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A β-diketonate–europium(III) complex-based time-gated luminescence probe for selective visualization of peroxynitrite in living cells

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ABSTRACT

Herein, we designed and synthesized a new β-diketonate–Eu^{3+} complex-based luminescent probe for peroxynitrite ($ONOO^-$), $Eu(hdph)_3(tpy)$, by using a 2,4-dimethoxyphenyl-substituted β-diketone ligand 1,1,1,2,2,3,3-hetafluoro-6-(2,4-dimethoxyphenyl)-4,6-hexanedione (hdph) and a coligand 2,2′,2″-terpyridine (tpy). The probe exhibits strong fluorescence with a great quantum yield of 25.6%. Upon the addition of $ONOO^-$, the luminescence of $Eu(hdph)_3(tpy)$ at 607 nm was dramatically quenched due to the interaction of the 2,4-dimethoxyphenyl moiety with $ONOO^-$, which leads to the deactivation of the excited Eu^{3+} complex and a 71-fold luminescence decrease. The dose-dependent luminescence decrease showed good linear relationship against the $ONOO^-$ concentration with the detection limit at nanomolar concentration level. In addition, the luminescence response of $Eu(hdph)_3(tpy)$ to $ONOO^-$ displayed high specificity over various reactive oxygen/nitrogen species. Taking advantage of the high sensitivity and specificity, $Eu(hdph)_3(tpy)$ was applied for the time-gated luminescence imaging of $ONOO^-$ in HepG2 cells. The results demonstrated the practical applicability of $Eu(hdph)_3(tpy)$ as a luminescent probe for the long-term visualization of intracellular $ONOO^-$.

1. Introduction

Peroxynitrite ($ONOO^-$), as a strong oxidant and efficient nitrating agent, is *in vivo* generated by the immediate reaction of nitric oxide (NO) and superoxide ($O_2^{\bullet-}$) without the involvement of enzymes [1]. In contrast to its beneficial role in signal transduction and immunogenic response against microbial invasion [2–4], abnormal $ONOO^-$ levels can have harmful effects on a number of bioactive species in cells, including nucleic acids, proteins, and lipids. Moreover, $ONOO^-$ can be subsequently decomposed into metastable radicals, such as hydroxyl radical ($^{\bullet}OH$), nitrogen dioxide ($^{\bullet}NO_2$) and carbonate radicals ($CO_3^{\bullet-}$), which will further react with biomolecules and cause irreversible damages. Increasing evidences have shown that the overproduction of $ONOO^-$ is responsible for a variety of diseases, such as ischemia–reperfusion injury, neurodegeneration, inflammation, cardiovascular diseases, and cancer [5]. Therefore, development of sensitive and selective methods for detecting intracellular $ONOO^-$ is very conducive for the exploration of its physiological and pathological roles in disease diagnosis.

Many techniques have been employed for $ONOO^-$ detection, such as UV–vis absorbance spectroscopy, electron paramagnetic resonance spectroscopy, immunohistochemistry, and chemiluminescence [6–10]. Unfortunately, all these methods are not biocompatible with living biosystems, and cannot measure the dynamic changes of the $ONOO^-$ concentrations within cells. Owing to the short half-life and low concentration of $ONOO^-$ under physiological conditions, the development of an accurate detection method for intracellular $ONOO^-$ remains a challenge. Recently, fluorescent probe technique has been developed as a powerful tool for detecting $ONOO^-$ *in vivo* due to the superiorities of high sensitivity, high spatial resolution, and real-time and non-destructive detection [11–14]. Several $ONOO^-$-specific fluorescent probes have been synthesized by exploiting the $ONOO^-$-triggered recognition reactions including aromatic nitration [15], organoselenium/organotellurium oxidation [16,17], phenol derivatives oxidation [18–21], and others [22,23]. However, such probes still have some limitations including poor photostability and strong background fluorescence interference.

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Time-gated luminescence imaging technique using lanthanide (mainly Eu^{3+} and Tb^{3+}) complexes as probes has been widely used for *in vitro* and *in vivo* detection of various bioactive species [24–30]. Compared to conventional organic fluorophore, the lanthanide luminescent probes possess super-long luminescence lifetime and large Stokes shift, which enable the usage of time-gated detection mode to effectively eliminate the short-lived background fluorescence from the complicated biosamples [31–33]. Recently, a lanthanide complex-based ratiometric probe with a 2,4-dimethoxyphenyl-substituted terpyridine polyacid derivative ligand, 4′-(2,4-dimethoxyphenyl)-2,2′:6′,2″-terpyridine-6,6″-diyl-bis(methylenenitrilo)tetrakis(acetate)-Eu^{3+}/Tb^{3+} (DTTA-Eu^{3+}/Tb^{3+}), has been demonstrated to work well for the time-gated luminescence imaging of $ONOO^-$ in living cells [34]. Nevertheless, the tedious synthesis procedures and low quantum yields of these terpyridine polyacid–Eu^{3+} complexes limit their broader applications [35]. It is well known that β-diketonate–Eu^{3+} complexes have significantly high quantum yields, and thus several β-diketonate–Eu^{3+} complex-based luminescent probes have been synthesized and used for the detection of diverse biological molecules and metal ions [31,36,37].

For simplifying the synthesis and improving the luminescence properties of the Eu^{3+} complex-based luminescent probe for $ONOO^-$, in this work, we designed and synthesized a new β-diketonate–Eu^{3+} complex-based luminescent probe for $ONOO^-$, $Eu(hdph)_3(tpy)$ by using a 2,4-dimethoxyphenyl-substituted β-diketone ligand 1,1,1,2,2,3,3-hetafluoro-6-(2,4-dimethoxyphenyl)-4,6-hexanedione (hdph) and a coligand 2,2′,2″-terpyridine (tpy) for the time-gated luminescence detection of intracellular $ONOO^-$. The probe was highly luminescent with a great quantum yield. Upon addition of $ONOO^-$, the luminescence intensity of $Eu(hdph)_3(tpy)$ at 607 nm was dramatically decreased. The luminescence response of the probe to $ONOO^-$ was investigated in aqueous media, and a good linear correlation was obtained between the luminescence intensity and the $ONOO^-$ concentration. To evaluate the applicability of the probe for cell imaging, the $Eu(hdph)_3(tpy)$-loaded HepG2 cells were prepared and used for the luminescence imaging detection of $ONOO^-$, and the photostabilities of the $Eu(hdph)_3(tpy)$-loaded cells were also examined. The results proved that the probe $Eu(hdph)_3(tpy)$ could be a very useful tool for the long-term monitoring of $ONOO^-$ in living cells.

2. Experimental

2.1. Reagents and materials

2′,4′-Dimethoxyacetophenone, 3-(4,5-dimethyl-2-thiazol-yl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and 3-morpholinosydnonimine (SIN-1, a $ONOO^-$ donor) were purchased from Sigma-Aldrich. 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene (NOC-13, a NO donor) was synthesized using a previously reported method [38]. Triton X-100 was purchased from Acros Organics. Anhydrous diethyl ether (Et_2O) and tetrahydrofuran (THF) were used after appropriate distillation and purification. Cultured HepG2 cells were obtained from Dalian Medical University. The isotonic saline solution consisting of 140 mM NaCl, 10 mM glucose and 3.5 mM KCl was prepared in our laboratory. Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

2.2. Physical measurements

^1H NMR spectra were measured on a Bruker Avance spectrometer (400 MHz). Mass spectra were recorded on a HP1100LC/MSD electrospray ionization mass spectrometer (ESI-MS). Elemental analysis was carried out on a Vario-EL analyser. Absorption spectra were measured on a Perkin-Elmer Lambda 35 UV–vis spectrometer. Time-gated luminescence spectra were measured on a Perkin-Elmer LS 50B luminescence spectrometer with the following conditions: delay time, 0.2 ms; gate time, 0.4 ms; cycle time, 20 ms; excitation slit, 10 nm; and emission slit, 10 nm. Luminescence lifetimes were measured on an FS5 spectrofluorometer of Edinburgh Instruments. Relative luminescence quantum yields (Φ_1) of $Eu(hdph)_3(tpy)$ and its reaction product with $ONOO^-$ were measured in 0.05 M borate buffer of pH 7.4 containing 0.25% cremophor C040, and calculated according to the equation $\Phi_1 = I_1\varepsilon_2C_2\Phi_2/I_2\varepsilon_1C_1$, with the Eu^{3+} complex, Eu^{3+}-(4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo)tetrakis-(acetate), as a reference ($\Phi_2 = 0.16$, $\varepsilon_{335\ nm} = 14300\ M^{-1}\ cm^{-1}$) [39]. In the equation, I_1 and I_2, ε_1 and ε_2, and C_1 and C_2 are the luminescence intensities, molar absorption coefficients, and concentrations for the measured complex and the standard complex, respectively. All bright-field and luminescence imaging measurements were carried out on a laboratory-use true-color time-gated luminescence microscope [40].

2.3. Synthesis of hdph

To 70 mL of anhydrous diethyl ether were added 4.84 g (89.6 mmol) of $NaOCH_3$, 4.84 g (20 mmol) of $C_3F_7COOC_2H_5$, and 3.6 g (20 mmol) of 2′,4′-dimethoxyacetophenone with stirring. After the solution was stirred at room temperature for 24 h, 100 mL of 15% H_2SO_4 was added, and the mixture was further stirred for 30 min. After evaporation to remove diethyl ether, the obtained sticky solid was washed with water and dissolved in 200 mL of boiling ethanol. The solution was filtered while hot, and the filtrate was condensed to 15 mL for recrystallization. The target compound was obtained as pale yellow crystal (2.8 g, 37.2% yield). ^1H NMR (400 MHz, $CDCl_3$): δ 3.89 (s, 3H), 3.93 (s, 3H), 6.48 (d, 1H), 6.59–6.62 (m, 1H), 7.00 (s, 1H), 8.01–8.03 (d, 2H). ESI-MS (*m*/*z*): 375.0 ($[M - H]^-$, 100%, calcd 375.1). Elemental analysis calcd (%) for $C_{14}H_{11}F_7O_4$ (hdph): C 44.69, H 2.95; found (%): C 44.82, H 2.87.

2.4. Synthesis of $Eu(hdph)_3(tpy)$

To 10 mL of ethanol were added 260 mg (0.75 mmol) of hdph, 92 mg (0.25 mmol) of $EuCl_3 \cdot 6H_2O$, 60 mg (0.25 mmol) of 2,2′:6′,2″-terpyridine, and 0.75 mL of aqueous solution of 1.0 M NaOH with stirring. After the mixture was refluxed for 1 h, the solution was cooled to room temperature. The precipitate was collected, and washed with water containing 10% ethanol. After drying, the product was recrystallized from ethanol, and white powder was obtained (196 mg, 47.6% yield). ESI-MS (*m*/*z*): 1533.4 ($[M + Na]^+$, 100%, calcd 1534.1). Elemental analysis calcd (%) for $C_{57}H_{41}EuF_{21}N_3NaO_{12}$ ($NaEu(hdph)_3(tpy)$): C 44.63, H 2.69, N 2.74; found (%): C 45.12, H 2.28, N 2.77.

2.5. Reaction of $Eu(hdph)_3(tpy)$ with $ONOO^-$

To 3 mL of 0.1 M carbonate buffer of pH 10.5 containing 0.25% cremophor C040 was added 60 μL of 1 mM $Eu(hdph)_3(tpy)$ in dimethyl sulfoxide (DMSO). After the solution was stirred for 5 min, different concentrations of $ONOO^-$ were added, and the solutions were stirred for 30 min at room temperature. The solutions were 10-fold diluted with 0.05 M borate buffer of pH 7.4 containing 0.25% cremophor C040, and then used for the time-gated luminescence