Live-Cell Imaging

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Luminescent Terbium Protein Labels for Time-Resolved Microscopy and Screening**

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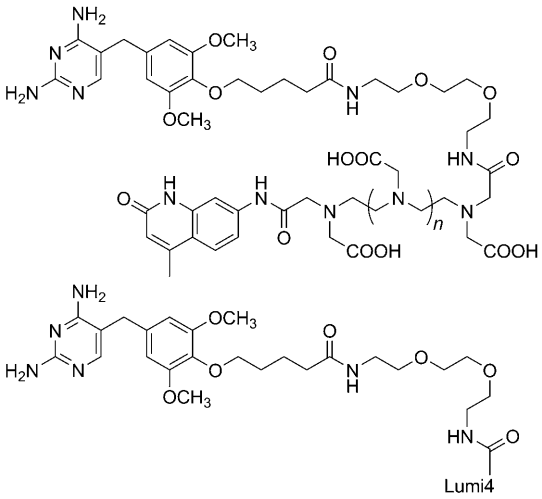
Lanthanide luminescence offers several advantages for fluorescence-based biological assays: 1) large Stoke's shifts (>150 nm) and multiple narrow emission bands (< 10 nm at half-maximum) allow efficient spectral separation of emission signals; 2) long luminescence lifetimes (microsecond to millisecond) enable time-resolved detection methods to remove scattering and autofluorescence background; and 3) relative insensitivity to photobleaching allows for prolonged detection.[1] Terbium and europium probes typically incorporate the metal ion into an organic chelating ligand that contains a sensitizing chromophore. When excited with near-UV light in the absorption band, the chromophore transfers energy by intersystem crossing to the triplet excited state and intramolecular transfer to the emissive level of the chelated metal.[1,2] Direct conjugation of lanthanide probes to antibodies, oligonucleotides, and proteins has enabled the development of sensitive, time-resolved fluorescence resonance energy transfer (TR-FRET) assays of biomolecular interactions in purified biochemical preparations, cellular extracts, and on cell surfaces.[3–8] Recent efforts have sought to develop lanthanide probes for live-cell imaging applications using time-resolved microscopy with pulsed, near-UV single photon excitation or two-photon excitation.[9–15]

Herein we report a facile method of imparting terbium luminescence to proteins for in vitro TR-FRET-based assays and live-cell imaging applications. We prepared heterodimeric molecules consisting of a protein-binding ligand, trimethoprim (TMP), linked to a series of luminescent terbium complexes, including carbostyril-124-linked polyaminocarboxylates and a 2-hydroxyisophthalamide-based complex (Scheme 1). The TMP-terbium complex conjugates (TMP-TCs) exhibit characteristic terbium luminescence and bind with high affinity to *Escherichia coli* dihydrofolate reductase (eDHFR) fusion proteins in vitro. The specific labeling of eDHFR expressed on the surface of living mammalian cells with TMP-TCs could be visualized using a sensitive time-resolved, fluorescence microscope capable of rapid image acquisition.



Scheme 1. Conjugates of trimethoprim (TMP) to sensitized terbium chelate complexes. Top: cs124-polyaminocarboxylates ($n=1$, diethyl enetriamine pentaacetic acid (TMP-cDTPA); $n=2$, triethylenetetra amine hexaacetic acid (TMP-cTTHA)). Bottom: Lumi4-Tb, a proprietary 2-hydroxyisophthalamide terbium complex (TMP-Lumi4).

The specific high-affinity ($K_D \approx 1$ nM) interaction between TMP and eDHFR has been exploited to develop the LigandLink Universal Labeling Technology (Active Motif, Inc., Carlsbad, CA) that makes it possible to tag eDHFR fusion proteins in wild-type mammalian cells with cell-permeable TMP-fluorophore conjugates.[16,17] We sought to utilize the TMP-eDHFR interaction for labeling proteins with lanthanide probes. For the first generation of TMP-TCs, we selected terbium complexes that were known to have good brightness (high extinction coefficients and quantum yields), could be conjugated without disrupting their terbium binding characteristics or luminescence, and could be synthesized relatively easily. Selvin and co-workers have developed and extensively characterized complexes of the chromophore carbostyril-124 (cs124) linked to diethylenetriamine pentaacetic acid (DTPA) and triethylenetetraamine hexaacetic acid (TTHA).[18–21] Terbium complexes of cs124-DTPA and cs124-TTHA have relatively high extinction coefficients ($\varepsilon \approx 10\,000\ \text{M}^{-1}\text{cm}^{-1}$ at 343 nm) and quantum yields in water

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of 0.32 and 0.40, respectively.[21] Moreover, the complexes exhibited similar brightness and lifetimes when conjugated to peptides or proteins.[18,20] Therefore, we prepared heterodimers of cs124-DTPA and cs124-TTHA linked to TMP using a 15-atom linker (Scheme 1), reasoning that the conjugation would preserve the essential characteristics of the parent complexes. Raymond and co-workers reported an extremely bright ($\varepsilon \approx 28000\,\text{M}^{-1}\text{cm}^{-1}$ at 354 nm, quantum yield = 0.59) multidentate 2-hydroxyisophthalamide (IAM) terbium chelate.[22] We covalently linked TMP to a proprietary analogue of the IAM complex (Lumi4) that has similar brightness and a luminescence lifetime (ca. 2.7 ms) and remains unchanged upon conjugation to proteins.[23] Each of the TMP-TCs that we prepared exhibited characteristic terbium luminescence when complexed with the metal (Supporting Information, Figure S1).

For both in vitro and live-cell applications, TMP-TCs must necessarily bind with high affinity to eDHFR fusion proteins. To determine whether the TMP-TCs could bind to eDHFR and serve as FRET donors to green fluorescent protein (GFP), we titrated a purified eDHFR-GFP fusion protein against a fixed concentration (20 nM) of the different TMP-TCs. Using a time-resolved fluorescence plate reader, we detected sensitized, long-lifetime (> 100 μs) emission of GFP that increased with increasing protein concentration (Figure 1). Addition of excess TMP substantially reduced the signal, indicating that intramolecular TR-FRET occurred between the eDHFR-bound conjugates and GFP. The relative intensities of sensitized GFP emission at binding saturation were positively correlated to the reported quantum yields of the complexes. A nonlinear, least-squares fit of the data showed the dissociation constants for binding to eDHFR of TMP-cDTPA, TMP-cTTHA, and TMP-Lumi4 to be (9 ± 1.3) nM, (22 ± 3.0) nM, and (1.8 ± 0.3) nM, respectively. The measured affinities were higher than a previously reported value for binding of a TMP-fluorescein conjugate to eDHFR ($K_D \approx 30$ nM)[17] and approach the value of free TMP.

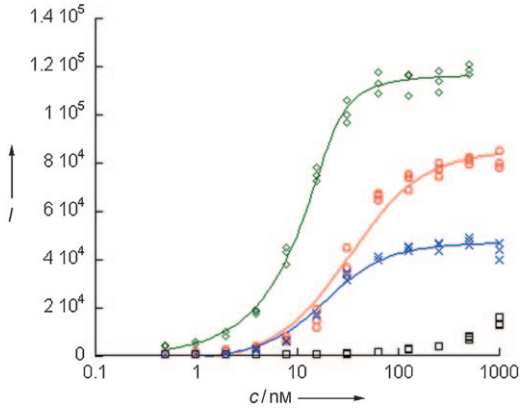

Figure 1. Intramololecular, time-resolved, fluorescence resonance energy transfer (TR-FRET) between eDHFR-bound TMP-TCs and GFP. Increasing concentrations (*C*) of purified eDHFR-GFP were titrated against a constant concentration (20 nM) of each compound. Sensitized GFP emission (520 nm) was detected after a time delay of 100 μs, upon pulsed excitation with near-UV light (ca. 340 nm): TMP-Lumi4 (◇), TMP-cTTHA (○), TMP-cDTPA (×). Addition of 1 μM TMP reduced the signal (TMP-cTTHA, □), confirming FRET. Lines represent nonlinear least squares fit to the data.

We next sought to determine whether TMP-TCs could be used to label eDHFR fusion proteins in living mammalian cells. NIH3T3 fibroblast cells were transiently co-transfected with two plasmid DNA vectors; one that expressed plasma membrane targeted eDHFR and another that expressed nucleus-localized cyan fluorescent protein (CFP), included as a positive control for transfection. The cells were incubated in growth medium containing 100 μM TMP-cTTHA for 20 hours, washed, and imaged using an epi-fluorescence microscope capable of pulsed UV excitation and time-resolved detection. No specific labeling of plasma membrane-localized eDHFR was observed in cells that expressed nucleus-localized CFP (Figure 2 a,b). Nonspecific luminescence was detected in all cells, possibly indicating endocytosis of the compound and trapping in lysosomes. When similar experiments were performed with lower concentrations and or shorter incubation times, long-lifetime luminescence could not be detected in cells incubated with any of the TMP-TCs.

While intracellular labeling of eDHFR with the TMP-TCs was not possible, we were able to successfully label eDHFR expressed on the cell surface. NIH3T3 fibroblasts were co-transfected with the nucleus-localized CFP expression plasmid and a vector that expressed eDHFR on the extracellular surface of the plasma membrane (pDisplay-eDHFR). 24 hours after transfection, the cells were incubated in growth medium containing 1 μM TMP-Lumi4 for 10 minutes. Subsequently, the cells were washed and imaged. A distinct membrane luminescence was observed only in cells that expressed nucleus-localized CFP when the cells were imaged in time-resolved mode (Figure 2 c,d). Owing to the dissociation of TMP-Lumi4 from eDHFR and diffusion into the medium, the membrane fluorescence could only be detected for approximately 20 minutes. A control experiment established that the membrane fluorescence was dependent on the specific labeling of the eDHFR fusion protein with TMP-Lumi4. Pre-incubation of the cells expressing membrane-targeted eDHFR in medium containing 10 μM TMP, followed by incubation in medium containing 1 μM TMP-Lumi4 resulted in no membrane staining. We were only able to detect cell-surface labeling of eDHFR with TMP-Lumi4, and not with TMP-cDTPA or TMP-cTTHA.

Herein we have shown that the high-affinity noncovalent interaction between TMP and eDHFR provides an effective means for imparting terbium luminescence to recombinant fusion proteins. TMP-TCs exhibited characteristic luminescence and high affinity for eDHFR, and they proved to be efficient sensitizers of GFP emission in an intramolecular TR-FRET assay. TMP-Lumi4 was particularly effective, binding to eDHFR-GFP with about 2 nM affinity and exhibiting more than 100-fold increase in FRET signal upon binding saturation. As FRET donors to GFP, TMP-TCs could be used to detect interactions between eDHFR and GFP fusion proteins. This would be particularly useful when protein-specific antibodies are unavailable, or in situations where direct conjugation of proteins with terbium complexes is problematic, such as assays of cell lysates. As prepared, the TMP-TCs reported herein are cell-impermeable, and can only be used to

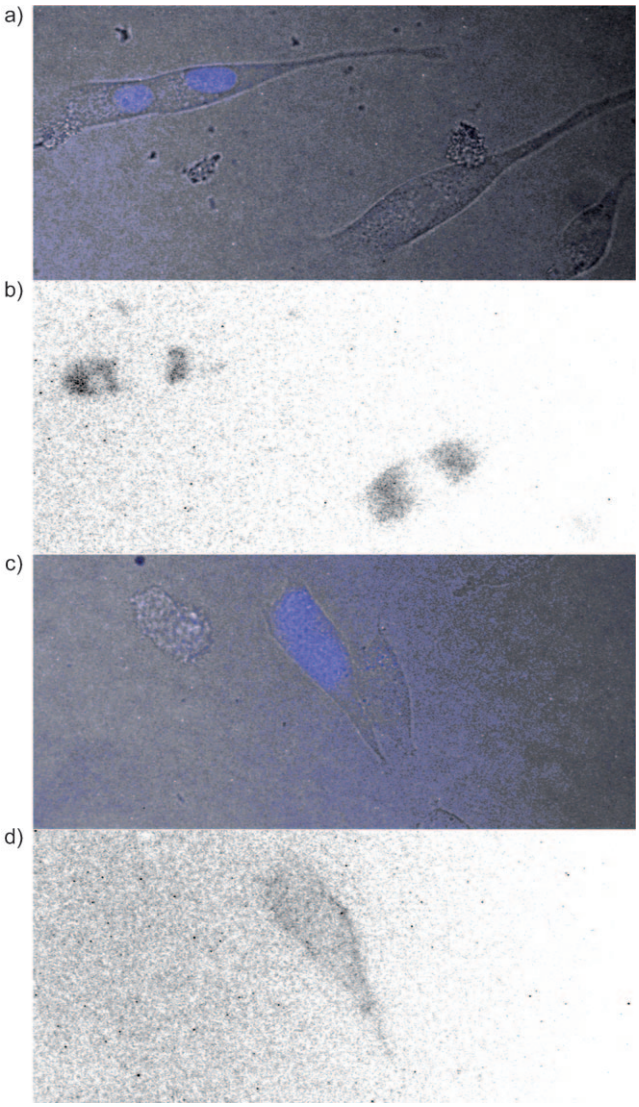

Figure 2. Time-resolved microscopy of NIH3T3 cells treated with TMP-TCs. a) Overlay of bright field and prompt fluorescence ($\lambda_{ex} = (480 \pm 20)$ nm, $\lambda_{em} = (535 \pm 25)$ nm) images of cells transiently expressing nucleus-localized CFP and plasma membrane-localized eDHFR. b) Inverse time-resolved fluorescence image of cells in (a) showing nonspecific luminescence. Cells were incubated in media containing TMP-cTTHA (100 μM) for 20 h, washed with PBS, mounted in media without compound, and imaged in time-resolved mode ($\lambda_{ex} = (350 \pm 25)$ nm, $\lambda_{em} = (550 \pm 10)$ nm, delay = 80 μs, exposure time = 1420 μs, number of exposure cycles = 660). c) Overlay image of cells transiently expressing nucleus-localized CFP and cell-surface-localized eDHFR. d) Inverse time-resolved fluorescence image of cells in (c) showing membrane luminescence in transfected cell. Cells were incubated in media containing 1 μM TMP-Lumi4 (10 min), washed, and imaged as in (b).

label proteins on cell surfaces. However, physical methods, such as scrape loading or bead loading, that are commonly used to load macromolecules into living cells could conceivably be used to deliver TMP-TCs to intracellularly expressed eDFHR fusion proteins.[24]

Experimental Section

The complete details of TMP-LC syntheses and characterization, plasmid vector construction, eDHFR-GFP fusion protein expression and purification, cell culture conditions as well as the instrumental configurations and experimental details of binding assays, and cellular microscopy are reported in the Supporting Information.

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