Water-soluble neutral calix[4]arene lanthanide complexes; synthesis and luminescence properties
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Water-soluble calix[4]arenes 10a,b with chromophores ("antenna") attached to the lower rim via a short spacer are described. In the neutral lanthanide complexes of 10a,b photoexcitation of the antenna induces lanthanide emission via intramolecular energy transfer. Calix[4]arene 10b with a chrysene moiety as sensitizer shows strong lanthanide emission for Eu³⁺ with an excitation maximum at λ = 363 nm.

Introduction

There is considerable interest in the synthesis of molecular probes for imaging or bioassay purposes. Particularly interesting are lanthanide probes because of their unique characteristics like exceptionally large Stoke shifts (~200–300 nm), narrow emission line spectra, long-wavelength emission (~500–600 nm), and long luminescence lifetimes (~600–1000 ms). This explains the strong motivation for the synthesis of ligands for these lanthanide ions that form complexes with high kinetic stability and that are soluble in water for coupling reactions to biomolecules.

Various calix[4]arene derivatives are suitable for the complexation of trivalent cations, but in most cases they are not soluble in water. Only Sabbatini et al. reported the encapsulation of a lanthanide ion by a tetraamide calix[4]arene to give a water-soluble system, while Shinkai et al. prepared lanthanide complexes via complexation of the phenolic oxygen atoms of water-soluble calix[n]arenes. However, such lanthanide complexes based on calix[4]arenes are positively charged, which might lead to specific binding in biological systems.

Recently, we described calixarene derivatives that are substituted with three carboxylic acid groups and which form neutral complexes with lanthanide cations. When we attached a triphenylene antenna chromophore to such calix[4]arenes they showed a strong sensitizing ability toward Eu³⁺ and Tb³⁺, allowing the excitation of Eu³⁺ and Tb³⁺ with wavelengths extending to 350 nm.

For a simple coupling to biomolecules, these calix[4]-arene lanthanide probes should be water-soluble. In the literature, different types of water-soluble calix[4]-arenes have been reported that have hydrophilic substituents such as SO₃⁻, COO⁻, PO₄⁻, SO₄(N(CH₃)₂CH₂OH)₂, N₃, Me₃, or polyethyleneoxy chains. Recently, we have reported neutral water-soluble calix[4]arenes by...
the attachment of polyalcohol residues at the upper rim of the calix[4]arene.27

In this paper, we report the synthesis and photophysical characterization of neutral, water-soluble Eu3+ and Tb3+ complexes substituted with two aromatic sensitizer groups (chrysene or 2-pyridyl disulfide).

Results and Discussion

Synthesis. The synthesis of water-soluble ionophores 10a,b is depicted in Scheme 1. The water-solubility is introduced only in the last step of the synthesis because this facilitates handling and purification of the intermediates during the synthesis.

Reaction of triester monoacid chloride calix[4]arene 5b, prepared from the monocarboxylic acid 5a,19 with 2,2,2-trichloroethanol28 in CH2Cl2 gave the trichloroethyl ester 5c in 96% yield. Reaction of trichloroethyl ester 5c with chlorosulfonic acid in CHCl3 afforded the tetrasulfonyl calix[4]arene 6 in 61% yield.29

N-[Tris-[(tert-butyldimethylsilyl)oxy]methyl]methane(3)27 (Chart 1) was coupled to calix[4]arene 6 to afford the tetrasulfonylamine 7 in 82% yield. This intermediate can be used to introduce different amino-substituted residues. Substitution31 of the chlroroethyl group in 7 with 6-(aminomethyl)chrysene (1f) or S-(2-pyridylthio)cysteamine (2)32 (Chart 1) and DBU as a base in acetonitrile gave the corresponding amines 8a,b in 63 and 67% yield, respectively. These sulfonamides (8a,b) were purified by chromatography over a NaCl-saturated silica column.33

6-(Aminomethyl)chrysene (1f) was prepared from the known 6-(bromomethyl)chrysene34 (1d) via the phthalimide 1e. A Gabriel reaction of 1d afforded the phthalimide 1e, which after deprotection with hydrazine monohydrate gave the desired 6-(aminomethyl)chrysene (1f) in an overall yield of 95%. The precursor of 1d, 6-(hydroxymethyl)chrysene (1c),35 was obtained in 94% yield.

Scheme 1

Chart 1

1a: R = H
1b: R = C(O)H
1c: R = CH3OH
1d: R = CH3Br
1e: R = CH3N(C6H4O2)
1f: R = CH3NH2

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6-(Aminomethyl)chrysene (1f) was prepared from the known 6-(bromomethyl)chrysene34 (1d) via the phthalimide 1e. A Gabriel reaction of 1d afforded the phthalimide 1e, which after deprotection with hydrazine monohydrate gave the desired 6-(aminomethyl)chrysene (1f) in an overall yield of 95%. The precursor of 1d, 6-(hydroxymethyl)chrysene (1c),35 was obtained in 94% yield.

in an alternative way via reduction of 6-chrysencarboxaldehyde (1b)\(^{36}\) with BH\(_3\)-THF.

The selective hydrolysis of the three ethyl esters without cleavage of the amide bonds in 8a,b, was achieved with K\(_2\)CO\(_3\) in refluxing MeOH–H\(_2\)O–THF (5:1:2) to give the triacid derivatives 9a,b in quantitative yield. Removal of the TBDMS-protective groups in 9a,b with trifluoroacetic acid (TFA) gave the water-soluble derivatives 10a,b in quantitative yield. Satisfactory mass spectra could be obtained with FRIT-FAB mass spectrometry. The \(^1\)H NMR spectra of compounds 10a,b are broad at room temperature but exhibit sharp signals at 80 °C, showing the characteristic AB system of the methylene bridges. This points to aggregation of the calix[4]arenes 10a,b at room temperature.\(^{37,38}\) The maximum solubility of 10a in water, determined by UV spectroscopy, is 8.0 mM at room temperature, and this is sufficient to couple 10a to biomolecules.\(^{20}\)

**Photophysical Properties.** In order to investigate the sensitizing ability of 10a and 10b in aqueous solution, equivalent amounts of lanthanide ions (in the form of Eu(NO\(_3\))\(_3\):5H\(_2\)O or Tb(NO\(_3\))\(_3\):5H\(_2\)O) were added to a 1.5 × 10\(^{-4}\) M solution of 10a or 10b in Tris–HCl buffer pH 8.0. After deoxygenation by purging with argon for 15 min, the excitation spectra of these solutions were measured with the detection set at the emission wavelength of the lanthanide ion (615 nm for Eu\(^{3+}\) with the detection set at the emission wavelength of the lanthanide ion occurs from the antenna triplet. This effect of adding Eu(NO\(_3\))\(_3\):5H\(_2\)O to an aqueous solution of 10a,b was found that 10b does not allow sensitized luminescence to be observed. This again can readily be explained from the energy diagram depicted in Figure 2, assuming that energy transfer to the lanthanide ion occurs from the antenna triplet.

Interestingly, in the case of 10b the effect of complexation with lanthanide ions is not restricted to quenching of the chrysene triplet. This becomes evident from the effect of adding Eu(NO\(_3\))\(_3\):5H\(_2\)O to an aqueous solution of 10b (8.5 × 10\(^{-5}\) M) on the total luminescence spectrum (see Figure 3a). In the absence of Eu\(^{3+}\), 10b displays the typical fluorescence spectrum of the chrysene unit (Φ\(_{fus}\) = 0.15, Φ\(_{SC}\) = 0.85 reported for chrysene\(^{39}\)). Upon addition of Eu\(^{3+}\), the sensitized lanthanide emission appears, and its concentration dependence (see Figure 3b) nicely confirms formation of a 1:1 complex between 10b and Eu\(^{3+}\). However, at the same time, the chrysene fluorescence diminishes to about 10% of its original intensity. This fluorescence quenching seems to occur via nonspecific (dynamic) quenching and is not limited to a 1:1 stoichiometry. For the fluorescence quenching both direct energy transfer from the chrysene singlet excited state to the lanthanide ion and enhanced intersystem crossing of chrysene under the influence of a “heavy atom effect” of the lanthanide are viable mechanisms.\(^{40}\) However, charge-transfer interaction between the singlet state of chrysene and the lanthanide also may be involved. The excited-state oxidation potential of chrysene is estimated to be \(E_{ox}^* = -2.1\) V (calculated as the ground state oxidation potential \(E_{ox} = 1.35\) V vs mtophore around 365 nm (see Figure 1). As we discussed before,\(^{19}\) this value must come close to the longest excitation wavelength possible for sensitization of Eu\(^{3+}\), since after excitation of the chromophore first intersystem crossing to the lowest triplet state is assumed to occur, which can subsequently give energy transfer to the luminescent lanthanide level (see Figure 2). For organic (poly)aromatic systems, a minimal single–triplet energy gap (\(E_{00} - E_{01}\)) of 5000 cm\(^{-1}\) may be assumed (in chrysene this is 7800 cm\(^{-1}\)), while the triplet must still have high enough energy to make the energy transfer fast and to exclude possible (thermal) back-energy transfer. For chrysene the triplet energy is 20 000 cm\(^{-1}\),\(^{39}\) only 2500 cm\(^{-1}\) above the luminescent Eu\(^{3+}\) level, implying that in compound Eu(10b) probably already some back energy transfer occurs at room temperature.

Not unexpectedly, with Tb\(^{3+}\) it was found that 10b does not allow sensitized luminescence to be observed. This again can readily be explained from the energy diagram depicted in Figure 2, assuming that energy transfer to the lanthanide ion occurs from the antenna triplet.

Interestingly, in the case of 10b the effect of complexation with lanthanide ions is not restricted to quenching of the chrysene triplet. This becomes evident from the effect of adding Eu(NO\(_3\))\(_3\):5H\(_2\)O to an aqueous solution of 10b (8.5 × 10\(^{-5}\) M) on the total luminescence spectrum (see Figure 3a). In the absence of Eu\(^{3+}\), 10b displays the typical fluorescence spectrum of the chrysene unit (Φ\(_{fus}\) = 0.15, Φ\(_{SC}\) = 0.85 reported for chrysene\(^{39}\)). Upon addition of Eu\(^{3+}\), the sensitized lanthanide emission appears, and its concentration dependence (see Figure 3b) nicely confirms formation of a 1:1 complex between 10b and Eu\(^{3+}\). However, at the same time, the chrysene fluorescence diminishes to about 10% of its original intensity. This fluorescence quenching seems to occur via nonspecific (dynamic) quenching and is not limited to a 1:1 stoichiometry. For the fluorescence quenching both direct energy transfer from the chrysene singlet excited state to the lanthanide ion and enhanced intersystem crossing of chrysene under the influence of a "heavy atom effect" of the lanthanide are viable mechanisms.\(^{40}\) However, charge-transfer interaction between the singlet state of chrysene and the lanthanide also may be involved. The excited-state oxidation potential of chrysene is estimated to be \(E_{ox}^* = -2.1\) V (calculated as the ground state oxidation potential \(E_{ox} = 1.35\) V vs mtophore around 365 nm (see Figure 1). As we discussed before,\(^{19}\) this value must come close to the longest excitation wavelength possible for sensitization of Eu\(^{3+}\)

\(^{36}\) 6-Chrysencarboxaldehyde (1b) was prepared in 71% yield from chrysene (1a) and 1,1,2,2-tetrachlormethyl methyl ether/TICl\(_3\). An alternative to prepare the aldehyde 1b is the oxidation of 6-(hydroxymethyl)-chrysene. Akiyama, S.; Nakagawa, M. Bull. Chem. Soc. Jpn. 1972, 45, 259.


SCE minus the 0-0 energy $E_{00} = 3.45$ V), which allows electron transfer to Eu$^{3+}$ ($E_{\text{red}} = -0.6$ V vs SCE) to occur. This conclusion is supported by the fact that the fluorescence of 10b is quenched to a much lesser degree by Tb$^{3+}$ (see Figure 3b). Tb$^{3+}$ is very hard to reduce ($E_{\text{red}} = -3.9$ V vs SCE) and also very hard to oxidize ($E_{\text{ox}} = 2.9$ V vs SCE), making photoinduced electron transfer between Tb$^{3+}$ and chrysene impossible.

We have reported previously on sensitized luminescence of calix[4]arene/lanthanide complexes containing an antenna chromophore attached to the lower rim of the calixarene cage, to a complexed lanthanide ion, and employing three carboxylic acid groups as the complexing moieties, which resulted in overall neutral lanthanide complexes. Depending on the chromophore used, excitation wavelengths up to 350 nm could be achieved. However, these complexes could only be studied in organic solvents (e.g., methanol) because of their insolubility in aqueous media.

The present lanthanide complexes combine long-wavelength excitation (i.e., 10b) and overall electroneutrality with water solubility via introduction of substituents at the upper rim of the calix[4]arene cage. The luminescent lifetime for Eu(10b) is 0.11 and 0.14 ms in aerated and deoxygenated aqueous solutions, respectively. These lifetimes are shorter than those observed previously for calix[4]arene/Eu$^{3+}$ complexes in methanol (0.23-1.3 ms), thus demonstrating the stronger quenching ability of water. However, the present luminescence lifetimes are also certainly useful in schemes employing time-gated emission measurements to suppress the influence of the general background fluorescence of biological material, which usually has a lifetime in the nanosecond region.

While the 2-pyridyl disulfide group in 10a only allows short wavelength excitation, it can be used as a coupling reagent toward SH functionalities in biological material.

Figure 3. (a) Total luminescence spectra of 10b (8.5 × 10$^{-5}$ M) upon addition of increasing amounts of Eu(NO$_3$)$_3$·5H$_2$O in Tris–HCl buffer (pH 8.0); deoxygenated, $\lambda_{\text{ex}} = 363$ nm. (b) Relative luminescence intensities of the chrysene fluorescence (384 nm) and Eu$^{3+}$ luminescence (614 nm) upon addition of increasing amounts of Eu(NO$_3$)$_3$·5H$_2$O or Tb(NO$_3$)$_3$·5H$_2$O to 10b in Tris–HCl buffer (pH 8.0) for both aerated (ox) and deoxygenated (deox) solutions; $\lambda_{\text{ex}} = 363$ nm.

Conclusions

We have developed a facile route to neutral, water-soluble calix[4]arene-based lanthanide complexes. Moreover, our approach has the potential for variation of substituents in the carboxamido fragment at the lower rim. Chrysene gave high energy transfer from a 1:1 complex ligand/Eu$^{3+}$ at wavelengths extending to 363 nm. Currently, we are investigating the possibility of introducing both a sensitizer and a handle for coupling to biomolecules in the synthesis route.

Experimental Section

Synthesis. General Procedure. Melting points are uncorrected. 'H NMR and 13C NMR spectra were recorded in CDCl3 with MeSi as the internal standard unless stated otherwise. Preparative column chromatography separations were performed on Merck silica gel 230–400 mesh, while precoated silica gel plates (Merck, 60 F254) were used for analytical TLC. FAB mass spectra were performed with m-nitrobenzyl alcohol as a matrix unless stated otherwise. CHCl3, used in the chlorosulfonylation reaction, was flushed over an Al2O3 column and dried on molecular sieves (4 Å). All other chemicals were analytically pure and were used without further purification. Hexane refers to the fraction with bp 40–60 °C. All reactions were carried out under an argon atmosphere. Standard workup means that the organic layers were finally washed with water, dried over magnesium sulfate (MgSO4), filtered, and concentrated in vacuo. The presence of solvents in the analytical samples was confirmed by 'H NMR spectroscopy or potentiometric titrations. Fast atom bombardment (FAB) mass spectrometry for the water-soluble calix[4]arenes 10a,b was carried out in a matrix solution (glycerol, thioglycolic acid, m-nitrobenzyl alcohol) onto a stainless steel probe and bombarded with xenon atoms with an energy of 3 keV. During these high-resolution FTR-MS measurements a resolving power of 10,000 (10% valley definition) was used. Cesium iodide and/or glycerol were used to calibrate the mass spectrometer. S-(2-Pyridylthio)lysine hydrochloride,22 tert-butylated calix[4]arene, and calix[4]arene tetraethyl ester were prepared according to literature procedures.22

Chrysene-6-carboxaldehyde (1b). A solution of chrysene (1a) (2.0 g, 8.76 mmol) and freshly distilled TiCl4 (3.30 g, 20.8 mmol) in dry THF (75 mL) was added BH3·SMe2 (9.1 mmol) in dry THF (5 mL), and the solution was stirred for 2 h at rt. The mixture was added to the residue. The organic layer was washed with 2 M HCl (2 × 100 mL), followed by standard workup. The crude reaction product was purified by flash column chromatography (CHCl3:hexane, 50:50). The product was crystallized from CH2Cl2/hexane to obtain a yellow colored solid: yield 71%; mp 165–168 °C (lit.26 mp 164–166 °C).

6-(Hydroxymethyl)chrysene (1c). To a solution of 1b (1.0 g, 3.9 mmol) in dry THF (75 mL) was added BH3·SMe2 (0.325 g, 6.46 mmol) and freshly distilled TiCl4 (3.30 g, 20.8 mmol) in dry THF (3 mL). The reaction mixture was stirred at rt for 30 min. The reaction was quenched by the addition of crushed ice (15 g). The organic layer was washed with 2 M HCl (2 × 100 mL), followed by standard workup. The crude reaction product was purified by flash column chromatography (CHCl3:hexane, 50:50). The product was triturated with CH2Cl2 to give pure 1c: yield 94%; mp 200–203 °C (lit.26 mp 200–202 °C).

6-(Phthalimidomethyl)chrysene (1e). A solution of 1d (1.5 g, 5.7 mmol) in DFU (25 mL) was added pyridinephthalamide (0.32 g, 1.72 mmol) in dry DFU (35 mL) was added dropwise (choloromethyl) ether (10.0 g, 86.8 mmol) at −10 °C. The reaction mixture was stirred at rt for 30 min. The reaction was quenched by the addition of crushed ice (15 g). The organic layer was washed with 2 M HCl (2 × 100 mL), followed by standard workup. The crude reaction product was purified by flash column chromatography (CHCl3:hexane, 50:50). The product was crystallized from CH2Cl2/hexane to obtain a yellow colored solid: yield 71%; mp 165–168 °C (lit.26 mp 164–166 °C).

6-(Aminomethyl)chrysene (1f). A solution of 1e (0.50 g, 1.29 mmol) in EtOH (15 mL) was refluxed for 2 h. The solvent was removed in vacuo, and H2O (50 mL) and CH2Cl2 (50 mL) were added to the residue. The organic layer was washed with NaHCO3 (saturated aqueous solution, 50 mL), followed by standard workup: yield 95%; mp 204–206 °C; 'H NMR δ 8.48 (d, 2 H, J = 8.4 Hz), 8.73 (d, 2 H, J = 8.4 Hz), 8.70 (d, 1 H, J = 8.4 Hz), 7.75 Hz), 8.22 (d, 1 H, J = 7.5 Hz), 8.00 (d, 2 H, J = 8.3 Hz), 7.8–7.5 (m, 4 H), 4.52 (s, 2 H), 1.69 (broad s, 2 H); 13C NMR δ 144.79; mass spectrum (EI) m/z 257.2 (M+1, calcld 257.1). Anal. Calcd for C16H12N2O: C, 86.20; H, 5.49; N, 5.12. Found: C, 86.20; H, 5.49; N, 5.12.

7.4
4.6 (m, 16 H), 4.22 (q, 6 H, J 2 H), 3.4

m spectrum

combined layers were dried over MgSO₄, filtered, and concentrated in vacuo.

1 H, J = 4.2 Hz), 8.05 (broad s, 1 H), 7.7—7.5 (m, 3 H), 7.4—7.1 (m, 8 H), 6.69 (broad s, 4 H), 6.58 (broad s, 4 H), 5.0—4.6 (m, 16 H), 4.22 (q, 6 H, J = 8.3 Hz), 3.81 (s, 24 H), 3.69 (m, 2 H), 3.4—3.1 (m, 12 H), 2.99 (t, 2 H, J = 6.7 Hz), 1.3—1.2 (m, 9 H), 0.83 (s, 108 H), 0.00 (s, 72 H); FAB mass spectrum (ONPOE) m/z 3238.0 (M⁺, calc 3237.5), 3128.6 (M⁺ — S-pyridine). Anal. Calcd for C₁₄₆H₂₇₃N₉O₃₅S₄Si₁₂: C, 53.44; H, 8.36; N, 4.30. Found: C, 53.30; H, 8.51; N, 4.17.

5, 11, 17, 23-Tetrakis[(tert-butyldimethyl-silyloxy)methyl]amino]carbonyl]methoxy]calix[4]arene (8b) was prepared similarly as compound 8a starting from the trichloroethyl ester 7 (1.65 g, 0.5 mmol), 6-(aminomethyl)chrysene (1f) (0.15 g, 0.66 mmol) and DBU (0.71 g, 4.6 mmol). The crude solid was purified by column chromatography (silica gel saturated with NaCl, ethyl acetate/CH₂Cl₂, 1:3) and eluted with ethyl acetate/H₂O to obtain a white pure solid: yield 99%; during melting point determination the compound decomposed; 1H NMR (D₂O, 80 °C) δ 7.1 Hz), 0.85 (s, 108 H), 0.01 (s, 72 H); FAB mass spectrum (glycerol) m/z 3333.6 (M⁺ — S-pyridine). Anal. Calcd for C₁₄₅H₂₆₈N₁₀O₃₅S₆Si₁₂: C, 53.30; H, 8.51; N, 4.17.

General Procedure for the Preparation of Triacid Calix[4]arenes 9a, b (0.5 g) in trifluoroacetic acid (TFA):H₂O (9:1, 25 mL) was stirred overnight, whereupon toluene (50 mL) was added. The solution was concentrated in vacuo. Again, toluene (50 mL) was added, and the mixture was concentrated in vacuo. This procedure was repeated three times. The crude white solid was dissolved in a minimum amount of H₂O (5 mL). To this solution was added dropwise acetonitrile (10a) or acetone (10b) until the product started to precipitate. After the suspension was stirred for 30 min, the product was filtered. The product was freeze-dried and analyzed. Prior to the luminescence measurements, solutions of compounds 10a, b in water were dialyzed (benzoylated cellulose membrane) to remove excess trifluoroacetic acid and again freeze-dried.

5, 11, 17, 23-Tetrakis[(hydroxyethyl)methyl]amino]carbonyl]methoxy]calix[4]arene (10a). A solution of tricapid calix[4]arene (8a, b) starting from the trichloroethyl ester 7 (5 mmol) in MeOH:H₂O 5:1 (50 mL) and a minimum amount of THF, for solubility reasons, was added K₂CO₃ (50 mmol). After the solution was refluxed for 15 min, CH₂Cl₂ (50 mL) was added, and the solution was acidified with 1 M HCl to pH 7. The aqueous layer was extracted with CH₂Cl₂ (2 × 50 mL). The combined layers were dried over MgSO₄, filtered, and concentrated in vacuo to give the pure products.

The characterization of the derivatives 9a, b with ¹H NMR spectroscopy was difficult because of line broadening. Additionally, the relative intensity of the ¹H CH₃ resonances (TBDMs) compared to the other ¹H-resonances is large. No satisfactory mass spectrum (FAB and FRIT-FAB) could be obtained for the triacid derivatives 9a, b. Therefore, characterization was done by melting point determination and elemental analysis.

**Figure 4.** Corrected emission spectra of Eu(10a) (- - ; λex = 250 nm) and Eu(10b) (aerated: -----; deoxygenated: ---; λex = 363 nm) in Tris—HCl buffer (pH 8.0) at room temperature.
7.2 (m, 19 H), 5.4–4.8 (m, 18 H), 4.69 (broad s, 24 H), 3.82 (d, 4 H, J = 9.3 Hz), 3.27 (d, 4 H, J = 11.5 Hz); FAB mass spectrum (glycerol) m/z 1857.8 ([M + H]+, calcd 1856.8); exact FRIT-FAB m/z 1856.4735 [M+, calcd 1856.4761]; the fragmentation pattern corresponds with the proposed structure. Anal. Calcd for C79H93N9O35S4·4TFA: C, 45.10; H, 4.01; N, 5.43. Found: C, 45.18; H, 4.23; N, 5.45.

**Luminescence Measurements.** Continuous emission and excitation spectra were recorded on a Spex Fluorolog 2 spectrofluorimeter. Figure 4 gives representative results of such spectra.

Time-resolved emission spectra were obtained using a Lumonics EX700 XeCl excimer laser (308 nm) as excitation source. The resulting luminescence was observed by a gated, intensified CCD camera from Princeton Instruments. Spectra were taken with a typical gatewidth of 25 µs, an initial delay of about 0.5 µs relative to the laser pulse maximum, and 50 µs increment delay between spectra. At each delay, spectra were averaged over 50 laser shots to improve the signal to noise ratio. From these data, luminescent lifetimes were calculated by fitting the wavelength integrated signal in time. Monoexponential decay was observed in all cases.

All measurements were done in Tris–HCl buffer (0.05 M, pH 8.0).

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