A new europium chelate-based phosphorescence probe specific for singlet oxygen†

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The first Eu^{3+} chelate-based phosphorescence probe specific for singlet oxygen has been designed, synthesized and characterized. The probe is highly sensitive, selective and water soluble for time-resolved luminescence detection of singlet oxygen with a detection limit of 2.8 nM.

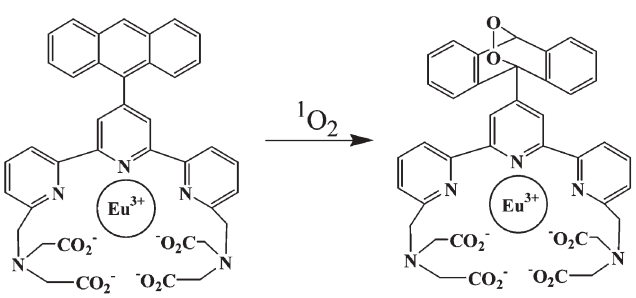
Singlet oxygen ($^1O_2$), an excited state of molecular oxygen, is a useful oxidant in organic synthesis and has an important biological reactivity. As an intermediate species in the detrimental oxidation of biomolecules, it can react with many kinds of biomolecules, such as DNA, protein and lipids.[1] Furthermore, it plays an important role in the cell cascade and induction of gene expression.[2] Since oxygen is ubiquitous and efficiently quenches electronically excited states, $^1O_2$ is likely to be formed following irradiation in countless situation and involved in various chemical, biological, and several disease processes.[3]

Due to the outstanding importance of $^1O_2$ in photochemical and photobiological processes, several methods for $^1O_2$ detection have been developed. Monitoring the direct emission of $^1O_2$ at 1270 nm is a specific and noninvasive method, but this method suffers from weak signal, and quantitative detection of very small amounts of $^1O_2$ is currently not possible in any medium.[4] Chemical trapping by spectroscopic probes is also found to be specific and much more sensitive than the detection of the 1270 nm luminescence. A commonly used $^1O_2$ trap, 9,10-diphenylanthracene (DPA), can react specifically with $^1O_2$ to form a thermostable endoperoxide at a rate of $k = 1.3 \times 10^6$ $M^{-1}s^{-1}$ accompanied by the decrease in absorbance at 335 nm as a signal of $^1O_2$ production.[5] However, this method is less sensitive because the detection is based on the measurement of absorbance. Nagano's group has synthesized two fluorescence probes for $^1O_2$ by conjugating fluorophore of fluorescein with 9,10-diphenylanthracene or 9,10-dimethylanthracene.[6] These probes can react with $^1O_2$ to yield the corresponding endoperoxides giving sensitive fluorescence responses. Recently, a chemiluminescence probe for $^1O_2$ by incorporating an electron-rich tetrathiafulvalene unit into a reactive luminophore of anthracene has been developed.[7] This probe exhibits strong chemiluminescence response and high selectivity for $^1O_2$ with a detection limit of 76 nM. The main drawback of this probe is its lower water solubility, a buffer containing 50% THF is necessary to dissolve the probe, which makes it rather unsuitable for use with biosystems.

Time-resolved fluorometry combined with the use of lanthanide chelate-based luminescence probes has provided an excellent way for developing highly sensitive bioaffinity assays. The applications of lanthanide luminescence probes for time-resolved fluoroimmunoassay, DNA hybridization assay, and luminescence microscopy bioimaging have been extensively investigated.[8] Different from organic fluorescence probes, lanthanide luminescence probes have the properties of long luminescence lifetime, large Stokes shift and sharp emission profile, which makes them suitable for use in microsecond time-resolved luminescence measurement to minimize the interference caused by background noises associated with biological samples, scattering lights (Tyndall, Rayleigh and Raman scatterings) and the optical components (cuvettes, filters and lenses).[9]

In the present work, a novel Eu^{3+} chelate-based phosphorescence probe specific for time-resolved luminescence detection of $^1O_2$, [4′-(9-anthryl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo) tetrakis(acetate)-Eu^{3+} (ATTA-Eu^{3+}) was designed and synthesized. In this chelate, the 9-anthryl group was used as a specific reactive moiety for $^1O_2$,[5–7] and (2,2′:6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo) tetrakis(acetate)-Eu^{3+}, as a fluorophore. The almost non-luminescent chelate can specifically react with $^1O_2$ to yield its endoperoxide (EP-ATTA-Eu^{3+}) with a great increase of the phosphorescence intensity, which can be used for time-resolved luminescence measurement (Scheme 1).

The new ligand ATTA was synthesized following the eight-step reaction (S1 in supporting information†). The corresponding endoperoxide of its Eu^{3+} chelate was synthesized by reacting ATTA-Eu^{3+} with chemically generated $^1O_2$ (MoO_4^{2-}/H_2O_2),[10] and the production of EP-ATTA-Eu^{3+} was confirmed by mass spectrum detection (S2 in supporting information†). The phosphorescence properties of ATTA-Eu^{3+} and EP-ATTA-Eu^{3+} are listed in Table 1. The excitation and emission maximum



Scheme 1 Reaction of ATTA-Eu^{3+} with $^1O_2$.

† Electronic supplementary information (ESI) available: synthesis, experimental detail and characterization of ATTA and EP-ATTA-Eu^{3+}. See http://dx.doi.org/10.1039/b503980k

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Table 1 Phosphorescence properties of ATTA-Eu^{3+} and EP-ATTA-Eu^{3+}[a]

Chelate	$\lambda_{ex,max}$ (nm)	$\lambda_{em,max}$ (nm)	$\varepsilon_{335\ nm}$ ($cm^{-1}M^{-1}$)	ϕ (%)	τ (μs)
ATTA-Eu^{3+}	289,335	615	17200	0.58	989
EP-ATTA-Eu^{3+}	289,335	615	14500	10.0	1209

[a] All data were obtained in 0.05 M borate buffer of pH 9.1.

wavelengths ($\lambda_{ex,max}$, $\lambda_{em,max}$) are not changed (there is no significant difference in the UV spectrum patterns between ATTA-Eu^{3+} and EP-ATTA-Eu^{3+}, which is similar to the Nagano's probe[6]), and molar absorption coefficients (ε) and luminescence lifetimes (τ) are changed slightly from ATTA-Eu^{3+} to EP-ATTA-Eu^{3+}. However, the luminescence quantum yield (ϕ) is drastically increased after the formation of EP-ATTA-Eu^{3+}. The luminescence quantum yield of EP-ATTA-Eu^{3+} is 17 times higher than that of ATTA-Eu^{3+}, so it can be said that ATTA-Eu^{3+} itself is almost non-luminescent, while EP-ATTA-Eu^{3+} is strongly luminescent.

When the EP-ATTA-Eu^{3+} complex was challenged with a five-fold excess of ethylenediamine tetraacetic acid, a conditional stability constant was measured to be $\sim 10^{20}$ by using the Verhoeven's method.[11] Moreover, no decrease of the phosphorescence intensity of EP-ATTA-Eu^{3+} was observed after several days at room temperature. Using the luminescence lifetimes of ATTA-Eu^{3+} and EP-ATTA-Eu^{3+} in H_2O and D_2O buffers, the average number (q) of water molecules in the first coordination sphere of Eu^{3+} ion was calculated from the equation of $q = 1.2\ (1/\tau_{H2O} - 1/\tau_{D2O} - 0.25)$ to be 0.05 and 0.04, respectively.[12] These results show that the probe has a high kinetic and thermodynamic stability, and the increase of the probe's phosphorescence intensity is not caused by the decrease of the number of the coordinated water molecules.[13]

Within a general paradigm, the overall luminescence quantum yield (ϕ_{tot}) of a lanthanide complex upon exciting of the chromophore of ligand is determined by the efficiency of the sensitization (η_{sens}) and by the quantum yield (ϕ_{Ln}) of the lanthanide luminescence step ($\phi_{tot} = \eta_{sens}\phi_{Ln}$).[14] In the present case, the excited state lifetime and the chemical surroundings of the ion of the EP-ATTA-Eu^{3+} are very similar to the ones of ATTA-Eu^{3+}, so the significant increase of luminescence quantum yield can be considered due to an increase in the efficiency of the population of the excited state of the Eu^{3+} ion. During photosensitised luminescence of the Eu^{3+} ion by ligand, the triplet state of terpyridine chromophore (CT_1, E = 22400 cm^{-1})[15] transfers the energy to $^5D_0$ energy level of Eu^{3+} (E = 17374 cm^{-1})[16] after the excitation of the terpyridine chromophore at 335 nm. However, the strong triplet (CT_1) – triplet (anthracene, AT_1, E = 14900 cm^{-1}) quenching blocks the effective energy transfer of $CT_1 \rightarrow {}^5D_0$,[17] so the phosphorescence of ATTA-Eu^{3+} is very weak. After the formation of EP-ATTA-Eu^{3+}, the triplet–triplet quenching between CT_1 and AT_1 disappears, thus the Eu^{3+} chelate becomes strongly luminescent.[18]

The effects of pH on the phosphorescence intensity and lifetime of EP-ATTA-Eu^{3+} have been investigated (S3 in supporting information†). In contrast to the rapid decrease of fluorescence intensity of fluorescein-based probes at pH < 7,[6] the phosphorescence intensity of EP-ATTA-Eu^{3+} is stable at pH > 3. This result indicates that ATTA-Eu^{3+} is very useful as a luminescence probe for $^1O_2$ in weakly acidic, neutral and basic buffers.

The reaction of hydrogen peroxide disproportionation catalyzed by molybdate ions was used as a chemical source of $^1O_2$ for the detection of $^1O_2$ production using ATTA-Eu^{3+} as the probe. The reaction was performed in 0.1 M carbonate buffer of pH 10.5, since the MoO_4^{2-}/H_2O_2 system only works under basic conditions.[10] A series of H_2O_2 solutions were added to the buffer solutions containing 10 μM of ATTA-Eu^{3+} and 1 mM of Na_2MoO_4. After the reaction, the solutions were 10-fold diluted (final probe concentration = 1.0 μM) with 0.05 M borate buffer of pH 9.1, and the excitation and emission spectra were measured with a time-resolved mode. As shown in Fig. 1, the phosphorescence signals show a good response with the increase of H_2O_2 concentration, and the emission of the probe consists of several discrete bands between 580 and 710 nm corresponding to the $^5D_0 \rightarrow {}^7F_J$ (J = 0–4) transitions of Eu^{3+}. This emission feature indicates that the emission of the probe can be monitored at

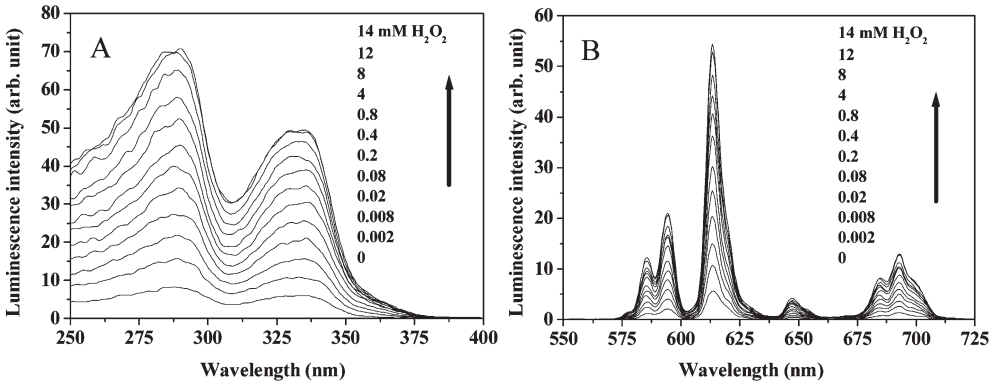

Fig. 1 Time-resolved excitation (A) and emission (B) spectra of ATTA-Eu^{3+} in the reaction with $^1O_2$ generated from a MoO_4^{2-}/H_2O_2 system. The conditions of delay time, 0.2 ms, gate time, 0.4 ms, cycle time, 20 ms, excitation slit, 10 nm, and emission slit, 5 nm were used for the measurements. Excitation spectra were recorded with λ_{em} = 615 nm and emission spectra with λ_{ex} = 335 nm.

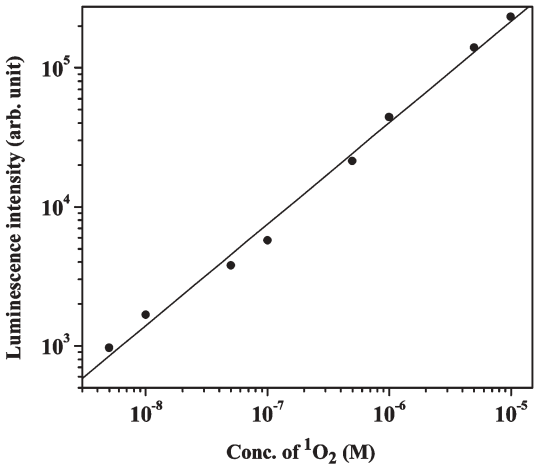

Fig. 2 Calibration curve for $^1O_2$. The curve was derived from the luminescence intensity of the H_2O_2/MoO_4^{2-}/ATTA-Eu^{3+} reaction in 0.1 M carbonate buffer of pH 10.5 with 100 nM of ATTA-Eu^{3+}, 10 mM of Na_2MoO_4 and a series of standard H_2O_2 solutions.

several discrete points between 580 and 710 nm. The significant increase of phosphorescence signal of the probe was also observed by using the photosensitization of rhodamine B as a $^1O_2$ source[19] in a 0.05 M Tris-HCl buffer of pH 7.4. When azide, a quencher of $^1O_2$,[20] was added to MoO_4^{2-}/H_2O_2/ATTA-Eu^{3+} system, the change of the probe's phosphorescence intensity can not be observed. These results distinctly indicate that the increase of phosphorescence intensity is caused by the reaction of the probe with $^1O_2$.

Because of quantitative generation of $^1O_2$ from MoO_4^{2-}/H_2O_2 system (one $^1O_2$ molecule can be formed quantitatively by the reaction of two H_2O_2 molecules),[10] this system was used for the quantitative detection of $^1O_2$. As shown in Fig. 2, the dose-dependence of phosphorescence intensity of the probe on $^1O_2$ concentration shows a good linearity. The detection limit for $^1O_2$, calculated as the concentration corresponding to three standard deviations of the background signal, is 2.8 nM, which is ~28 times lower than that of the chemiluminescence method.[7]

The reactions of ATTA-Eu^{3+} with different reactive oxygen species (H_2O_2, $^{\bullet}OH$, O_2^{-}, and $^1O_2$) were investigated to examine its selectivity. In the same buffer, the phosphorescence intensities of 100 nM ATTA-Eu^{3+} upon reactions with 10 μM H_2O_2, 10 μM H_2O_2 + 10 μM ferrous ammonium sulfate ($^{\bullet}OH$),[7] 10 μM KO_2 (O_2^{-})[21] and 10 μM H_2O_2 + 10 mM Na_2MoO_4 ($^1O_2$) were increased 46%, 76%, 6% and 1246%, respectively. These results indicate that the probe ATTA-Eu^{3+} is highly specific for $^1O_2$.

In summary, the first Eu^{3+} chelate-based phosphorescence probe specific for time-resolved luminescence detection of $^1O_2$ has been designed, synthesized and characterized. The photophysical characterization and the mechanism of the phosphorescence of the probe were discussed. The properties of high sensitivity, selectivity and water solubility of the new probe for $^1O_2$ detection suggest that the probe should be widely useful for the luminescence detection of $^1O_2$ in many chemical and biological systems. Perhaps, the time-resolved luminescence microscopy imaging of $^1O_2$ in biological systems using the new probe would be a favorably useful technique for visualizing the temporal and spatial distribution of $^1O_2$ in biological samples.

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