Lifetime- and Color-Tailored Fluorophores in the Micro- to Millisecond Time Regime

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Abstract: We report the development of compound dyes which have tunable emission wavelengths, from 520 to 680 nm, and simultaneously tunable excited-state lifetimes, ranging from 50 to 500 μs. Future work is expected to extend these ranges yet further. The compound dyes are made of a lanthanide donor with a millisecond lifetime, which transfers energy to an acceptor with nanosecond lifetime, the latter made from an organic fluorophore. By varying the distance between the two, energy transfer from donor to acceptor results in a variable donor lifetime and emission, primarily from the acceptor. Emission color of the complex is therefore determined by choice of acceptor, and the lifetime decay of acceptor emission following an excitation pulse is determined by the donor's lifetime, which feeds the acceptor's delayed emission. By utilizing both the time-domain and wavelength-domain, these probes have the potential for quadratically increasing the number of detectable probes on a single sample.

Multi-color fluorescence is widely used in biosciences for the simultaneous or sequential detection of multiple targets. By means of conventional techniques, approximately five different colors can be detected on a single sample, and by using combinatorial methods, detection of more than 24 pseudo-colors on a single sample has been achieved.[1] Multi-lifetime fluorescence is another possible method for discrimination of signals.[2] However, the excited-state range of organic-based fluorophores is limited, generally in the 1−10 ns range, and not systematically tunable. Metal−chelate complexes can extend the lifetime regime to microseconds,[3] and lanthanides can have millisecond lifetimes.[4] These long-lived probes have the advantage that background fluorescence, which is typically nanosecond in lifetime, can be readily discriminated against. Another advantage is that instrumentation is considerably simpler in the micro- and millisecond time regimes than in the nanosecond regime. However the number of long-lived luminescent probes is limited, and millisecond probes have inherently limited emission rates (~1000 photons/sec/molecule).

Recently it has been shown that using compound dyes in which a fluorescent donor molecule transfers energy to a nearby acceptor dye can yield enhanced emission characteristics including a single excitation wavelength and multiple emission wavelengths.[5] To date, enhanced lifetime characteristics have not been shown. In this contribution we report the development of compound dyes which have both tunable emission wavelengths, from 520 to 680 nm, and simultaneously tunable excited-state lifetimes, ranging from 50 to 500 μs. Future work is expected to extend these ranges yet further. We call these lifetime and color-tailored fluorophores (LCTF). By utilizing both the time-domain and wavelength-domain, these probes have the potential for quadratically increasing the number of detectable probes on a single sample.

To construct an LCTF, a lanthanide donor molecule (lifetime $\tau_o \approx$ msec in the absence of acceptor) transfers energy to an organic acceptor fluorophore, which then re-emits (Figure 1). The color and lifetime of this re-emission (called sensitized emission), are tunable. The color is tunable because, when energy transfer (E) is chosen to be large, emission color is largely determined by the acceptor fluorophore, which can be chosen for the desired emission color. The lifetime of the complex is tunable because it is determined by the amount of energy transfer between the donor and acceptor. Specifically, the donor's lifetime (τ) is reduced by energy transfer $\tau = \tau_o (1 - E)$. The acceptor has an intrinsic fluorescence lifetime of a few nanoseconds, but because it is continually being excited via energy transfer from the donor, its emission intensity decays with a lifetime that follows the donor's lifetime, τ. The lifetime is readily tuned by altering the distance (R), and hence energy transfer efficiency, between donor and acceptor: according to Förster's theory, which we have shown to be applicable to lanthanide-based resonance energy transfer,[6,7] $E = (1 + R/R_o)^{-1/6}$, where R_o depends on the spectral and emission properties of the donor−acceptor pair and is the distance at which energy transfer is 0.5. As proof of principle of these lifetime and color-tailored compounds, here we use oligonucleotide duplexes as a rigid, yet distance-adjustable scaffold, for donor−acceptor pair attachment. The DNA also acts as a means of attaching

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a

5'-Ln-(CH$_2$)$_6$-N$_1$...N-Fl-N-N$_{20}$...-3'
3'-N$_1$...N-N-N-N$_{20}$...-5'

b

5'-Ln-(CH$_2$)$_6$-N...N-N-N-3'
3'-N...N-N-N-R-5'

R = Fl or Cy5
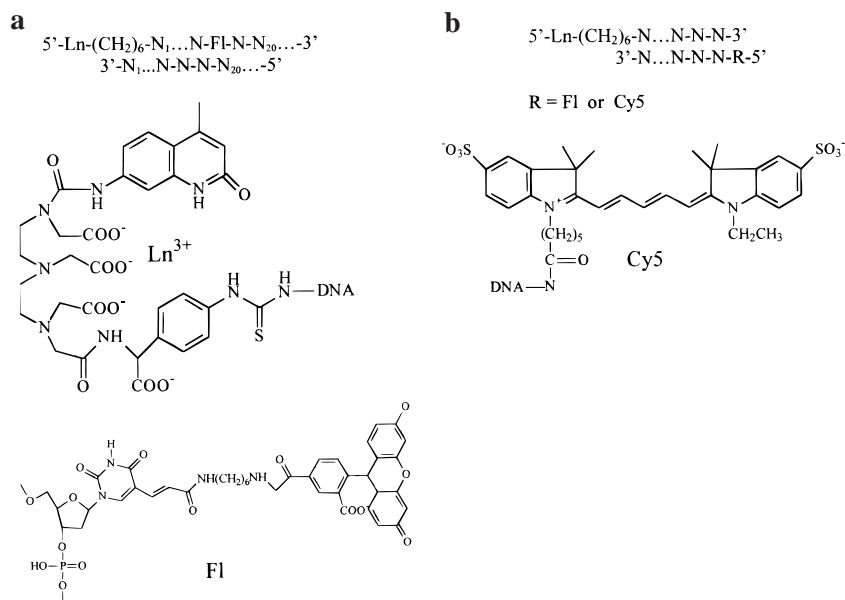
Ln^{3+}

DNA

Cy5

Fl

Figure 1. Structure of LCTF made from a single (a) DNA strand or complementary pair (b) of DNA strands. The lanthanide donor chelate is placed on the 5'-end of the DNA. The fluorescein acceptor is internally labeled a number of bases (here either 6, 8, or 10 bases) away from the donor on the same strand (a) or placed on the opposite complementary strand (b), shown here at the 5'-position. The acceptor is fluorescein in (a), an acceptor for terbium, and CY5, an acceptor for europium, is used in (b).
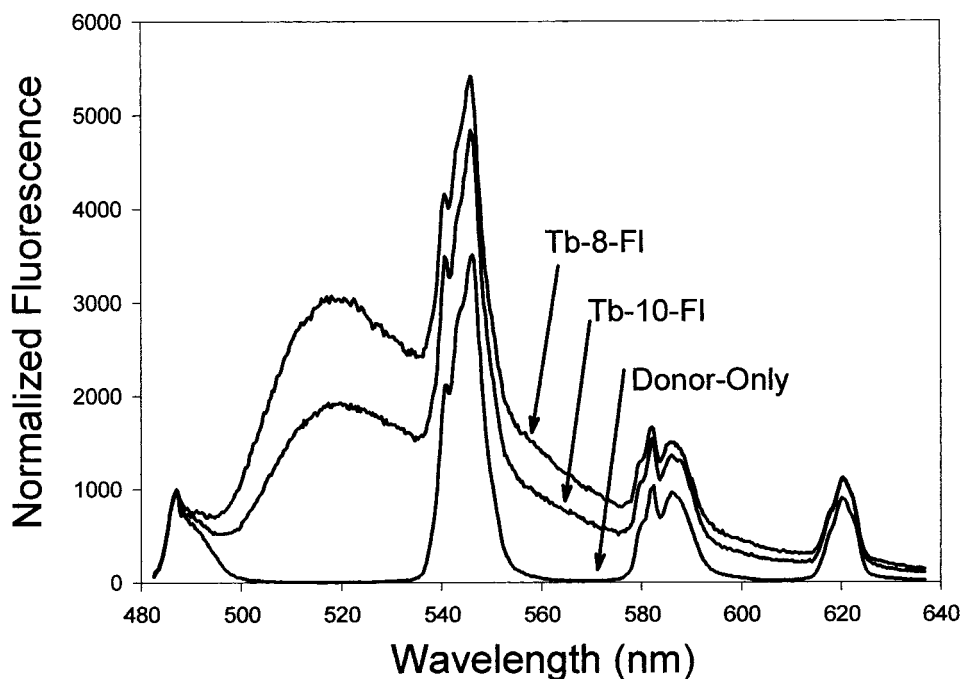

Figure 2. Time-delayed emission spectra of terbium-fluorescein LCTF placed on the same DNA strand, separated by either 8 or 10 base pairs, and hybridized to unlabeled complementary strand. Emission is collected 30 μs after the excitation pulse; all fluorescein emission (peak at 520 nm) is due to energy transfer from terbium. Energy transfer is larger for the Tb-8-Fl, yielding a larger amount of fluorescein-sensitized emission.

(hybridizing) the LCTF to target DNA. In principle, the potential exists for making LCTFs for attachment to protein and other targets.

We have attached donor−acceptor pairs to DNA in two ways. One method links a lanthanide chelate donor on the 5'-end of a DNA strand and a fluorescein acceptor in the middle of the *same* strand via C-5 deoxyuridine tethering (Figure 1a). In this method, by choosing an appropriate DNA sequence, the LCTF can bind and label a unique complementary DNA sequence (in a target DNA, for example) with a probe of defined color *and* lifetime−for example, in fluorescence in situ hybridization. The other method (Figure 1b) tethers a lanthanide chelate donor on

the 5'-end of one DNA strand and places the acceptor on the 5'-end of a complementary strand, as described previously.[6−8] This attachment mechanism may be useful in chip-based DNA diagnostics and sequencing, because an oligonucleotide can be conveniently synthesized from a solid support with an incorporated acceptor fluorophore, or the support itself may contain an acceptor. The lanthanide chelate, DTPA-cs124 (diethylenetriaminepentacetic acid-carbostyril 124) has previously been shown to be an excellent energy-transfer donor when bound to terbium or europium.[6,7] The donor-only lifetimes are 1.5 ms for Tb^{3+} and 0.62 ms for Eu^{3+} in H$_2$O-based solutions.

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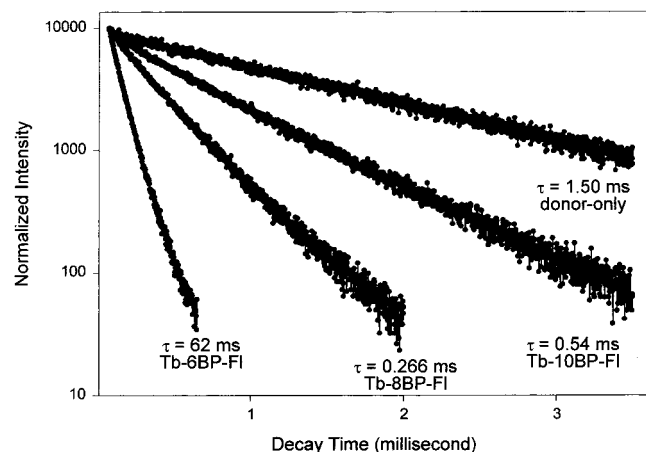*J. Am. Chem. Soc., Vol. 122, No. 4, 2000* 659

Figure 3. Lifetime decay of fluorescein-sensitized emission (520 nm) after receiving energy from terbium donor. The lifetime can be tailored depending on distance between donor and acceptor. Donor and acceptor placed on same strand and hybridized to unlabeled complementary strand.

Figure 2 shows the delayed emission spectra of terbium and fluorescein placed on the same strand, separated by either 8 or 10 base pairs (Tb-8-FL, Tb-10-FL, respectively), and hybridized to a complementary unlabeled DNA target. Also shown is the terbium-only spectra. The data are collected beginning 30 μs after pulsed excitation of the sample, and consequently, any prompt (nsec) fluorescence of the acceptor has decayed away. The large broad peak around 520 nm is therefore due to fluorescein emission arising only from energy transfer. With greater energy transfer, the Tb-8-FL displays more sensitized emission than the Tb-10-FL. (Tb-6-FL, data not shown, has a lifetime sufficiently short so that a significant fraction of the signal was lost in the 30 μs delay time.)

Figure 3 shows the temporal decay of the sensitized fluorescein emission at 518 nm for the Tb-6-FL, Tb-8-FL, Tb-10-FL, and Tb-DNA-donor-only reference. These decays were curve-fit to give coefficient-weighted averages of 62, 266, 540, and 1500 μs lifetimes for Tb-6-FL, Tb-8-FL, Tb-10-FL, and donor-only, respectively. This result clearly shows that the sensitized emission lifetime can be experimentally tuned in the μs time regime. Each LCTF is best fit to a 3-exponential decay, likely caused by the fluorescein acceptor experiencing three different local conformations. Despite this complexity, the average lifetimes are extremely reproducible, with repeat measurements on the same sample within 1% and between different samples within 10%. Hence, a target DNA labeled with one of these LCTFs can readily be distinguished from a target DNA labeled with a different LCTFs simply based on lifetimes.

Figure 4 shows that different emission colors with similar lifetimes can also be generated by appropriate choice of acceptors, donors, and donor–acceptor distances. Two sets of LCTFs were synthesized. One has Eu^{3+}-chelate as donor on the 5′-end of one strand and Cy5 as the acceptor on the 5′-end of the complementary strand, with 10 base pairs separating the donor and acceptor. The other has a Tb^{3+}-chelate as donor on the 5′-end of one strand and fluorescein as the acceptor on the 5′-end of the complementary strand, with 8 base pairs separating the donor and acceptor. (The fluorescein was also placed internally on the complementary strand using an abasic fluorescein phosphoramidite with similar results (data not shown).) Figure 4 shows that the two sensitized emissions have very similar lifetimes, 250 μs for Eu-10-Cy5 pair and 270 μs for Tb-8-Fl pair. However, Cy5 fluoresces around 668 nm (red
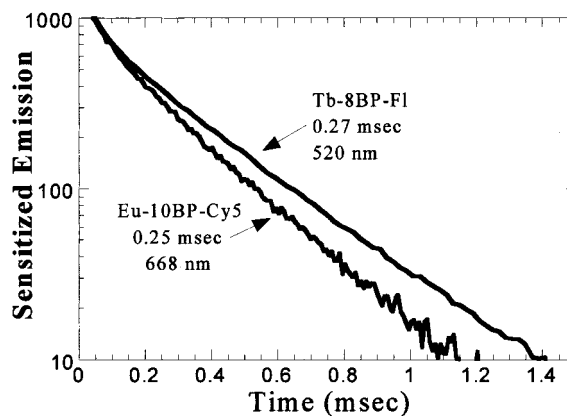

Figure 4. Similar lifetimes with different emission colors can be generated. The donor was placed on the 5′-end of DNA, the acceptor on the 5′-end of complementary DNA and the DNAs hybridized.
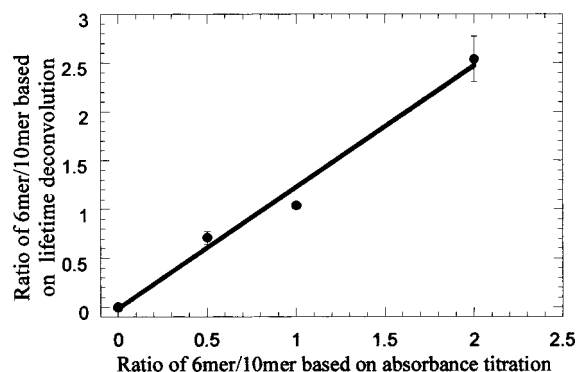

Figure 5. Two probes with the same emission color (520 nm) but different lifetimes can be independently identified and their populations quantified. Stock solutions of Tb-6-Fl and Tb-10-Fl were mixed at different ratios (determined by absorbance at 260 nm). The sensitized emission lifetimes of the mixtures were fit to a sum of the Tb-6-Fl decay and Tb-10-Fl decay and the relative populations determined. The best fit line is: $y = -0.016 + 1.24x$; $r = 0.993$.

color), whereas fluorescein emits around 520 nm (green color). These results demonstrate that probes with similar lifetimes, but different emission colors, can be generated.

Figure 5 shows the ability to discriminate between a mixture of two probes with the same emission color (detection at 520 nm) but different lifetimes. A Tb-6-Fl DNA ($\langle\tau\rangle = 62$ μs) was mixed with various amounts of Tb-10-Fl ($\langle\tau\rangle = 540$ μs) and the resulting multi-exponential decay was curve-fit to a sum of 6-mer-only and 10-mer-only decays. The population of each species was determined by the relative amplitudes of each component of the fit, and compared to the known amount determined by a simple titration based on absorption measurements at 260 nm. The linearity is quite good ($r = 0.993$). The slope differs from unity by 24%, but this is within the expected variability due to uncertainty in DNA concentration (approximately ±10% for each strand) based on absorbance titration.

The lifetime- and color-tailored dyes reported here open up the possibility of quadratically increasing the number of resolvable probes on a single sample, relying not just on conventional color discrimination but also on both color and excited-state lifetime discrimination. If, for example, four colors and four lifetimes can be detected, a target DNA could potentially be labeled with 16 probes, each binding to a unique site and each probe/sequence uniquely identified by a combination of color and lifetime discrimination. These probes also

extend fluorescence lifetime-based detection from the nanosecond range into the tens-to-hundreds of microseconds time domain. This time-scale is long enough to enable facile and nearly complete discrimination against short-lived background fluorescence and scattering, yet short enough such that emission

rate (saturation) is not a problem in many applications.

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