tethering cyclooctatetraene (COT, a highly conjugated system with low triplet energy) to the fluorophore of interest via an alkyl linker.[6,8]

Upon revisiting the literature, we reasoned that we could alternatively exploit T-T energy transfer to ligand field states in the coordination complexes of transition metal ions such as Ni^{2+}.[17−19] Ni^{2+}, among other transition metal ions, has been previously reported to quench excited triplet states of various organic chromophores. We reasoned that old tricks learned from mechanistic studies conducted with benzophenone,[17] anthracene,[17,20,21] naphthalene,[22,23] porphyrins,[21] and azo dyes[18,24] could then be applied toward enhancing the photostability of single-molecule fluorophores in aqueous solution via a physical mechanism. Here we report on the photoprotection of Cy3, a common fluorophore for single-molecule studies, mediated by Ni^{2+} ions. We describe single-molecule fluorescence studies and mechanistic ensemble fluorescence and transient absorption studies that show that Ni^{2+} is a suitable photoprotective agent that operates via physical quenching of the triplet excited state of Cy3. Our studies show that photoprotection by Ni^{2+} ions, at concentrations in the hundred micromolar range, is comparable to, if not better than, that of ME at concentrations in the hundred millimolar range, providing a much desired chemically inert alternative to the standard chemically active triplet quenchers prevalent in single-molecule studies.

RESULTS AND DISCUSSION

Single-Molecule Fluorescence Studies. To evaluate Ni^{2+} as a potential triplet quencher and stabilizer for extended single-molecule fluorescence studies, we tested its effect on the photostability of Cy3 tagged to a surface-immobilized DNA duplex (Figure 1a,b). The complementary strand in the duplex was functionalized with a biotin moiety at the 3′ end to enable its immobilization on polyethylene glycol (PEG)-coated glass coverslips via biotin−streptavidin interaction. A total internal reflection fluorescence microscope (TIRFM) was used for sample excitation and imaging (see Figure 1d and Supporting Information for detailed experimental setup and methods).[25−27] Different concentrations of Ni^{2+} ions were flowed with an imaging buffer containing a glucose oxidase/catalase (GOX/CAT) oxygen scavenging system.[2] We quantified the effect of Ni^{2+} on the photostability of Cy3 by measuring the photobleaching time for each single molecule and next building an ensemble survival time histogram (Figure 2a). Fitting the histograms to monoexponential decay functions allowed us to obtain the average survival time for Cy3 under various different conditions and in the presence and absence of Ni^{2+} ions.

Comparison of the survival time histograms acquired in the presence of 0.1 mM Ni^{2+} and oxygen scavenger versus those recorded with oxygen scavenger alone (Figure 2a) reveals that Ni^{2+} substantially increased the photostability of single Cy3 dyes attached to DNA. Given that single molecules displayed on average the same intensity per unit time both with and without Ni^{2+} (see Figure 2b), the increased survival time directly translated into proportional increases in the total number of emitted photons and in the Cy3 photostability. At a Ni^{2+} concentration of 0.1 mM, we recorded a 5-fold increase in the average survival time of Cy3 compared to that under conditions with oxygen scavenger alone prepared with 6 units/mL of GOX and 800 units/mL of CAT (Figure 2c). No blinking was observed in the single-molecule intensity versus time trajectories recorded at 5 frames per second (200 ms time

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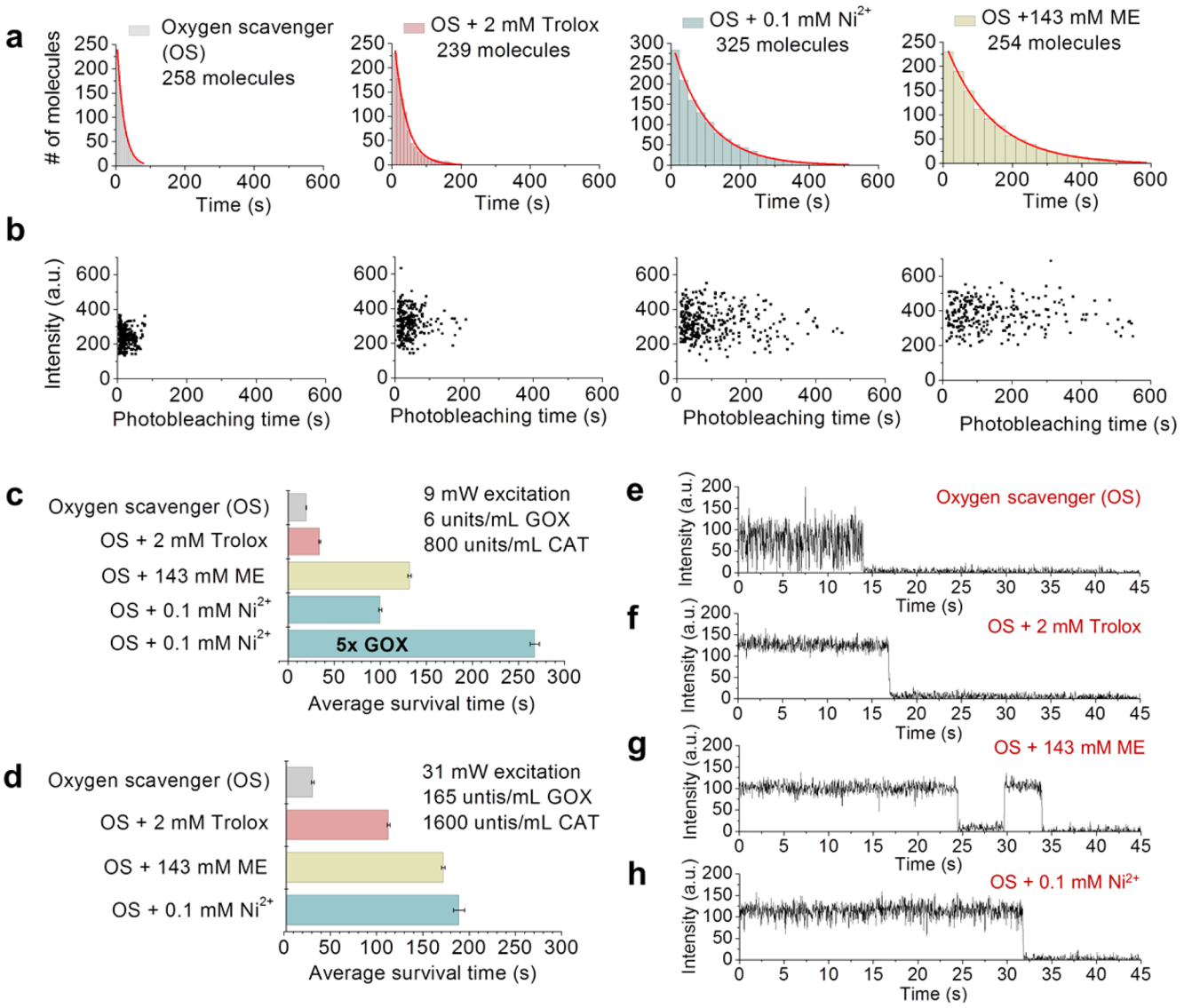

Figure 2. (a) Survival time histograms assembled from the photobleaching time recorded for the single molecules imaged in the TIRFM experiment. Imaging was conducted in the presence of oxygen scavenger and either no other additive, or 2 mM Trolox, or 0.1 mM Ni^{2+}, or 143 mM ME (going from left to right, respectively). (b) Average fluorescence intensity per unit time for single Cy3 molecules plotted versus their corresponding photobleaching times; conditions same as above. (c) Summary of the average Cy3 survival times (for data shown in panels a and b) when either no additive, or Trolox, or Ni^{2+}, or ME is used as triplet quencher. The data were acquired at low excitation powers (9 mW) and low enzyme concentrations (6 units/mL of GOX, 800 units/mL of CAT, unless specified otherwise). (d) Comparison of the effects of Ni^{2+}, Trolox, and ME on the photostability of Cy3 acquired at higher laser excitation powers (31 mW) and increased enzyme concentrations (165 units/mL of GOX, 1600 units/mL of CAT). All experiments were performed in the buffer containing 50 mM Tris-HCl, 40 mM NaCl, and 3% w/v of α-D-glucose. (e–h) Intensity versus time trajectories for a single Cy3-labeled DNA duplex at high excitation powers (conditions as in panel d) obtained with 30 ms time resolution.

bin). Concentrations of Ni^{2+} above 0.1 mM resulted in a decreased average intensity of Cy3, consistent with Ni^{2+} acting as a quencher of the singlet excited state (vide infra).

In order to compare our results obtained with Ni^{2+}, we also performed single-molecule photobleaching experiments with other commonly used triplet quenchers, including β-mercaptoethanol (ME, 143 mM) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 2 mM). These two triplet quenchers were used at those concentrations commonly reported in the literature.[12] Trolox was freshly prepared and contained no purposely oxidized fraction.[2,12] No additional advantages were observed using Trolox in a mixture with its oxidized form[28] (see Figure S1 for details). Figure 2c compares the photobleaching times for all three triplet quenchers used. In the presence of 2 mM Trolox, we observed only 1.7-fold enhancement in Cy3 photostability versus oxygen scavenger alone. This is consistent with previous reports where Cy3 photostability was enhanced 1–2.5-fold when Trolox was used a triplet quencher.[12] Trolox has been shown to have a large effect on the photostability of single-molecule dyes that absorb in the red region of visible light (such as Cy5), but only a marginal effect on Cy3 and other green fluorophores used for single-molecule studies.[9] The enhancement of Cy3 photostability obtained in the presence of ME was comparable to that in the presence of Ni^{2+} ions. When present at 143 mM (1% v/v), ME increased the Cy3 photostability around 6.5-fold.

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Importantly, a 3 orders of magnitude drop in the concentration of triplet quencher additive—143 mM for ME versus 0.1 mM for Ni^{2+}—yielded the same overall extended Cy3 lifetime.

Surprisingly to us, a 5-fold increase in the amount of glucose oxidase present in the oxygen scavenger system (up to 30 units/mL) led to an even higher (13-fold) enhancement of Cy3 photostability in the presence of Ni^{2+} ions (Figure 2c, bottom). A GOX/CAT oxygen-scavenging system has been shown to achieve a steady-state oxygen concentration within a few minutes;[29] thus, increasing the enzyme concentration should not have made any difference. This observation may indicate that our single-molecule chambers are not perfectly sealed, and it takes a longer time to achieve a steady-state oxygen concentration. A longer time required to achieve equilibration at lower enzyme concentrations was also observed by Stein et al.[30] Thus, care was taken to acquire all photobleaching data after the same time (20 min) had elapsed from the introduction of the oxygen scavenger to the imaging chamber. Overall, Ni^{2+} when present at 0.1 mM increased the photostability of Cy3 attached to DNA duplex 5−13-fold, depending on the amount of glucose oxidase (6 units/mL vs 30 units/mL, respectively) present in the imaging buffer, upon exciting at 532 nm at an excitation power of 9 mW (Figure 2c).

We further compared the effect of the above three triplet quenchers on the photostability of Cy3 at an increased laser power (31 mW). At these higher excitation powers, Ni^{2+} yielded significantly better intensity versus time trajectories for similar extended photostability when compared to ME. Higher enzyme concentrations (165 units/mL glucose oxidase, 1600 units/mL catalase), as suggested by a recent protocol,[2] were used in order to estimate the extended photostability of Cy3 that can be achieved at these increased excitation powers. Figure 2d shows that, under these conditions, 0.1 mM Ni^{2+} increased the photostability of Cy3 attached to DNA up to 6.5-fold compared to that with oxygen scavenger alone. Almost no blinking (less than 3% of the molecules) was observed in the single-molecule intensity versus time trajectories recorded at up to 33 frames per second (30 ms time bin). A similar increase in Cy3 photostability was obtained in the presence of 143 mM ME; however, in this latter case we observed fluctuations (blinking) in the Cy3 intensity versus time trajectories (see Figure 2, panel h for Ni^{2+} and panel g for ME, also Figure S2). Such blinking behavior, as shown in Figure 2g, was observed in approximately 30% of the molecules analyzed. At this higher excitation rate and increased enzyme conditions, Trolox increased the photostability of Cy3 by 3.6-fold, and it did not perform nearly as well as Ni^{2+}.

Ensemble Fluorescence Studies. It is known that sufficiently large concentrations of transition metal ions, including Ni^{2+}, may potentially quench a fluorophore singlet excited state. Therefore, we next determined the maximum concentration of Ni^{2+} that may be utilized toward enhancing Cy3 photostability by quenching the Cy3 triplet state without, however, significantly reducing Cy3 fluorescence intensity.[31−37] Steady-state and time-resolved fluorescence quenching studies were thus performed on the Cy3-tagged DNA construct (Figure 1a) with increasing concentrations of Ni^{2+}.

Figure 3 illustrates the results from our quenching studies. Here we correlated the increasing concentration of Ni^{2+} with the experimentally obtained decay rate constant for Cy3 fluorescence decay, calculated as the inverse of the intensity-weighted average fluorescence lifetimes, given that Cy3 exhibits biexponential decay kinetics with decay lifetimes of 0.49 ns

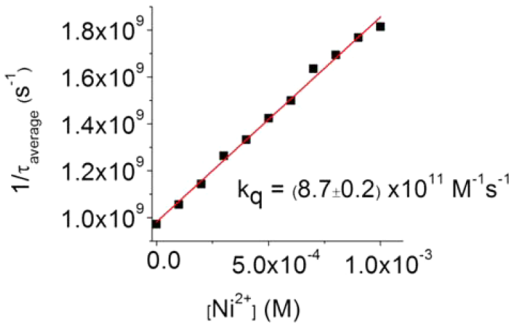

Figure 3. Linear correlation between Ni^{2+} concentration and the experimentally obtained decay rate constant recorded for Cy3 singlet excited state. Rate constants were obtained from the inverse of the intensity-weighted average fluorescence lifetime of Cy3 (50 mM Tris-HCl, 40 mM NaCl).

(39%) and 1.37 ns (61%) (similar results have been obtained for other Cy3-DNA constructs[38]). From these experiments, we conclude that Cy3 fluorescence is quenched by Ni^{2+} in a dynamic fashion, where the experimentally observed dynamic quenching rate constant is $k_q \cong 1 \times 10^{12}\ M^{-1}\ s^{-1}$. Given the above k_q value, under our experimental single-molecule imaging conditions with $Ni^{2+} \leq 0.1$ mM, less than 10% fluorescence quenching is expected.

Importantly, we may infer that the effective concentration of Ni^{2+} around Cy3 is much larger than that in bulk solution since the "apparent" k_q value estimated in Figure 3 (and based on the Ni^{2+} concentration in solution) is 2 orders of magnitude larger than the one expected for a diffusion-controlled reaction. The higher effective concentration is relevant to both Cy3 singlet excited-state quenching and reduced emission intensity, as discussed in the above paragraph, but also to Cy3 triplet excited-state quenching and the increased Cy3 photostability, as reported in this work. A high Ni^{2+} effective concentration is not surprising, as Ni^{2+} can bind to the negatively charged DNA backbone as well as coordinate to DNA bases.[39] We propose that the dynamic quenching is due to Ni^{2+} binding and hopping on the negatively charged DNA backbone, as has been shown by Atherton et al.[31]

Transient Absorption Studies. To demonstrate that the observed photoprotection of Cy3 by Ni^{2+} is the result of Cy3 triplet excited-state quenching by this ion, we subsequently performed mechanistic time-resolved absorption studies. We used aqueous solutions of sulfo-Cy3-NHS ester (Figure 1C) in lieu of Cy3-labeled DNA duplex because high concentrations as well as large sample volumes are required to visualize transient species in the laser flash photolysis (LFP) setup. Phenalenone (1*H*-phenalen-1-one) was chosen as a triplet sensitizer,[40,41] as it allowed us to minimize the direct excitation of Cy3: phenalenone absorbs at 355 nm, where Cy3 has negligible absorbance. Furthermore, since phenalenone is water-soluble, it enabled us to perform the LFP studies under conditions similar to those utilized in single-molecule experiments. Upon laser excitation, phenalenone undergoes rapid intersystem crossing to yield its triplet excited state (Figure S3).[41] In the presence of Cy3, it next undergoes T-T energy transfer, yielding in turn Cy3 in its triplet manifold (Scheme 2, steps 1 and 2). The effect of Ni^{2+} as a quencher of Cy3 triplet excited state was next studied and the quenching rate constant ($k_{q\text{-}T}$) determined (Scheme 2, step 3).

Figure 4a displays the transient absorption spectra acquired at different time intervals following excitation of phenalenone

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Scheme 2. Cy3 Triplet Sensitization by Photoexcited Phenalenone and Its Subsequent Quenching by Ni^{2+}

$$\text{Phenalenone} \xrightarrow{h\nu} \xrightarrow{k_{ISC}} {}^{3*}\text{Phenalenone} \quad (1)$$

$$^{3*}\text{Phenalenone} + \text{Cy3} \xrightarrow{k_{ET}} \text{Phenalenone} + {}^{3*}\text{Cy3} \quad (2)$$

$$^{3*}\text{Cy3} + Ni^{2+} \xrightarrow{k_{q\text{-}T}} \text{Cy3} + Ni^{2+} \quad (3)$$

in an Ar-saturated solution containing 5 μM sulfo-Cy3-NHS. A band centered at 490 nm due to the T-T absorption of phenalenone[40] is clearly observed in the first time window (see also Figure S3), which partially overlaps with the depletion band of the Cy3 ground state. Also observed is the bleaching of ground-state Cy3, with a prominent negative peak at 550 nm and the appearance of a new band centered at 640 nm that we assign to Cy3 T-T absorption in water. A similar transient was reported upon direct excitation in water of Cy3B,[42] a rigidified trimethine cyanine dye with a structure similar to that of Cy3. The temporal evolution of the 510 and 640 nm bands and the 550 nm bleaching is shown in Figure 4b at early times following excitation. It may be appreciated that the decay of phenalenone triplet occurs concomitant with the depletion of Cy3 ground state and the appearance of the Cy3 triplet state at 640 nm, consistent with the mechanism proposed in Scheme 2. The 5 μM Cy3 concentration employed ensured not only a rapid formation of Cy3 triplet to perform the next triplet quenching studies, but also a low enough concentration of Cy3 in the triplet manifold to minimize any effect on Cy3 triplet dynamics arising from, e.g., T-T annihilation.

Figure 5 displays the results following quenching of Cy3 triplet by Ni^{2+}, monitored at 640 nm under Ar-saturated conditions. The experimentally obtained decay rate constant for the Cy3 triplet excited state ($k_{exp\text{-}T}$) is observed to increase with increasing concentrations of Ni^{2+}, consistent with Ni^{2+} quenching this transient species (addition of Ni^{2+} did not affect the shape of the transient absorption spectrum of either ^{3}*Cy3 or ^{3}*phenalenone; see Figures 4a, S3, and S4). Under the pseudo-first-order kinetic conditions employed, one may obtain the triplet quenching rate constant ($k_{q\text{-}T}$) and the triplet decay rate constant in the absence of the quencher Ni^{2+} ($k^{o}_{dec\text{-}T}$) from the slope and intercepts, respectively, of the correlation between $k_{exp\text{-}T}$ and $[Ni^{2+}]$ (see Figure 5). We obtained values for $k^{o}_{dec\text{-}T} = 1.5 \times 10^{4}$ s^{-1} and $k_{q\text{-}T} = 4.6 \times 10^{7}$ M^{-1} s^{-1} (see also Figures S5 and S6 and discussion therein for data analysis). The dynamic quenching constant of Cy3 triplet by Ni^{2+} is 2 orders of magnitude smaller than for the diffusion-controlled reaction, pointing to an activation-controlled quenching mechanism. From the analysis of the above rate constants and upon inspection of Figure 5, we may realize that Ni^{2+} at a concentration of 0.1 mM is not an efficient triplet quencher for sulfo-Cy3-NHS. We would expect no more than 30% quenching for a DNA-free Cy3 triplet state under these conditions. However, and as discussed for the time-resolved fluorescence studies, the large effective concentration of Ni^{2+} when complexed to the DNA backbone ensures an efficient quenching by Ni^{2+} under single-molecule imaging conditions.

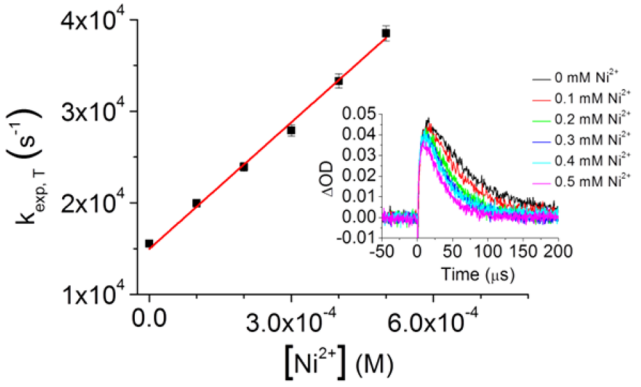

Figure 5. Linear correlation between Ni^{2+} concentration and the experimentally obtained decay rate constant recorded for Cy3 triplet excited state. Measurements were performed in Ar-saturated aqueous solutions. The inset shows decay traces recorded at 640 nm for Cy3 triplet excited state with increasing concentrations of Ni^{2+}.

It is interesting to consider the mechanism of triplet deactivation by Ni^{2+} ions and compare it with those of other triplet-state quenchers commonly used in single-molecule fluorescence studies. ROXS systems rely on depopulating triplet excited states by electron-transfer reactions with oxidants or reductants and next recovery of the radical ions that are formed. Such electron-transfer reactions, in the case of Ni^{2+} as a triplet quencher of Cy3, would be difficult, as neither Ni^{2+} ions nor Cy3 are a good reducing species; i.e., in the presence of Ni^{2+} ions, the triplet excited state of Cy3 may not act as either an electron donor or an electron acceptor. Furthermore,

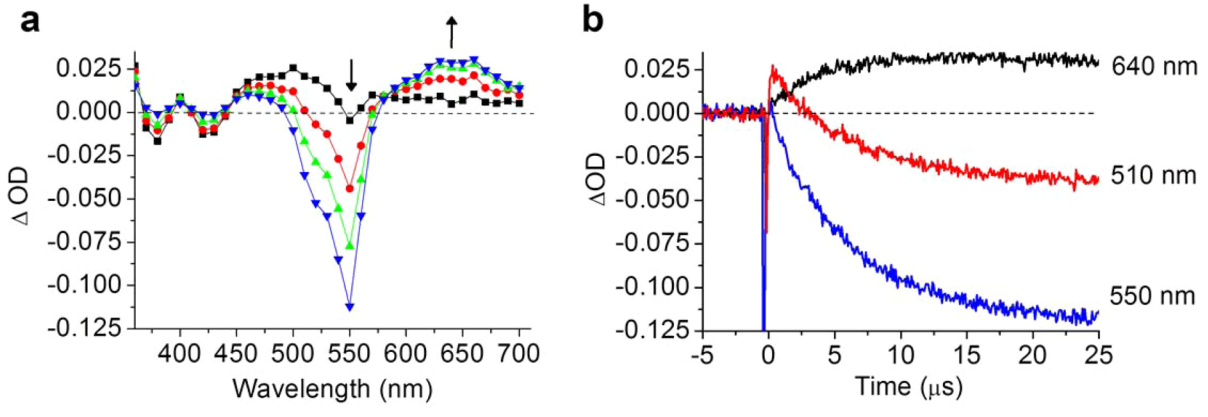

Figure 4. (a) Transient absorption spectra acquired upon 355 nm excitation of an aqueous solution of phenalenone (absorbance at 355 nm = 0.3) containing 5 μM sulfo-Cy3-NHS ester. Spectra were recorded (black ■) 1 μs, (red ●) 4 μs, (green ▲) 6 μs, and (blue ▼) 18 μs after excitation. (b) Temporal evolution of the absorbance recorded at 510, 550, and 630 nm.

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considering previous quenching studies of organic excited-state triplets by transition metal ions, including Ni^{2+}, Co^{2+}, Mn^{2+}, and Cu^{2+},[20,22,32] the quenching abilities of the metal ions do not correlate well with their ionization potentials. Similarly, no correlation seems to exist between the quenching rate constants and the atomic numbers of the ions or their multiplicity, as should be expected if spin−orbit coupling or magnetic perturbation occurred due to heavy-atom effect. A considerable number of previous studies suggest energy transfer via electron exchange as the most feasible mechanism for quenching of organic triplets by Ni^{2+} ions.[17,18,20,22,23,32] (However, other mechanisms could be plausible, as suggested by Butler and Pilling when noting that electron exchange may be difficult to unequivocally prove.[43])

Triplet-state quenchers that operate via a physical mechanism, such as Ni^{2+} or COT,[8] preserve intact the structure of the chromophore, as they do not rely on the photoinduced formation of long-lived, potentially reactive transient species as is the case for ROXS-based strategies. In addition, Ni^{2+} is compatible with redox-sensitive substrates, whereas the oxidizing and reducing agents employed in ROXS may lead to the chemical modification of the biomolecules under study, via thiol reduction (ME) or Michael addition (quinones).

We note that, for the system described in this work, where the Cy3 dye is attached to a DNA helix, a relatively low concentration of Ni^{2+} ions in solution is sufficient to achieve good photostabilizing results, given a high effective concentration of Ni^{2+}. One could even envision designing systems that have Ni^{2+} anchored in the close proximity to a dye, e.g., via a chelator such as nitrilotriacetic acid (NTA), similar to the concept of self-healing fluorophores.[10] Such a protocol would have an additional benefit, namely minimizing any toxicity in live cell studies potentially arising from free Ni^{2+}. Very recent work by Tampé and co-workers has demonstrated that ATTO565 or AlexaFluor 647 covalently labeled Ni-NTA can be successfully applied to target His-tagged proteins in live cells without any observable toxicity.[44]

Preliminary results in our group show similar levels of photoprotection for a lipid-soluble analogue of Cy3, DiI, where the cyanine dye backbone is decorated with long alkyl chains to ensure its anchoring to a lipid membrane (Figure 6a). When DiI is embedded within the membrane of liposomes prepared from the negatively charged lipid 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), the dye shows substantially enhanced photostability with sub-millimolar concentrations of Ni^{2+} (see Figure 6b). In turn, with liposomes prepared with the zwitterionic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), the effect of Ni^{2+} on photostability of DiI is not as large, and higher concentrations are required to achieve the same effect on photostability (Figure 6c). Regardless, these concentrations are vastly smaller than those required if, for example, ME is used. Considering the detrimental effect other small-molecule triplet quenchers may have on lipid membranes,[45] once again Ni^{2+} offers a desirable alternative for single-molecule studies relying on lipid membrane encapsulation or the study of membrane-bound biomolecules. It is important to emphasize that much higher concentrations of Ni^{2+} ion will be required to quench triplet states for systems where the concentration of Ni^{2+} around the fluorophore is that of bulk solution, i.e., no high effective concentration. Nevertheless, we believe that it is important to bring attention to Ni^{2+} as a suitable triplet-state quencher, whether it would be for photostabilization of single-molecule dyes or for other possible applications requiring suppressing triplet states.

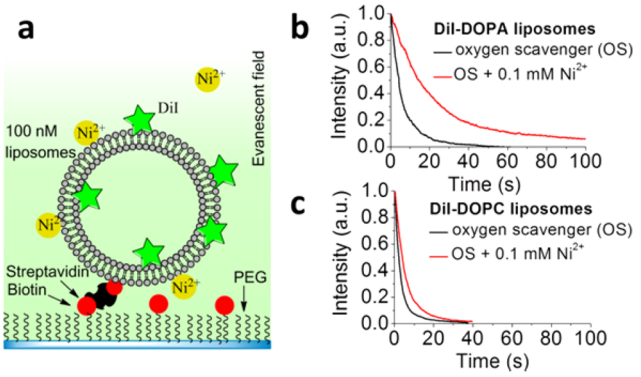

Figure 6. (a) Cartoon illustrating the setup of the single-molecule experiment conducted with liposomes stained with DiI, a lipid-soluble analogue of Cy3. Either DOPA or DOPC liposomes stained with DiI were immobilized to a PEG-coated glass coverslip via biotin−streptavidin interactions.[46] Different concentrations of Ni^{2+} were flowed with the oxygen scavenging buffer (50 mM Tris pH 8, 40 mM NaCl, 3% glucose, 30 units/mL GOX, 800 untis/mL CAT). The sum of the fluorescence intensity recorded for all liposomes within a time frame is plotted versus time in panels b (DiI-DOPA) and c (DiI-DOPC).

CONCLUSIONS

In conclusion, our results demonstrate that Ni^{2+} ions are efficient photostabilizing agents suitable for single-molecule fluorescence studies. The results obtained from transient absorption spectroscopy studies confirm that the stabilizing mechanisms involves quenching of the triplet excited state of Cy3 dye, plausibly through a physical pathway. To our knowledge, the beneficial effect of Ni^{2+} has not been brought to attention for applications in single-molecule fluorescence imaging. In contrast, transition metal ions are commonly regarded as typical fluorescence quenchers to avoid. We demonstrate that, if used at concentrations where singlet quenching is not prevalent, Ni^{2+} ions can efficiently quench triplet excited states, dramatically enhancing the photostability of a fluorophore without the formation of undesired radical intermediates. Ni^{2+} provides a much desired chemically inert alternative to Cy3 triplet excited-state quenching in single-molecule studies.

ASSOCIATED CONTENT

Supporting Information

Materials and experimental procedures including single-molecule imaging, single-molecule intensity versus time trajectories, and LFP studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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