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Rapid Exchange Luminescence: Nitroxide Quenching and Implications for Sensor Applications

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Sensitized terbium (Tb^{3+}) luminescence has become a very valuable tool in biotechnology applications.[1−3] Sensitization of the excitation process via energy transfer from a chromophore is needed for such applications in order to overcome the extremely low extinction coefficient of the ion itself. The major utility of sensitized Tb^{3+} luminescence stems from its long lifetime (ca. milliseconds), permitting easy time-gated detection and thus suppression of stray light, sensitizer and endogeneous fluorescence, and Raman scattering. Luminescent sensor applications are generally based on an equilibrium of the form $S + A \leftrightarrows S^*A$, where a nonemissive form, S, is converted to an emissive form, S*, on binding to an analyte, A. In such cases, the detection limit for A is limited by the equilibrium process $S \leftrightarrows S^*$, which contributes a background signal in the absence of A. Decreasing the equilibrium constant for this reaction so as to reduce the background level also reduces the affinity of the sensor for the analyte. The unwanted residual signal from S* and that from the analyte S*A will have very similar lifetimes in a fluorescence-based sensor. Here we show that due to dynamic averaging this will not be the case for a luminescence probe because the $S \leftrightarrows S^*$ interchange process is often much faster than the luminescence emission. Because of this, the use of time-gated detection can fully suppress the background from the $S \leftrightarrows S^*$ equilibrium and will thus permit very high sensitivity detection of the analyte A.

This dynamic effect on emission properties has previously been demonstrated for Eu^{3+} ions, where the excitation spectrum of the unsensitized emission of the ion is shifted due to complexation.[4,5] This involves $Eu^{3+}(L)_{n-1} + L \leftrightarrows Eu^{3+}(L)_n$-type equilibria, where the lifetimes of the two Eu^{3+} species differ. Slow exchange results in two decays with limiting values, rapid exchange results in a single average decay, and intermediate exchange results in two component decays whose amplitudes and lifetimes depend on the exchange rates. These fundamental observations are applied here to the analysis of a system that, because it involves sensitized excitation, has potential applications as a sensor species.

A luminescent species L may be quenched by addition of a species Q via either a collisional process or by formation of a complex LQ.[6,7] The former will exhibit Stern−Volmer behavior, while the second will saturate when [Q] exceeds the dissociation constant. For weak association, these two processes are difficult to distinguish. However, it is well-known that complex formation will have a larger effect on quenching as measured by steady-state processes than it does when measured by the effect of Q on the emission lifetime. Because of this, a Stern−Volmer plot for the steady-state quenching will exceed that for the lifetime decrease. Here we show that the reverse is observed due to rapid exchange for the case of quenching of a sensitized Tb^{3+} complex.

The system investigated here is the cs124−DTPA (carbostyril 124 diethylenetriamenepentaacetic acid) complex shown in Figure 1.[8] Edidin and co-workers have shown that the nitroxide TEMPO (TEMPO = 2,2,6,6-tetramethylpiperidinooxy, free radical) spin labels quench Tb^{3+} emission.[9] Their work involved steady-state and time-resolved measurements of Tb^{3+} complexed to EDTA. In this study, we present time-gated and time-resolved data for Tb^{3+}−cs124−DTPA/TEMPOL (4-hydroxy-TEMPO). The two studies show very similar Stern−Volmer quenching behavior for Tb^{3+}-complex/TEMPOL. In each study, there is a difference between the time-averaged and time-resolved results. The previous work dismissed this difference; our work is focused on the quantitative interpretation of this difference. This leads to different conclusions as to the mechanism of quenching that is of general interest.

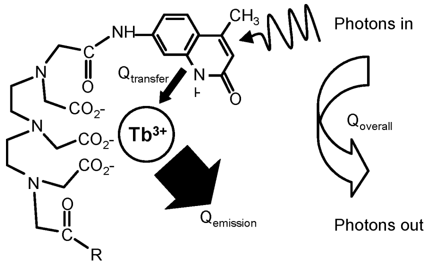

Figure 1. Tb^{3+}−cs124−DTPA chelate and mechanism of energy transfer.

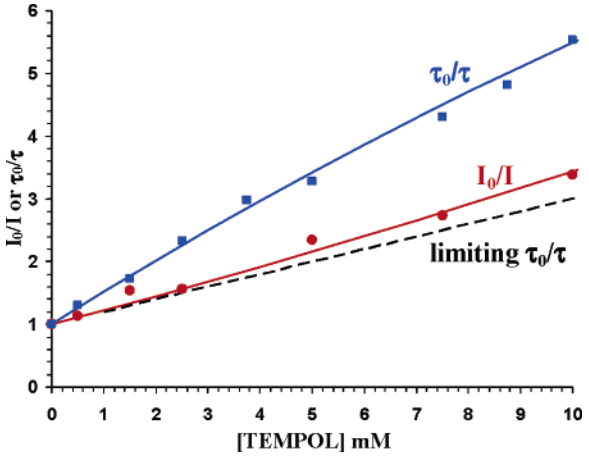

Figure 2. Stern−Volmer plot of Tb^{+3}−cs124−DTPA quenching by TEMPOL in 10 mM Tris buffer (pH 8). The time-averaged data were obtained by collecting a signal between 120 and 5000 μs.

Figure 2 is a Stern−Volmer plot of time-averaged (I_0/I) and time-resolved (τ_0/τ) quenching of Tb^{3+}−cs124−DTPA by TEMPOL. Figure 3 shows the individual time decays, where, in all cases, the data are adequately described by a single exponential.

The feature of interest is that the time-resolved quenching data of Figure 2 are higher than those for the time-averaged behavior. This result is in qualitative agreement with previously reported results for the Tb^{3+}/EDTA complex. However, in that work, the data were plotted in different figures, and it was only noted that the two results differed by 13%. No comment was made on the sign of the difference in that work. In our case, the time-resolved

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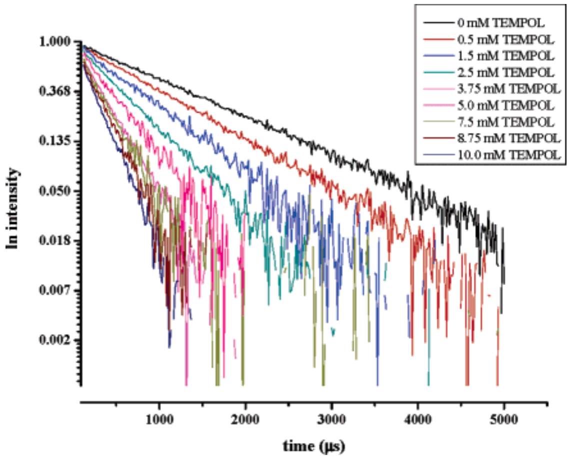

Figure 3. Time-resolved quenching of Tb^{3+}−cs124−DTPA by TEMPOL.

data are roughly a factor of 2 higher than the time-averaged data. The direction of this effect is opposite to that expected if static quenching is present. Inclusion of the small effect of inner and outer filtering or quenching of the sensitizing fluorophore would lower the I_0/I curve and thus enlarge this discrepancy.

A model developed to describe this result requires recognition of the importance of temporal averaging on the time scale of a chemical reaction. In this case, the chemical reaction in question is postulated to be formation of a complex between the terbium chelate and the quenching nitroxide. The notation $F + Q \leftrightarrows FQ$ with formation constant $K = [FQ]/[F][Q]$ is used. If the complex FQ is nonfluorescent, we have the usual static quenching situation. We propose here that the complex is fluorescent with a low quantum yield and a correspondingly short emission lifetime. This is the expected result for electron-transfer quenching in a complex where separation after transfer is precluded. In the usual case of short-lived fluorescence, this would result in elevation of the time-averaged Stern−Volmer data from the purely collisional value and introduction of a biphasic emission time dependence corresponding to the two species F and FQ. If the time decay of species FQ is too short to resolve or is simply ignored, then the concentration dependence of the observed long-time dependence reflects purely collisional quenching. This is, again, simply the usual case of static quenching modified to have a finite decay time for the complex.

What is new in the present case is that the time scale for the chemical interchange $F + Q \leftrightarrows FQ$ is shorter than the emission process. The relaxation rate, k_R, for a bimolecular reaction is the sum of the forward and reverse rates, $k_R = k_f\,[Q] + k_r$. This is on the order of microseconds for weak binding, very much longer than the usual nanosecond fluorescence, but very much shorter than terbium luminescence. The terbium ion complex, when excited, interchanges between free and complex states many times before emission, resulting in a single exponential decay with a dynamically average value. This behavior is reminiscent of diffusion-enhanced energy transfer observed for the same ion.[10]

The equation describing the τ_0/τ behavior in the rapid exchange limit is then simply $1/\tau = f_F\,(1/\tau_F) + f_{QF}\,(1/\tau_{QF})$, where f_F is the fraction of the species that is uncomplexed, $f_F + f_{QF} = 1$, and the decay times, τ_F and τ_{QF}, for each species are, in principle, subject to reduction due to collisional quenching, but the fast decay suggests that this will not be observable for the complex. This is consistent with the observations. The time-resolved (τ_0/τ) data and time-averaged (I_0/I) data in Figure 2 are fit by a rapid exchange model (see Supporting Information) with the Stern−Volmer quenching constant, $K_{SV} = 200\ M^{-1}$ for F and 0 for QF, an equilibrium constant $K = 20\ M^{-1}$, and a relative fluorescence of the FQ complex of 0.05. Parallel studies of 4-amino-TEMPO lead to $K_{SV} = 250\ M^{-1}$ and $K = 15\ M^{-1}$ with a relative fluorescence of 0.05 for the complex. The dashed line in Figure 2 shows the behavior expected for the time-resolved data if the emission rate were faster than the chemical relaxation rate. The steady-state data are above that line due to the contribution from static quenching. The elevation of the time-resolved data above the dashed line is due to the dynamical averaging effect observed and described here. The slight curvature of the τ_0/τ curve reflects the small value of K. The asymptotic value reached at very high quencher concentration is the reciprocal of the relative fluorescence, $1/0.05 = 20$.

The importance of these observations stems from their relevance to sensor design, as discussed above. Two conformational states of a sensor, S and S*, that are in rapid equilibrium will have a uniform, short lifetime and can be suppressed by time-gating so as to emphasize the presence of the long-lived S*A complex, which is kinetically isolated from these components.

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Supporting Information Available: Experimental procedures and equations, and their derivation. This material is available free of charge via the Internet at http://pubs.acs.org.

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