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Time Gating Improves Sensitivity in Energy Transfer Assays with Terbium Chelate/Dark Quencher Oligonucleotide Probes

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Abstract: Lanthanides are attractive as biolabels because their long luminescence decay rates allow time-gated detection, which separates background scattering and fluorescence from the lanthanide emission. A stable and highly luminescent terbium complex based on a tetraisophthalamide (TIAM) chelate is paired with a polyaromatic-azo dark quencher (referred to as a Black Hole Quencher or BHQ) to prepare a series of 5′TIAM(Tb)/3′BHQ dual-labeled oligonucleotide probes with no secondary structure. Luminescence quenching efficiency within terbium/BHQ probes is very dependent on the terbium−BHQ distance. In an intact probe, the average terbium−BHQ distance is short, and Tb → BHQ energy transfer is efficient, decreasing both the terbium emission intensity and lifetime. Upon hybridization or nuclease digestion, which spatially separate the Tb and BHQ moieties, the Tb luminescence intensity and lifetime increase. As a result, time-gated detection increases the emission intensity ratio of the unquenched probe/quenched probe due to the shorter lifetime of the quenched species. A 40-mer probe that has a 3-fold increase in steady-state luminescence upon digestion has a 50-fold increase when gated detection is used. This study demonstrates that time gating with lanthanide/dark quencher probes in energy transfer assays is an effective means of improving sensitivity.

Introduction

Lanthanide reporters, most commonly Tb^{3+} and Eu^{3+} complexes, are widely used for time-resolved measurements in diagnostic, research, and high-throughput screening assays.[1,2] The long emission lifetimes of lanthanides (milliseconds) eliminates background signal (from scattering and autofluorescence) that occurs on the nanosecond time scale by using time-gated detection. A wide variety of chelate structures and strategies for sensitizing lanthanide luminescence have been reported.[3,4] The present study uses a highly emissive and stable terbium complex based on a multidentate 2-hydroxyisophthalamide (TIAM) chelate.[5]

In most cases, conjugation of lanthanide chelates to oligonucleotides has been accomplished post oligonucleotide synthesis, with the notable exceptions of a chelate−phosphoramidite[6] and a europium cryptate−dUTP.[7] For sensitive, sequence-specific binding assays, Förster resonance energy transfer (FET or FRET) and, in the case of lanthanides, luminescence resonance energy transfer (LRET) are used to quench the luminescence of the unbound probe. The energy transfer efficiency is very dependent on the distance between the lanthanide donor and the acceptor dye. For efficient energy transfer, the absorption spectrum of the dye should have significant overlap with the emission lines of the lanthanide. In LRET assays, the acceptor dye is most often an organic fluorophore with a lifetime of nanoseconds. Upon Ln → dye energy transfer, the acceptor dye acquires the millisecond emission decay of the lanthanide.[8]

Many commercial assays such as real-time polymerase chain reaction (PCR) use dual-labeled oligonucleotide probes[9] that are covalently labeled at the 5′ end with a reporter dye and at the 3′ end with a quencher, preferably a dark quencher with no native emission. This report describes a series of 5′TIAM(Tb)/3′BHQ-2 dual-labeled probes (Figure 1). Upon nuclease digestion or hybridization, the increase in Tb emission intensity, both with and without time gating, and the corresponding increase in Tb emission lifetime are compared. It is shown that because the terbium to dark quencher energy transfer decreases not only the Tb luminescence intensity but also the Tb excited-state lifetime, gated detection can increase the dequenched/quenched emission intensity ratio and, therefore, assay sensitivity.

Results

Synthesis of the TIAM(Tb)/BHQ-2 Probes. The coordination chemistry and spectroscopic properties of this ligand

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(1) Mathis, G. *J. Biomol. Screen.* **1999**, *4*, 309−313.
(2) Dickson, E. F G.; Pollak, A.; Diamandis, E. P. *J. Photochem. Photobiol. B* **1995**, *27*, 3−19.
(3) Parker, D. *Coord. Chem. Rev.* **2000**, *205*, 109−130.
(4) Parker, D.; Williams, J. A. G. *J. Chem. Soc., Dalton Trans.* **1996**, 3613.
(5) Xu, J.; Melchior, M.; Petoud, S.; Raymond, K. N. To be submitted for publication.
(6) Hovinen, J.; Hakala, H. *Org. Lett.* **2001**, *3*, 2473−2476.
(7) Alpha-Bazin, B.; Bazin, H.; Guillemer, S.; Sauvaigo, S.; Mathis, G. *Nucleosides, Nucleotides Nucleic Acids* **2000**, *19*, 1463−1474.
(8) Chen, J.; Selvin, P. R. *J. Am. Chem. Soc.* **2000**, *122*, 657−660.
(9) Lee, L. G.; Connell, C. R.; Bloch, W. *Nucleic Acids Res.* **1993**, *21*, 3761−3766.

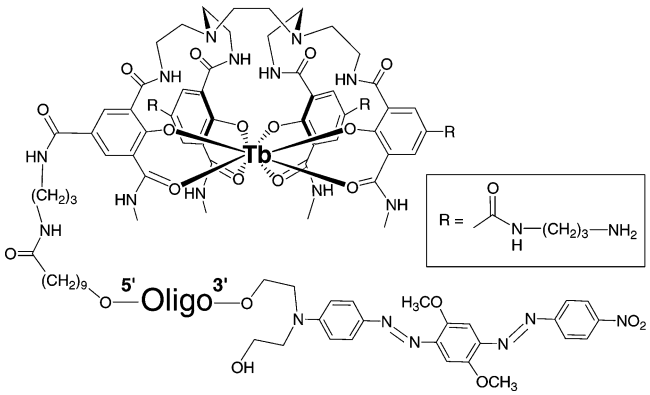

Figure 1. The 5′-TIAM(Tb) reporter and 3′-BHQ-2 dark quencher oligonucleotide probe.

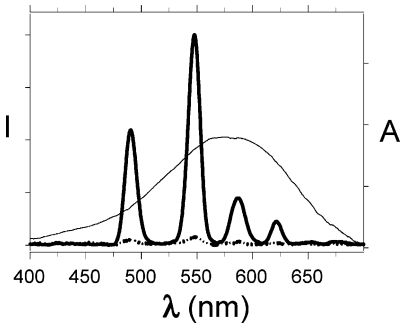

Figure 2. Spectral overlap of TIAM(Tb) emission with BHQ-2 absorption. Thick solid line, emission from 5′-TIAM(Tb) 10-mer; dashed thick line, emission from 5′-TIAM(Tb) 10-mer mixed with the complementary 5′-BHQ-2 probe; thin solid line, absorption spectrum of BHQ-2 labeled oligo.

system[4] and synthesis of the 2-hydroxyisophthalimide chelate have been reported elsewhere.[10] The dual-labeled probe shown in Figure 1 was synthesized by coupling the tetraamino TIAM ligand to a 5′-succinimidyl ester−oligo-3′BHQ-2 moiety that was still attached to the controlled pore glass (CPG) solid support. This conjugation was done via solid-phase synthesis to prevent cross-linking and also so that the excess TIAM could be washed away. The crude yield of the dual-labeled probe was estimated by HPLC to be 50%. All probes were dual-HPLC purified.

Digestion and Hybridization Assays with the Dual-Labeled 22-mer Probe. The absorption spectrum of BHQ-2 is overlaid with the luminescence spectrum of TIAM(Tb) in Figure 2. The significant spectral overlap indicates that the Tb → BHQ-2 energy transfer should be efficient. Scheme 1 depicts the hybridization and nuclease digestion assays that are used to investigate quenching within the dual-labeled probes, and Table 1 summarizes the corresponding data. The intact probe sample was split into three equal portions that were either left intact, digested, or hybridized. The same samples were used for both the intensity and lifetime measurements. Figure 3 shows the luminescence decay curves of the intact, digested, and hybridized probe samples. The decay of the intact probe closely matches the instrumental response curve in phosphorescence mode, and therefore only an upper limit of 2 μs can be determined for the lifetime.[11] A 5′-TIAM(Tb) 10-mer probe, with no BHQ-2, has an emission lifetime of 1.2 ms, while the

Scheme 1. Luminescence Is Released upon Hybridization or Enzymatic Digestion of a TIAM(Tb)/BHQ-2 Dual-Labeled Probe

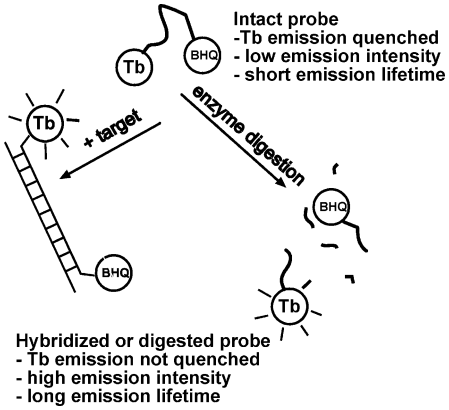

Table 1. Emission Lifetime and Intensity Data at 548 nm for the TIAM(Tb)/BHQ-2 22-mer Probe

sample	lifetime (μs)	rel steady-state em int	rel gated[a] em int
intact probe	<2	1	1
digested probe	800	94	250
hybridized probe	1600	140	670

[a] Delay 200 μs, gate 1600 μs.

digested and hybridized TIAM(Tb)/BHQ-2 22-mer probe samples have lifetimes of 0.8 and 1.6 ms, respectively. These decay curves were fit very well by single exponentials. The long lifetimes of the digested and hybridized 22-mer probe samples indicate that the BHQ-2 moiety is spatially far removed from the TIAM(Tb) and there is no quenching.

The increase in luminescence intensity upon hybridization of the 22-mer probe was higher than upon digestion in steady-state mode (140 and 94, respectively). It is expected that the hybridized probe, which has a longer emission lifetime, should also have higher luminescence intensity. When a delay of 200 μs and gate of 1600 μs were used, these ratios increased to 670 and 250 for the hybridized and digested probes, respectively. This increase in the nonquenched/quenched emission intensity ratio upon time gating can be attributed to the removal of the emission of the quenched probe. The emission of the quenched 22-mer probe, which has a lifetime of <2 μs, is removed by the delay time of 200 μs.

Dual-Labeled Poly-T Probes. The terbium luminescence of dual-labeled 5′TIAM(Tb)/3′BHQ-2 poly-T probes was investigated before and after nuclease digestion. The emission decay curves of dual-labeled T_{30} probe (with 30 T bases) and T_{40} probe (with 40 T bases) are multiexponential with average lifetimes of 16 and 42 μs, respectively (Table 2 and Figure 4). Thus, the average emission lifetime within the dual-labeled probes increases with probe length and the average TIAM(Tb)−BHQ-2 distance. The multiexponential decay shows that there is a range of TIAM(Tb) quenching rates and therefore a distribution of TIAM(Tb)−BHQ-2 distances within each probe. This is presumably due to the flexibility of the poly-T sequence. Upon nuclease digestion, the TIAM(Tb) emission decay of both samples is fit well by a single exponential giving a lifetime

(10) Petoud, S.; Cohen, S. M.; Bünzli, J.-C. G.; Raymond, K. N. *J. Am. Chem. Soc.* **2003**, *125*, 13324−13325.

(11) The emission intensity from the intact probe sample at 548 nm is very weak and below the detection limit of the PTI lifetime instrument in nanosecond mode which has limited excitation energy from a nitrogen-filled flashlamp.

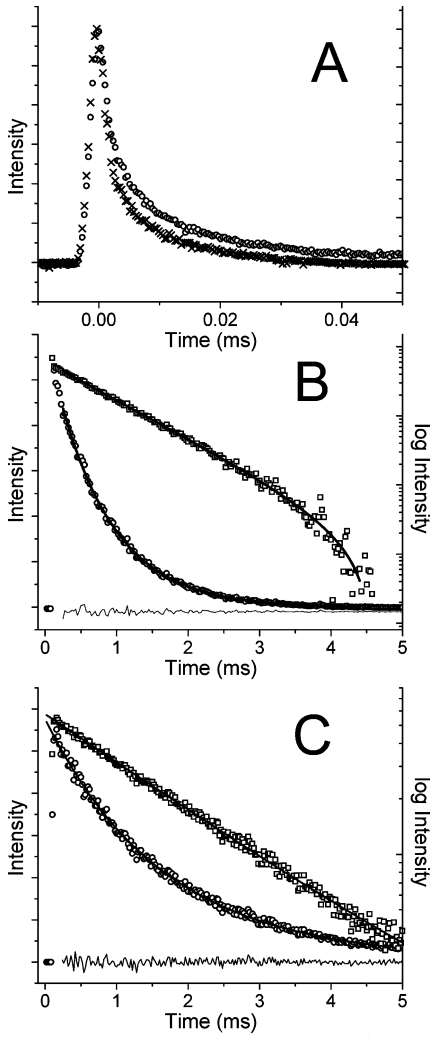

Figure 3. Luminescence decay at 548 nm for TIAM(Tb)/BHQ-2 22-mer probe samples. (A) Intact probe. Decay shown as circles and instrumental response shown as crosses. (B) Digested probe and (C) hybridized probe. Decay shown as circles in linear scale and as squares in log scale. Residuals and single-exponential fit shown as lines.

Table 2. Effect of Nuclease Digestion on Luminescence of TIAM(Tb)/BHQ Probes

sample	av em lifetime (μs)	int increase upon digestion, no gating	int increase upon digestion, with gating[a]
22-mer	<2	94	250
T_{30} probe	16	10	90
T_{40} probe	42	3	50

[a] Delay 200 μs, gate 1600 μs.

of 970 μs. This indicates that upon digestion the TIAM(Tb) and BHQ-2 are well separated and that no energy transfer occurs.

The increase in luminescence intensity without gating upon digestion is 10 and 3, respectively, for the T_{30} and T_{40} probes. This correlates with the lifetime data because the T_{40} probe which has less efficient Tb → BHQ-2 energy transfer should therefore have a lower increase in luminescence upon digestion. When a delay of 200 μs and a gate of 1600 μs are used, the increase in luminescence intensity is 90 and 50, respectively, for the T_{30} and T_{40} probes. Thus, the increase in luminescence when gated detection is used instead of steady state is more dramatic with longer probes.

Table 3. Effect of Nuclease Digestion on Steady-State Fluorescence of FAM/BHQ-2 Poly-T Probes

sample	increase in fluorescein fluorescence
T_{20}	4.9
T_{30}	2.2
T_{40}	1.6

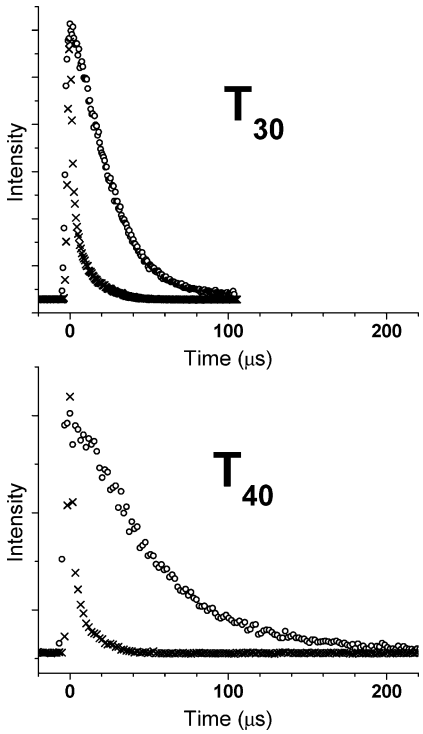

Figure 4. Luminescence decay at 548 nm for 5′TIAM(Tb)/3′BHQ-2 dual-labeled T_{30} and T_{40} probes. Decay shown as circles and instrumental response shown as crosses.

For comparison, the quenching within a series of fluorescein (FAM)/BHQ-2 poly-T probes (with 20, 30, and 40 T bases) was studied via nuclease digestion. The fluorescein reporter and BHQ-2 quencher are routinely used as a pair in fluorogenic dual-labeled oligonucleotide probes. As the sequence length and average FAM−BHQ-2 distance increase, the fluorescence released upon nuclease digestion decreases (Table 3). The digested probes all presumably have the same fluorescence intensity; therefore, the magnitude of the released fluorescence is due to the efficiency of the FAM−BHQ-2 quenching in the intact probes and, as expected, shorter probes have more efficient quenching. Even in steady-state detection mode, the terbium T_{30} and T_{40} probes have larger emission intensity increases upon digestion than the corresponding FAM probes.

Discussion

The luminescence intensity and lifetime of a terbium reporter has been studied in a series of dual-labeled oligonucleotide LRET probes. The luminescence decays of all of the dequenched samples (digested by nuclease, singly labeled 5′-TIAM(Tb) 10-mer probe, and hybridized) were fit very well by single exponentials. This shows that the TIAM(Tb) reporters within each sample experience the same environment. The emission lifetimes of these samples increase as there are more oligonucleotides near the TIAM(Tb). The oligonucleotide bases are not contributing to the sensitization of Tb because the

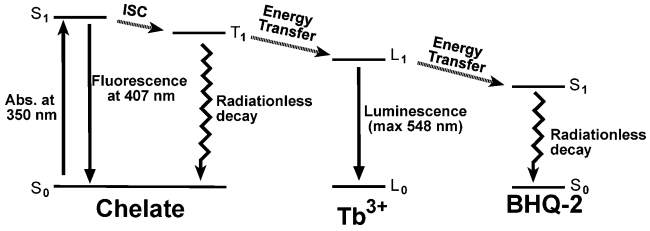

Figure 5. Electronic energy level diagram for the lanthanide chelate/BHQ-2 probe.

excitation wavelength is 350 nm. Perhaps the oligonucleotide bases shield the terbium ion from quenching by outer sphere water molecules.[3,12]

The photophysics occurring within the probe samples is depicted in Figure 5. Absorption at 350 nm generates the chelate excited state, which can return to the ground state through fluorescence at 407 nm; alternatively, a triplet state of the chelate is populated through intersystem crossing. It is well-known that sensitization of lanthanides occurs from triplet states; for terbium, which has a main emission energy level at 20 400 cm^{-1}, the triplet level of the sensitizer must be at least 3500 cm^{-1} higher in order to make energy transfer to the terbium fast and irreversible.[13] Other lanthanide biolabels generally have an organic sensitizer separate from the lanthanide chelate. The dual-function TIAM chelate is also the sensitizer; this should increase chelate to terbium energy transfer due to their close proximity and orbital overlap. After population of the terbium excited state, luminescence can occur; alternatively, if the BHQ-2 is nearby, energy transfer through the Förster mechanism can occur. BHQ-2, an aromatic azo dye, has very efficient radationless decay and no detectable fluorescence, and is therefore a dark quencher.[14]

Karvinen et al. recently studied a series of Eu−acceptor dye pairs on peptides in assays with hydrolyzing enzymes. Some Eu−acceptor pairs showed efficient quenching even with minimal spectral overlap; ionic interaction between the positive charge of the quencher and negatively charged chelate is an alternative quenching mechanism that is possible within flexible substrates.[15] According to this ionic interaction mechanism, one would expect a quenched conformer with a well-defined lifetime. In the TIAM(Tb)/BHQ-2 T_{20} and T_{30} probes, a range of lifetimes and quenching efficiencies was observed. Thus, a through-space energy transfer mechanism such as LRET accounts for the quenching of TIAM(Tb) by BHQ-2 in the dual-labeled probes.

The multiexponential decay curves suggest that each intact dual-labeled probe has a range of conformers and TIAM(Tb)−BHQ-2 distances. A large range of probe conformations may be sampled during the long excited-state lifetime of a TIAM-(Tb) reporter, and this would increase the possibility of quenching interactions with BHQ-2.[16] In the digested probe sample, only intermolecular quenching interactions are available. [A 10^{-6} M solution has an average intermolecular distance of 1200 Å, far beyond the distances of efficient LRET quenching.]

The Tb → BHQ-2 quenching efficiency decreases with increasing probe length and longer Tb−BHQ-2 distance in the 22-, 30-, and 40-mer 5′TIAM(Tb)/3′BHQ-2 probes. This is observed via the increasing TIAM(Tb) emission lifetimes and decreasing change in luminescence upon probe digestion. When digestion of a TIAM(Tb)/BHQ-2 40-mer probe is monitored by steady-state emission, the signal increases only 3-fold. [The corresponding signal increase for a 5′FAM/3′BHQ-2 40-mer probe was 1.6.] Such minimal changes in emission intensity limit sensitivity and precision.

Time gating increases this ratio of emission intensities because only the long-lifetime emission is sampled. Signal originating from the quenched probe is removed because it has a lifetime shorter than the 200 μs delay time. The signal increase upon probe digestion for steady-state vs gated detection is more dramatic with longer probes. Thus, time gating increases the sensitivity in energy transfer assays, especially in cases where the quenching efficiency is modest.

Conclusions

The efficient quenching of terbium luminescence by BHQ-2 not only decreases the emission intensity but also shortens the Tb excited state lifetime. Time-gated detection is widely used to separate long-lived lanthanide luminescence from short-lived background scattering and fluorescence. In an LRET assay, the change in Tb luminescence intensity indicates that an event such as hybridization or digestion has occurred. This report shows that the change in the luminescence intensity in such assays is increased with time-gated detection. This is due to removal of short-lived luminescence originating from the quenched species. Thus, time gating can be used to increase the sensitivity of LRET assays with lanthanide/dark quencher probes.

Experimental Section

Preparation of Oligonucleotide Probes. DNA sequences were made with a Biosearch 8700 DNA synthesizer using standard phosphoramidite reagents. The 5′ Tb 10-mer sequence is 5′Tb-d-CCT-AGA-GTG-G-3′. The DADE phosphoramidite (Trilink) was used to obtain a 5′-succinimidyl ester still attached to the CPG solid support. The tetra-amino Tb chelate (1.5 mg), dissolved in 0.2 mL of DMF with 15 μL of *N*-methylmorpholine, was passed over the solid support using two syringes over 12 h. After the reaction solution was rinsed away with DMF, the sample was treated with concentrated ammonia at 60 °C for 1.5 h to cleave and deprotect the oligonucleotide. During this step, the Tb ion dissociates from the chelate. In pH neutral buffer, the Tb ion is later inserted via terbium citrate.

The dual-labeled 22-mer probe sequence is 5′-d-CGA-TTC-TTC-ACA-CCA-TGT-TCA-G-3′. The complementary sequence was made with three extra T bases on each end. To make dual-labeled probes, BHQ-2 CPG solid support (Biosearch Technologies) was used. The 5′-FAM sequences were prepared using 6-carboxyfluorescein−aminohexyl amidite (Biosearch Technologies). All synthetic DNA samples were dual-HPLC purified by anion exchange (Dionex DNA Pac PA-100 column; solvent A, 0.038 M tris[hydroxymethyl]aminomethane, 15% acetonitrile; solvent B, solvent A with 1 M NaBr) followed by reversed-phase HPLC (Hamilton PRP-1 column; solvent A, 0.1 N TEAA; solvent B, acetonitrile). Sample purity was confirmed by

(12) Beeby, A.; Clarkson, I. M.; Dickins, R. S.; Faulkner, S.; Parker, D.; Royle, L.; de Sousa, A. S.; Williams, J. A. G.; Woods, M. *J. Chem Soc., Perkin Trans. 2* **1999**, 493−503.
(13) Steemers, F. J.; Verboom, W.; Reinhoudt, D. N.; van der Tol, E. B.; Verhoeven, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 9408−9414.
(14) Johansson, M. K.; Fidder, H.; Dick, D.; Cook, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 6950−6956.
(15) Karvinen, J.; Laitala, V.; Mäkinen, M.-L.; Mulari, O.; Tamminen, J.; Hermonen, J.; Hurskainen, P.; Hemmilä, I. *Anal. Chem.* **2004**, *76*, 1429−1436.
(16) Probe conformational changes are on the order of microseconds: Bonnet, G.; Krichevsky, O.; Libchaber, A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8602−8606.

analytical anion-exchange and reversed-phase HPLC. Micrococcal nuclease (USB) was used for the nuclease digestion assay.

Spectroscopic Measurements. All measurements were made in a buffer solution of 10 mM trizma hydrochloride, 50 mM KCl and 3.5 mM $MgCl_2$. Absorption spectra were recorded using an HP 8452 diode-array spectrophotometer. Fluorescence measurements were obtained with a Perkin-Elmer LS50b luminescence spectrometer and a Molecular Devices SpectraMax Gemini spectrofluorometer. Excitation at 488 nm, a 495 nm cutoff filter, and emission at 520 nm were used with the fluorescein probes. Excitation at 350 nm, a cutoff filter at 420 nm, and emission at 548 nm were used with the TIAM(Tb) probes. Additional fluorescence measurements were made using an S2000 Ocean Optics spectrometer with a CCD-array detector, PX-2 pulsed xenon lamp, and DT-1000 deuterium tungsten halogen light source.

Luminescence decay measurements were performed using a PTI TimeMaster spectrometer. Phosphorescence decay measurements were made using excitation from a pulsed xenon source (Xenoflash) passed through a monochromator at 350 nm onto the sample. Emission was channeled through a monochromator at 548 nm into a stroboscopic PMT detector. Instrumental response curves were generated using a scattering sample of dilute nondairy creamer and the emission wavelength of 350 nm.

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