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$[Os(bpy)_2(CO)(enIA)][OTf]_2$: A Novel Sulfhydryl–Specific Metal–Ligand Complex

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The synthesis and physical–chemical characterization of the metal–ligand complex $[Os(bpy)_2(CO)(enIA)][OTf]_2$ (where enIA = ethylenediamine iodoacetamide) with a sulfhydryl-specific functional group is described. The UV and visible absorption and luminescence emission, including lifetime and steady-state anisotropy, are reported for the free probe and the probe covalently linked to two test proteins. The spectroscopic properties of the probe are unaffected by chemical modification and subsequent covalent linkage to the proteins. The luminescence lifetime in aqueous buffer is approximately 200 ns and the limiting anisotropy is greater than 0.125, suggesting a potentially useful probe for biophysical investigations.

Introduction

During the past decade there has been a growing interest in metal−ligand complexes (MLCs) as luminescence probes for biophysical applications and chemical sensing.[1] Research has centered mainly on rhenium and ruthenium complexes because of their high quantum yields and high emission anisotropies. Less attention has been given to osmium derivatives because their quantum yields are typically lower. However, osmium complexes generally have significantly greater chemical and photochemical stability, a factor that can be critically important in biological applications. The quantum yield and chemical stability depend on the relative energy levels of the MLC. Excitation is a singlet−singlet transition, and there is efficient intersystem crossing from the excited singlet to the triplet (^{3}MLCT) state. The energy of the ^{3}MLCT state relative to the metal d−d state determines the nonradiative rate constants and hence regulates triplet quantum yields. Observable luminescence requires that the d−d state be at higher energy than the MLCT state. There is a reciprocal relationship between quantum yield and chemical stability. It has been noted[2] that as the gap between the d−d and MCLT states increases, quantum yield decreases and chemical stability increases, and vice versa. However, this energy gap is ligand dependent.

We have been exploring new $[Os(bpy)_2(CO)L]^{n+}$ as candidates for stable, high-quantum yield probes. Our current focus is on osmium-based MLC bioconjugates that are site-specific. Synthesis of site-specific bioconjugates requires selective chemical reactivity. For example, one strategy for generating single reactive sites in proteins is to prepare recombinant mutants that contain a single free cysteine residue. This provides a sulfhydryl group at a precisely defined site, which can be covalently modified with, for example, iodoacetamides. Other luminescent metal complexes for site-specific protein labeling have been reported in the literature.[3] These are ruthenium and rhenium-based MLCs, functionalized with a iodoacetamide or a maleimide group, that have been synthesized for binding Cys residues in proteins by Lo[3a] and Lakowicz.[3e−3f] In addition, a trinuclear heterobimetallic Ru(II)/Pt(II) complex was developed for targeting sulfhydryl-containing amino acids and peptides.[3b]

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(1) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Kluwer Accademic/Plenum Publishers: New York, 1999.

(2) Demas, J. N.; DeGraff, B. A. *Anal. Chem.* **1991**, *63*, 829A−837A.

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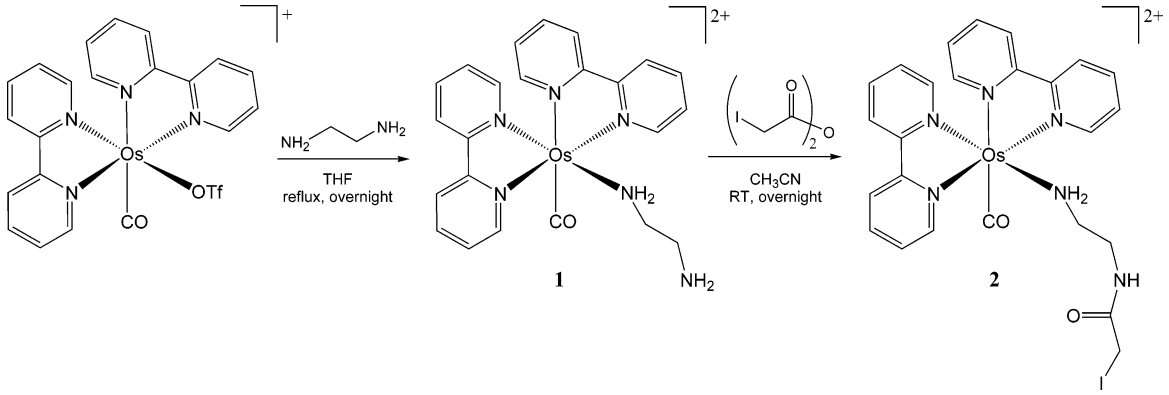
Scheme 1



Here we report the synthesis and characterization of the iodoacetamide derivative of $[Os(bpy)_2(CO)(en)][OTf]_2$ (where $OTf = CF_3CO_3^-$ and en = ethylenediamine) as a sulfhydryl-specific MLC (see Scheme 1). We then demonstrate covalent modification of two test proteins: human serum albumin (HSA) and a mutant of *E. coli* uracil DNA glycosylase (UDG), which each contain a single reactive cysteine residue.

Experimental section

Physical Measurement. NMR spectra were recorded on a JEOL EX 400 spectrometer ($B_0 = 9.4$ T, ^1H operating frequency 399.78 MHz). All compounds were dissolved in deuterated acetone and chemical shifts were internally referenced relative to the residual protons in the deuterated solvents. COSY and NOESY experiments were acquired using standard procedures. Absorption measurements were performed using an Uvikon 930 spectrometer (Kontron Instruments) with 2-nm spectral bandwidth. For quantum yield measurements, 10-mm path length fluorescence quartz cuvettes were used. Lucifer yellow was chosen as a *standard* because its absorption band (maximum at 430 nm) overlaps the excitation wavelength of our *test* sample, $[Os(bpy)_2(CO)(en)][OTf]_2$ (445 nm), and both compounds exhibit large Stokes shifts (100 vs 145 nm). Serial dilutions ($n = 6$) of the *standard* and the *test* samples were prepared in the absorbance interval from 0 to 0.1, and the UV−vis absorption and relative emission fluorescence spectra were recorded. The integrated fluorescence intensity vs absorbance were then plotted to obtain a straight line with slope m and intercept = 0. The slopes of the graphs are proportional to the quantum yield of the different samples, and the absolute value of the *test sample*'*s* quantum yield was calculated according to the following equation:[4]

$$\Phi_X = \Phi_{ST}\left(\frac{Grad_X}{Grad_{ST}}\right)\left(\frac{\eta_X^2}{\eta_{ST}^2}\right) \qquad (a)$$

where the subscripts ST and X refer to the *standard* and the *test* sample, respectively. Φ is the fluorescence quantum yield, Grad is the m gradient coefficient obtained from the plotted straight line, and η is the refractive index of the solvent. The error of the calculated quantum yield was within ±7% of the reported value, based upon the observed errors in the gradients of the straight lines from three different experiments ($0.9979 < r^2 < 0.9998$).

Steady-state fluorescence intensities and emission spectra were recorded using a PTI QuantaMaster C60/2000 spectrofluorometer with excitation and emission band-passes of 2 and 4 nm, respectively. Steady-state anisotropy measurements were performed by using two automated rotating Glen-Thomson polarizers placed in the excitation and in the emission path. The intensity of the parallel (vv) and the perpendicular (vh) components of the polarized fluorescence emission were then used to calculate the steady-state anisotropy, $\langle r \rangle$, as follows:

$$\langle r \rangle = I(vv)\cdot G - I(vh)/I(vv)\cdot G - 2I(vh) \qquad (b)$$

where G, the "grating" correction factor, is calculated as $G = I(hh)/I(vh)$.

Nanosecond time-resolved fluorescence measurements were obtained by the time-correlated single photon counting method using a model 5000U fluorescence lifetime spectrometer (IBH Consultants Ltd., Glasgow, U.K.). The instrument response function was 1.4 ns (fwhm) using a Hamamatzu R3235 photomultiplier. The data were collected into 1024 channels with a resolution of 0.103 ns per channel. The data were analyzed by nonlinear least-squares method assuming the fluorescence intensity decay as a sum of discrete exponential components, each described by a decay constant (lifetime, τ_i ns) and its relative contribution (amplitude, α_i) to the total fluorescence decay:[5]

$$I(t) = \sum \alpha_i \cdot e^{-t/\tau i} \qquad (c)$$

Infrared spectra were measured on samples in KBr pellets using a Bruker Equinox 55 FT-IR spectrophotometer with a resolution of 1 cm^{-1}. An accumulation of 64 scans was sufficient to provide high-quality spectra. Mass spectra were recorded using a MALDI-TOF Reflex III (Bruker Daltonics, Bremen, Germany).

Electrochemistry Measurements. Reactants for electrochemistry were prepared as reported: acetonitrile was distilled over calcium hydride just before use, and tetrabutylammonium hexafluorophosphate (TBA) was obtained as metathesis reaction between KPF_6 (Fluka) and tetrabutylammonium iodide (Aldrich), recrystallized three times from 95% ethanol, and dried in a vacuum oven at 110 °C overnight. Electrochemistry was performed with an EG&G PAR 273 electrochemical analyzer controlled by a PC. A standard three-electrode cell was designed to allow the tip of the reference electrode to closely approach the working electrode.

(3) (a) Lo, K. K.-W.; Hui, W.-K.; Ng, D. C.-M.; Cheung, K.-K. *Inorg. Chem.* **2002**, *41*, 40−46. (b) Chow, C.-F.; Chiu, B. K. W.; Lam, M. H. W.; Wong, W.-Y *J. Am. Chem. Soc.* **2003**, *125*, 7802−7803. (c) Murtaza, Z.; Herman, P.; Lakowicz, J. R. *Biophys. Chem.* **1999**, *80*, 143−151. (d) Youn, H. J.; Terpetschnig, E.; Szmacinski, H.; Lakowicz, J. R. *Anal. Biochem.* **1995**, *232*, 24−30. (e) Castellano, F. N.; Dattelbaum, J. D.; Lakowicz, J. R. *Anal. Biochem.* **1998**, *255*, 165−170. (f) Dattelbaum, J. D.; Abugo, O. O.; Lakowicz, J. R. *Bioconjugate Chem.* **2000**, *11*, 533−536.

(4) Williams, A. T. R.; Winfield, S. A.; Miller, J. N. *Analyst* **1983**, *108*, 1067−1071.

(5) Grinvald, A.; Steinberg, I. Z. *Anal. Biochem.* **1974**, *59*, 583.

The reference electrode was KCl 3 M Calomel Electrode, the auxiliary electrode was a platinum wire, and the working electrode was a glassy carbon (GC). Positive feedback *iR* compensation was applied routinely. All measurements were carried out under Ar in anhydrous deoxygenated solvents. Ferrocene (Fc) was used as an internal standard, and potentials are reported against the Fc(0/+1) redox couple (measured $E_{1/2}(0/+1) = 0.38$ V).

Materials. Reagent grade chemicals and solvents, obtained from commercial suppliers, were used as received.

Synthesis of $[Os(bpy)_2(CO)(en)]OTf_2$ (1). The complex $[Os(bpy)_2(CO)(OTf)]OTf$[6] was dissolved in dry tetrahydrofuran (THF), and an excess of ethylenediamine (1:100) was added. The solution was allowed to reflux overnight with constant stirring. The solvent was then evaporated and the remaining yellow solid was washed with ethyl ether. The reaction yield was about 70%. The reactivity of $[Os(bpy)_2(CO)(OTf)]OTf$ toward ethylenediamine was noted previously by Demas,[7] but no complete synthesis and characterization has been reported. The assignment of the ^{1}H spectrum was obtained by a NOESY experiment. ^{1}H spectrum in acetone-d_6 (ppm): 9.62 (m, 2H), 8.95 (d, 1H), 8.89 (d, 1H), 8.83 (d, 1H), 8.79 (d, 1H), 8.56 (t, 1H), 8.33 (t, 1H), 8.29 (t, 1H), 8.21 (t, 1H), 8.07 (m, 2H), 7.97 (t, 1H), 7.68 (d, 1H), 7.61 (t, 1H), 7.50 (t, 1H), 5.55 (broad t, 1H), 5.05 (broad t, 1H), 3.25 (m, 2H), 3.12 (m, 1H), 3.06 (m, 1H). ^{19}F spectrum in acetone-d_6 (ppm): − 79.32. ^{13}C spectrum in acetone-d_6 (ppm): 177.87, 159.54, 158.93, 156.76, 156.24 (shoulder), 154.48, 152.66, 149.05, 141.65, 140.83, 140.09, 139.38, 129.53, 128.76, 128.44, 128.33, 125.77, 125.54, 124.98, 124.56, 51.29, 50.38. IR Measurements (KBr, cm^{-1}): 3231 and 3147 (NH stretching), 2999 (CH stretching), 1943 (CO stretching), 1440, 1266, 1156 and 1101, 466. Maldi-MS: *m*/*z* 591 (M^+), 531 (M^+ − en).

Synthesis of $[Os(bpy)_2(CO)(enIA)]OTf_2$ (2). The complex $[Os(bpy)_2(CO)(en)][OTf]_2$ was dissolved in dried acetonitrile, and an excess of iodoacetic anhydride (molar ratio 1:10) was added. The solution was stirred overnight in the dark. The solvent was then evaporated; the residue was dissolved in acetone, and the iodoacetamide derivative was precipitated with ethyl ether. The reaction yield was about 40%. The assignment of the ^{1}H spectrum was obtained by a COSY and NOESY experiment. ^{1}H spectrum in acetone-d_6 (ppm): 9.59 (d, 2H), 9.44 (d, 1H), 8.92 (d, 1H), 8.89 (d, 1H), 8.81 (d, 1H), 8.76 (d, 1H), 8.55 (t, 1H), 8.33 (t, 1H), 8.28 (t, 1H) 8.20 (t, 1H), 8.08 (t, 1H), 8.01 (d, 1H), 7.97 (t, 1H), 7.90 (br, NH), 7.67 (d, 1H), 7.61 (t, 1H), 7.50 (t, 1H), 5.74 (br t, 1H), 5.18 (broad t, 1H) 3.65 (s, 2H COCH_2I), 3.28 (m, 2H, CH_2), 3.05 (br, 1H, CH_2), the other CH_2 proton is under the water peak. ^{19}F spectrum in acetone-d_6 (ppm): − 79.34. ^{13}C spectrum in acetone-d_6 (ppm): 177.52, 169.13, 159.34, 156.67, 156.27, 154.45, 152.45, 149.09, 147.62, 141.71, 140.80, 140.09, 139.43, 129.72, 128.94, 128.41 (double height), 125.81, 125.47, 124.94, 124.62, 50.45, 41.73, − 1.31. IR Measurements (KBr, cm^{-1}): 3228 and 3118 (NH stretching), 2960, 2979, 2880 (CH stretching), 1945 (CO stretching), 1660 (broad, NHCO), 1546 (broad, NHCO), 1251, 1156 and 1022. Maldi-MS: *m*/*z* 759 (M^+), 631 (M^+ − I), 531 (M^+ − enIA).

Results and Discussion

Products were fully characterized by 1D and 2D ^{1}H NMR, 1D ^{13}C- and ^{19}F NMR, IR spectroscopy, mass spectrometry, and cyclic and square-wave voltammetry (for details see Experimental Section and Supporting Information). The cyclic voltammetry (CV) of a solution of compound **1** in acetonitrile shows two subsequent reversible 1e reductions at half wave potentials of $E_{1/2}(+2/+1) = -1.54$ V and $E_{1/2}(+1/0) = -1.73$ V vs ferrocene/ferrocinium couple (Figure 1). The plots of the peak currents vs the square root of the scan rate are linear in the range 0.05−5 V/s, and the separation between the anodic and cathodic peaks is similar to that of the ferrocene/ferrocenium couple in the same conditions and close to the theoretical value of 60 mV for a Nernstian system. Square wave voltammetry (SWV) also was employed to obtain a better separation of the two reductions. SWV yielded the same half-wave potentials values. Two irreversible reductions are observed at more negative potentials (peaks at −2.38 and −2.62 V). On the oxidation side, there are two irreversible processes. The first, at +0.806 V, involves one electron, while the second, at +1.35 V, is a multielectron oxidation.

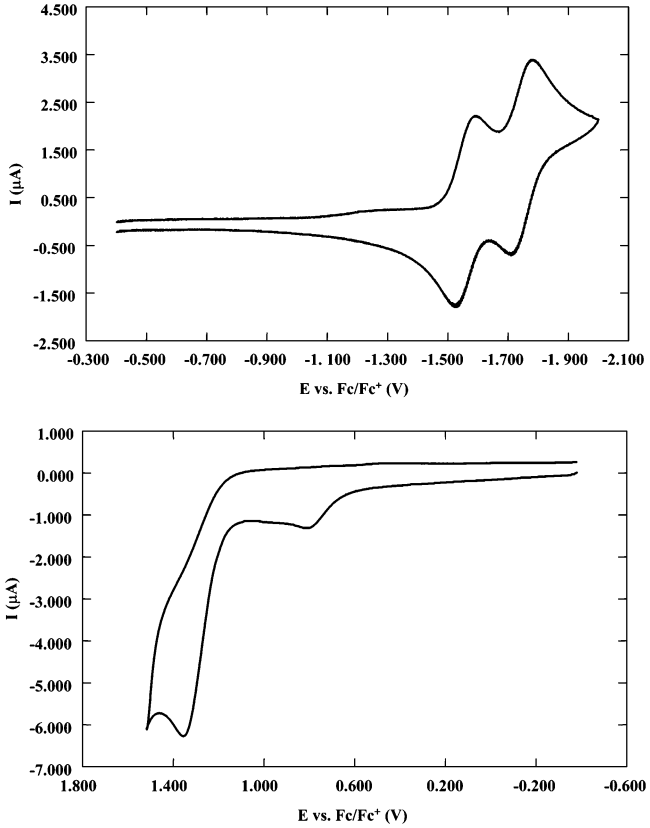


Figure 1. Cyclic voltammetry of a 1 mM solution of **1** in acetonitrile at 200 mV/s on glassy carbon electrode.

The absorption and the emission spectra of compounds **1** and **2** were measured in water, pH range 2 to 10, and in methanol. The absorption spectra, which were solvent independent, exhibited four well-resolved peaks in the wavelength region between 200 and 600 nm (Figure 2) at 205, 264, 300, and 445 nm. The 300 and 445 nm peaks exhibit 84.5% and 9.2%, respectively, of the extinction of the 264 nm peak. In addition, distinct shoulders can be noted at approximately 245, 310, and 325 nm. The luminescence spectra were also solvent independent. The emission exhibited a large Stokes shift of ∼5880 cm^{-1}, with a maximum

(6) Sullivan, B. P.; Caspar, V. J.; Johnson, R. S.; Meyer, J. T. *Organometallics* **1984**, *3*, 1241−1251.
(7) Sacksteder, L.; Demas, J. N.; DeGraff, B. A. *Inorg. Chem.* **1989**, *28*, 1787.

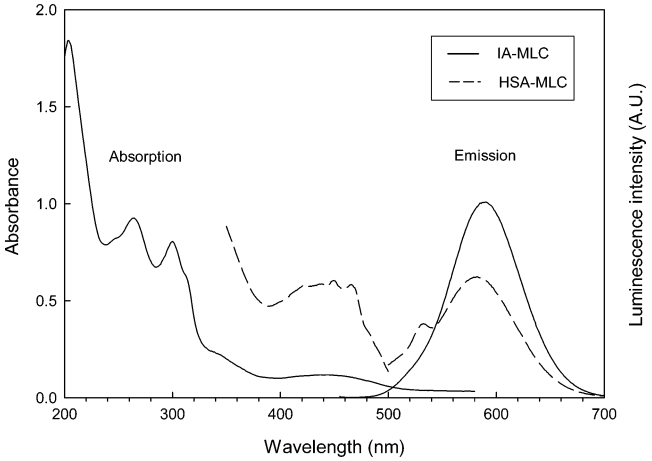


Figure 2. Absorption and emission (solid lines) of **2** in methanol, and excitation and emission (dashed lines) of **2**−HSA in 10 mM Tris buffer, pH 8.0.

near 590 nm; this shift is characteristic of this class of luminophores.[8] Using the fluorescence probe Lucifer Yellow (Molecular Probes) as a reference standard having a quantum yield of 0.21,[9] the relative quantum yield of the MLC in methanol was calculated to be about 0.006. The luminescence intensity decay of the iodoacetamide derivative in 10 mM Tris buffer (pH 8), measured by time-correlated single-photon counting, had an average lifetime[10] of approximately 200 ns, which is in the range expected for MLCs. The steady-state excitation anisotropy, averaged between 400 and 450 nm, was about 0.125 at −10 °C in 99:1 glycerol to methanol.

To evaluate the potential of the probe as a bioconjugate, we labeled two proteins having single free cysteine residues, HSA and a mutant of *E. coli* UDG. Wild-type UDG contains a single, buried cysteine residue, Cys31, which was mutated to serine to prevent its chemical modification. This mutation is isosteric, and it does not affect enzymatic activity. Then, from inspection of the X-ray crystal structure model of UDG,[11] we identified the solvent-exposed serine, S150, as a candidate for mutation to cysteine, and generated the double mutant C31S, S150C UDG, which also has wild-type activity.

To label HSA (Sigma-Aldrich, St. Louis, MO), the protein was dissolved at 2.5 mg/mL in 2 mL of 100 mM phosphate buffer containing 1 mM DTT at pH 7.3. After 3 h, the protein solution was dialyzed overnight against 100 mM phosphate buffer at pH 7.3. Conjugation was then accomplished by addition of compound **2**, previously dissolved in methanol, to the protein solution. The final molar concentration of compound **2** was 3-fold that of the protein; the resulting solution was 4% v/v methanol. The reaction proceeded overnight at 4 °C, followed by gel filtration chromatography on a Sephadex G-25 column (Amersham Biosciences,

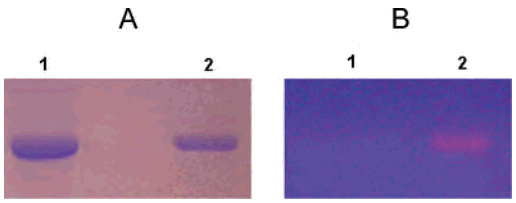


Figure 3. SDS−PAGE of HSA. 30 μL of non-conjugated and conjugated HSA were loaded on lanes 1 and 2, respectively: (A) the gel as stained with Coomassie Blue; and (B) the same gel before staining and imaged to capture fluorescence emission.

Uppsala, Sweden) equilibrated in 10 mM Tris buffer, pH 8.0. The purity and stability of the labeled protein was assayed by SDS−PAGE (13%). The ratio of dye-to-protein labeling was 0.6 to 0.8. We calculated the probe concentration using an extinction coefficient of 1830±93 $M^{-1}cm^{-1}$ at 450 nm that we had previously determined for the probe in water. The protein concentration was determined from the densitometric scan of the SDS−PAGE[12] gel that included known concentrations of unmodified HSA (Figure 3A) as a reference standard. The labeled protein also was visualized on SDS−PAGE gels by the bright luminescence band, which migrated at the same position as the unmodified, Coomassie-blue stained protein band (compare Figure 3A and B). We followed a similar procedure to label the UDG double mutant, and a similar sub-stoichiometric ratio of modification was obtained.

The average emission lifetime of MLC covalently bound to either UDG or HSA was about 200 ns, the same as that of the free probe. The steady-state anisotropy of MLC-labeled UDG was measured in 50% glycerol at 5 °C (viscosity ≈ 9 cp).[13] Under these conditions, the steady-state anisotropy was about 0.05. Assuming a limiting anisotropy of 0.2,[14] and rotation of the protein as a sphere, at a viscosity of 9 cp, the theoretical maximum steady-state anisotropy would be 0.064. On the other hand, a limiting anisotropy of 0.15, which is closer to the value of 0.125 that we obtained for the probe in a highly viscous medium, yields a steady-state anisotropy of 0.048, which is within the error (±0.006) of the value of 0.05 obtained for the labeled protein.

Conclusion

The long lifetime and significant anisotropy of this MLC probe can be useful for investigating the dynamics of proteins in assembling systems with molecular weights of several hundred KDa.[15] Furthermore, because the probability of Förster resonance energy transfer (FRET) is proportional to the lifetime of the donor,[1] this probe may also prove valuable in single-molecule experiments.[16] The relatively low quantum yield, however, may be a limitation, especially in single-

(8) Lakowicz, J. R.; Castellano, F. N.; Gryczynski, I.; Gryczynski, Z.; Dattelbaum, J. D. *J. Photochem. Photobiol. A: Chem.* **1999**, *122*, 95−101.
(9) Stewart, W. W. *J. Am. Chem. Soc.* **1981**, *103*, 7615.
(10) The average lifetime $\langle\tau\rangle = \sum\alpha_i\tau_i^2/\sum\alpha_i\tau_i$ where α_i is the amplitude and τ_i is the lifetime of the *i*th component.
(11) Xiao, G.; Tordova, M.; Jagadeesh, J.; Drohat, A. C.; Stivers, J. T.; Gilliland, G. L. *Proteins* **1999**, *35*, 13−24.
(12) Lamelli, U. K. *Nature* **1970**, *227*, 680−685.
(13) This is an extrapolated value [see *CEP*, Oct. 2002, p 36, www.cepmagazine.org] based on absolute viscosities at 30, 25, and 20 °C for 50% glycerol from *Lange's Handbook of Chemistry*; Handbook Publishers/McGraw-Hill: New York, 1949; p. 1633.
(14) Lakowicz, J. R.; Malak, H.; Gryczynski, I.; Castellano, F. N.; Meyer, J. T. *Biospectroscopy* **1995**, *1*, 163−168.
(15) Guo, X.-Q.; Castellano, F. N.; Li, L.; Lakowicz, J. R. *Anal. Chem.* **1998**, *70*, 632.
(16) Schwille, P.; Kettling, U. *Curr. Op. Biotechnol.* **2001**, *12*, 382−386.

molecule applications. Experiments are under way to evaluate the effects of other probe ligands on the quantum yield, lifetime, and anisotropy. Although elimination of triplet quenchers such as oxygen would improve the quantum yield, this is not a practical approach with biological systems, especially in vivo. For example, we are interested in using this class of probes to modify transporter proteins in living brain slices to study the dynamics of neurotransmitter transport; however, in this system, eliminating oxygen would cause hypoxia. We also plan to use these luminescent probes for other biophysical applications. In particular, the long lifetime and red emission is favorable for FRET investigations of protein interactions and assembly on membrane surfaces in processes such as initiation of blood coagulation.

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Supporting Information Available: NMR spectra for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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