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NO Fluorescence Sensing by Europium Tetracyclines Complexes in the Presence of H_2O_2

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Abstract The effect on the fluorescence of the europium:
tetracycline (Eu:Tc), europium:oxytetracycline (Eu:OxyTc)
and europium:chlortetracycline (Eu:ClTc) complexes in ap-
proximately 2:1 ratio of nitric oxide (NO), peroxynitrite
(ONOO$^-$), hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$)
was assessed at three ROS/RNS concentrations levels, 30 °C
and pH 6.00, 7.00 and 8.00. Except for the NO, an enhance-
ment of fluorescence intensity was observed at pH 7.00 for all
the europium tetracyclines complexes—the high enhance-
ment was observed for H_2O_2. The quenching of the fluores-
cence of the Tc complexes, without and with the presence of
other ROS/RNS species, provoked by NO constituted the
bases for an analytical strategy for NO detection. The quanti-
fication capability was evaluated in a NO donor and in a
standard solution. Good quantification results were obtained
with the Eu:Tc (3:1) and Eu:OxyTc (4:1) complexes in the
presence of H_2O_2 200 μM with a detection limit of about
3 μM (Eu:OxyTc).

Keywords Europium:tetracyclines complexes · Nitric oxide
quantification · Fluorescence · Hydrogen peroxide ·
Peroxynitrite · Superoxide

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Introduction

Fluorescence sensing uses synthetic organic fluorophores,
fluorophores of biological origin, metal-ligand complexes,
lanthanide complexes and more recently fluorescent
nanoparticles [1]. The demand for alternatives of organic
fluorophores with better characteristics is a continuous chal-
lenge. Some of the already existing alternatives, as the
lantanide complexes, presents specific characteristics that
make them more considered in fluorescence sensing [2–6].
The lanthanide complexes presents some interesting lumines-
cence characteristics, as higher emission wavelength, narrow
fluorescence emission band, large Stokes shift, long fluores-
cence lifetime and an intense fluorescence emission in the
presence of some compounds that make them especially ade-
quate for their use as fluorescence sensors [7–13]. The euro-
pium tetracyclines complexes have been widely used for the
detection and quantification of several biochemical species
namely hydrogen peroxide (H_2O_2) [14–19], urea hydrogen
peroxide [20], low density lipoproteins [21–23], cholesterol
[24], bilirubin [25, 26], bile acid [27], lysozyme [28], nucle-
oside phosphates [29, 30], NADP [31], coenzyme a [32],
superoxide dismutase [33], DNA [34], heparin [35], lecithin
[36], catalase [37] and glucose [38]. Most of these analytical
methodologies are based on the enhancement of the fluores-
cence intensity by these species [14–24, 28, 31–38]. Only
with bilirubin, bile acid and nucleoside phosphates the sensing
is based on the fluorescence quenching [25–27, 29, 30].
Catalase and glucose determination was done indirectly
thought hydrogen peroxide detection [37, 38].

Reactive oxygen (ROS) and nitrogen (RNS) species, object
of this study, are usually involved in several physiological and
pathological processes acting as signalling molecules and
induce cell damage [39]. The detection and/or quantification
of these species in biological samples is not straightforward

due to their high reactivity, short half-life, lower concentrations, rapid diffusion, possible antioxidant mechanisms and potential interferences. The main ROS/RNS are nitric oxide (NO), peroxynitrite ($ONOO^-$), superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2). Their detection is essentially done by electron spin resonance (ESR), fluorescence, chemiluminescence, colorimetric and electrochemical analytical techniques [40–42]. Some applications of lanthanide complexes in ROS/RNS sensing involved the determination of hydroxyl radical [43, 44], singlet oxygen [45, 46] and NO [47].

In this work, the detection of the main ROS/RNS and its mixtures, by the europium:tetracycline (Eu:Tc), europium:oxytetracycline (Eu:OxyTc) and europium:chlortetracycline (Eu:ClTc) complexes was studied at different pH values. At pH 7 the sensing of NO by the europium tetracyclines complexes was optimized and the detection and/or quantification capability was evaluated. The more adequate europium tetracyclines ratio for NO fluorescence sensing in the presence of H_2O_2 was obtained. In the optimized conditions, the NO detection capability was checked varying the NO concentration and the quantification capability was evaluated by the generation of NO with a rapid donor DiEthylAmine NOnoate (DEA/NO) and in standard solutions.

Experimental

Reagents

Europium, tetracycline, oxytetracycline, chlortetracycline, 3-(N-morpholino) propanesulfonic acid (MOPs), potassium superoxide, sodium nitrite, hydrogen peroxide and DiEthylAmine NOnoate diethylammonium salt (DEA/NO) were obtained from Sigma-Aldrich Química S.A. (Spain). Sodium hydroxide and hydrochloric acid were purchased from Merck, Darmstadt (Germany). Mili-Q water with resistivity of 18.2 MΩ.cm at 25 °C was used in all the experiments.

Solutions

All the solutions prepared from a solid powder were done by rigorous weighting to the required final concentration and were prepared daily. Buffer solutions of pH 6.00, 7.00 and 8.00 were obtained by adding, to the MOPs buffer solution previous prepared (pH 10.1), HCl 0.1 M in order to obtain the desired pH. The initially europium and tetracyclines solutions of the desired concentrations were prepared in MOPs buffer of pH 6.00, 7.00 and 8.00. The required europium tetracyclines complexes were then obtained mixing equal volumes of these europium and tetracyclines solutions previously prepared.

The $ONOO^-$ solutions were prepared in a refrigerated beaker under constant stirring by mixing 100 mL of $NaNO_2$ 600 mM, 100 mL of H_2O_2 600 mM in HCl 0.6 M and 100 mL of NaOH 3.6 M. The solution will turn yellow indicating the formation of peroxynitrite. The agitation is maintained until no O_2 is formed. Diluted solutions were done in NaOH 0.1 M to the desired concentrations. The saturated NO solutions (1.9 mM) were prepared from bubbling NO in deoxygenated water (bubbling argon during 15 min). Lower concentrations standard solutions were prepared with deoxygenated water by rigorous dilution. Also H_2O_2 and O_2^{-} solutions were prepared in water.

The DEA/NO stock solution was prepared in NaOH 10 mM and kept at −20 °C before use. The liberation of NO was done in deoxygenated MOPs pH 7.00.

Instrumentation

The absorbance and the fluorescence emission spectra were collected in a standard 1 cm fluorescence quartz cell respectively in a Jasco V-530 UV-Visible spectrophotometer and in a Jasco FP-6200 spectrofluorimeter. The absorption spectra were obtained in a wavelength range from 220 to 450 nm with a 2 nm interval, slit with 2 nm and wavelength scan rate medium; the Fluorescence emission spectra were obtained in a wavelength range from 420 to 650 nm with a 1 nm interval, slit with 10 nm, sensitivity response medium, response time fast and wavelength scan rate 1,000 nm/min.

All the determinations in the optimization and quantification stages were made at 30 °C using a standard 1 cm fluorescence quartz cell. The control of temperature was done by circulation of water at 30 °C by an external thermostat bath. Also a 395 nm LED, a sampling compartment (CUV-ALL-UV 4-way) and two 1.0 mm core diameter fiber optics (P1000-2-UV–VIS) from Ocean Optics were used. Of the two optical fibers, one guides the light from the source to the sampling compartment and the other guides the emitted light to the detector. The detection was done by a QE65000 charge-coupled device also from Ocean Optics. The reaction time profiles were obtained collecting the signal during 950 s at a wavelength of 616 nm, every 10 s with an integration time of 300 ms. For each reaction time profile the fluorescence intensity before the ROS/RNS addition (I_0) and after 900 s (I) was evaluated and a fluorescence intensity variation ($\Delta I = I - I_0$) was calculated.

Results and Discussion

Figure 1 presents the absorption and emission spectra of the europium, tetracyclines and europium tetracyclines complexes at the ratio and concentrations indicated (initially prepared solutions before the formation of the complexes). These concentrations and their complexes ratio were chosen attending to preliminary evaluations based in previous published results of the Eu:Tc complex with H_2O_2 [19].
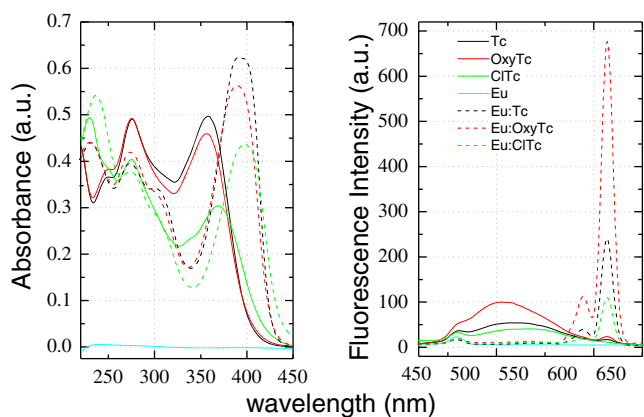
Fig. 1 UV-visible absorption and fluorescence emission spectra at excitation wavelength of 395 nm of Eu 65 μM; Tc, OxyTc and ClTc 30 μM; Eu:Tc, Eu:OxyTc and Eu:ClTc 130 μM:60 μM complexes

The tetracyclines and the corresponding europium tetracyclines complexes have similar absorption and emission spectral shapes with the exception of the absorption spectra of the ClTc and Eu:ClTc complex. The absorption spectra are characterized by two bands at about 276 and 360 nm for the three tetracyclines and at 274 and 390 nm for the related europium tetracyclines complexes. For the emission spectra the wavelength of the main bands are located at about 520 and 614 nm, respectively, for the tetracyclines and europium tetracyclines complexes. For the absorption and emission spectra the highest intensity was observed for the Tc and OxyTc and corresponding complexes.

ROS/RNS Analysis

The detection of the main ROS/RNS (NO, $ONOO^-$, H_2O_2, $O_2^{\cdot-}$), at three concentration levels of 25, 50 and 100 μM were assessed with Eu 130 μM and tetracyclines 60 μM (2:1 ratio, at a constant temperature of 30 °C) and at pH 6.00, 7.00 and 8.00—due to the basicity of the standard $ONOO^-$ solution the final pH of the higher concentration solutions were higher (pH 7.00, 8.00 and 12 with $ONOO^-$ 50 μM; and, of pH 7.60, 11.19 and greater than 12 with $ONOO^-$ 100 μM). The results are presented in Fig. 2.

The analysis of Fig. 2 shows that the presence of the ROS/RNS species affects differently the fluorescence of the Tc complexes and that this effect is dependent of the pH. At pH 7, the presence of the ROS species H_2O_2 and $O_2^{\cdot-}$ provoke an increase of the fluorescence intensity of the three Tc complexes and $ONOO^-$ has a similar behaviour for the Tc and ClTc complexes. The fluorescent enhancement observed at pH 7 with the H_2O_2 from 25 to 100 μM is for Eu:Tc about 115 times, Eu:OxyTc about 150 times and Eu:ClTc about 18 times. Depending of the europium tetracycline complex, these fluorescence enhancement is about 2 and
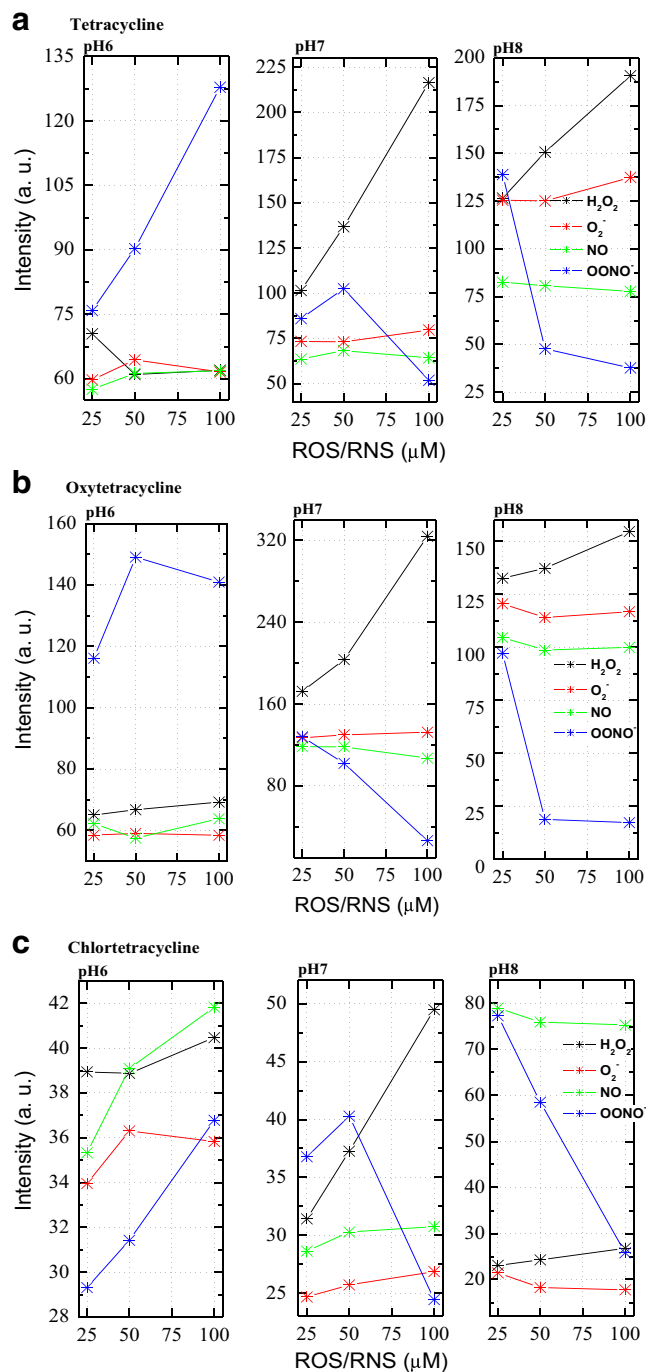

Fig. 2 ROS/RNS 25, 50 and 100 μM detection by Eu:Tc, Eu:OxyTc and ClTc complexes 130 μM:60 μM at a temperature of 30 °C and pH of 6.00, 7.00 and 8.00

19 times greater than the enhancement observed respectively with the $ONOO^-$ (pH 6) and $O_2^{\cdot-}$ (pH 7).

At pH 7.00, NO provokes quenching of the fluorescence intensity of the Eu:Tc and Eu:OxyTc complexes and has no significant effect on the fluorescence intensity of the Eu:ClTc complex. At pH 7.00 this quenching effect is more evident for the Eu:OxyTc complex. A small increase of the fluorescence intensity was also observed with the Eu:Tc

complex at pH 7.00 from 25 to 50 μM and with the Eu:ClTc complex at pH 6.00 and 7.00 from 25 to 100 μM.

NO Sensing Optimization

Due to the different NO effect on the fluorescence of the europium tetracyclines complexes the sensing of NO at pH

Fig. 3 Fluorescence intensity variation by addition of H_2O_2 100 μM at different Europium:tetracyclines ratios

7.00 was studied in detail. As shown in the ROS/RNS analysis the quenching effect of NO in the presence of the europium tetracyclines complexes suffers from a lack of sensitivity. To increase the sensitivity, the strategy followed was the NO sensing in the presence of H_2O_2—which would increase the background fluorescent signal. Moreover, the presence of a relatively high concentration of H_2O_2 would allow the detection of NO in systems where H_2O_2 is present. In order to achieve this objective the optimization of the europium tetracyclines complexes in the presence of H_2O_2 was performed and the NO detection in the presence of different levels of H_2O_2 analysed.

Europium:Tetracyclines Ratio

The first optimization step was to search for the concentrations of europium and tetracyclines and the ratio of these concentrations that in the presence of H_2O_2 results in a high fluorescence signal. The enhancement of the fluorescence upon addition of H_2O_2 100 μM to europium tetracyclines complexes with ratios for the Eu:Tc and Eu:OxyTc from 1 to 6 and for the Eu:ClTc from 1 to 10 was measured. Figure 3 shows the results obtained in this assessment.

The analysis of Fig. 3 shows that the ΔI increases and stabilises for a higher Eu:Tc ratio for the three Tc and that the intensity shows different trends for the three Tc—Eu:Tc 3:1 (Eu 80 μM:Tc 240 μM); Eu:OxyTc 4:1 (Eu 60 μM:Tc 240 μM); Eu:ClTc 6:1 (Eu 80 μM:Tc 560 μM). A lower I_0 is also found with all the europium tetracyclines complexes at these complex ratios and concentrations.

After this initial study, the response of the europium tetracyclines complexes in the presence of H_2O_2 was evaluated in the complexes ratio and concentrations previous found by variation of the H_2O_2 concentration. Figure 4 shows the typical time profiles and Table 1 the linear regression parameters obtained by addition of H_2O_2 of concentrations from 0.5 to 200 μM. The greatest ΔI variation upon addition of H_2O_2
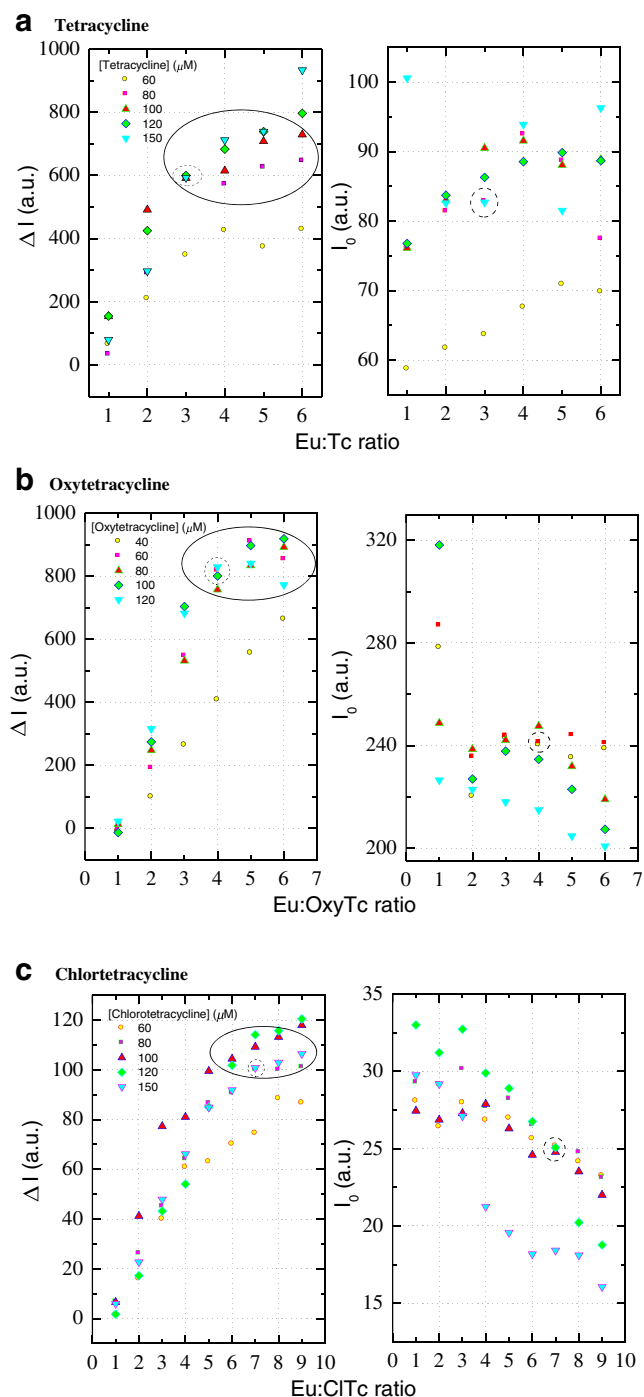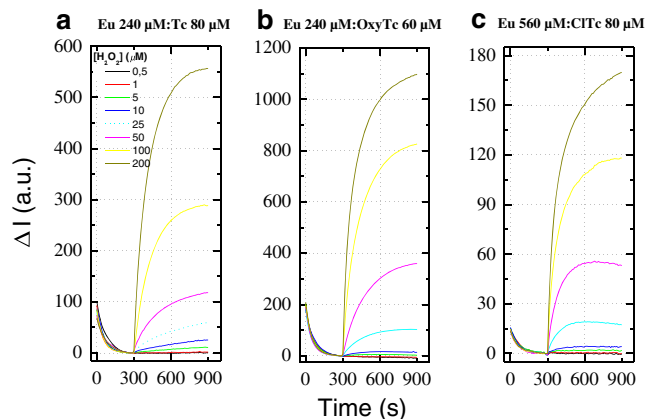

Fig. 4 Typical time profiles of the fluorescence intensity variation by addition of different H_2O_2 concentrations of the **a** Eu:Tc, **b** Eu:OxyTc and **c** Eu:ClTc complexes at the more adequate europium:tetracyclines ratio and concentrations

Table 1 Linear regression of the fluorescence intensity variation of the europium:tetracyclines complexes and the H_2O_2 concentration from 0.5 to 200 μM at the optimized ratio

$y = bx + a[\Delta I = f(C_{H2O2})]$	Eu 240 μM:Tc 80 μM (3:1)	Eu 240 μM:OxyTc 60 μM (4:1)	Eu 560 μM:ClTc 80 μM (7:1)
Lin. range	0.5–200 μM	5–100 μM	5–100 μM
m	8	5	5
a (s_a)	−4.94 (4.15)	−77.76 (22.05)	−8.71 (2.72)
b (s_b)	2.81 (0.05)	8.91 (0.43)	1.25 (0.05)
$s_{y/x}$	9.40	33.26	4.11
R	0.9990	0.9966	0.9973
p ANOVA	<0.0001	0.0002	0.0002

ΔI fluorescence intensity variation; *Lin. range* linear range; *m* number of calibration points; *a* intercept; s_a intercept standard deviation; *b* slope; s_b slope standard deviation; $s_{y/x}$ standard deviation of residuals; *R* linear correlation coefficient; *p ANOVA* ANOVA probability value (p) for a 5 % significance level of the linear regression

was obtained with Eu:OxyTc and the lowest with Eu:ClTc (Fig. 4). Table 1 shows that better linear fit results were obtained with Eu:Tc and generally worse results with the Eu:ClTc complex. Also, a greater sensitivity was found with Eu:OxyTc by variation of the H_2O_2 concentration. Attending to these results we expect identical quantification potential with a similar sensitivity for NO sensing by the Eu:Tc and Eu:OxyTc complexes in the presence of H_2O_2.

H_2O_2 Concentration

The quantification of NO in the presence of a concentration of H_2O_2 from 50 to 400 μM was studied. Table 2 presents the results obtained using the previously optimized europium tetracyclines complexes ratio and concentrations—linear regression of the variation of fluorescence intensity with the NO concentrations in the presence of H_2O_2. Good linear regression

Table 2 Linear regression of the fluorescence intensity variation of the europium:tetracyclines complexes and the NO concentrations from 1 to 200 μM at the optimized ratio in the presence of H_2O_2 from 50 to 400 μM

[H_2O_2] (μM)	$y = bx + a[\Delta I = f(C_{NO})]$									
	a	s_a	b	s_b	$s_{y/x}$	R	m	Lin. range	ΔI_{Lowest}	$\Delta I_{Greatest}$
a) Eu 240 μM:Tc 80 μM (3:1)										
50	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	−1.10	−31.57
100	−97.59	4.52	−1.46	0.10	8.15	−0.9914	6	1–100 μM	−88.74	−240.16
200	−163.22	11.43	−1.69	0.11	17.28	−0.9936	5	5–200 μM	−169.57	−491.59
300	−130.32	5.35	−1.15	0.05	6.22	−0.9984	4	25–200 μM	−154.03	−359.73
400	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	−859.33	−717.30
b) Eu 240 μM:OxyTc 60 μM (4:1)										
50	−47.37	0.83	−0.32	0.01	1.25	−0.9991	5	5–200 μM	−47.43	−110.77
100	−167.04	6.89	−1.07	0.07	12.22	−0.9908	6	5–200 μM	−169.27	−374.60
200	−142.07	4.72	−1.49	0.05	7.02	−0.9986	5	10–200 μM	−152.27	−436.04
300	−122.10	18.10	−2.23	0.16	22.43	−0.9950	4	10–200 μM	−130.77	−555.52
400	−945.70	7.08	−0.45	0.07	10.53	−0.9671	5	10–200 μM	−943.07	−1031.55
c) Eu 560 μM:ClTc 80 μM (7:1)										
50	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	−11.37	−11.49
100	−16.18	1.23	−0.24	0.02	1.53	−0.9923	4	5–100 μM	−17.32	−39.88
200	−15.17	0.32	−0.13	0.003	0.48	−0.9991	5	10–200 μM	−16.56	−40.33
300	−13.23	0.39	−0.14	0.004	0.60	−0.9989	5	5–200 μM	−13.59	−41.12
400	−5.76	0.79	−0.16	0.02	1.22	−0.9864	5	1–100 μM	−4.87	−20.85

n.p. not possible to fit; *a* intercept; s_a intercept standard deviation; *b* slope; s_b slope standard deviation; $s_{y/x}$ standard deviation of residuals; *R* linear correlation coefficient; *m* number of calibration points; *Lin. range* linear range; ΔI_{Lowest} difference of fluorescence intensity at the lowest NO concentration; $\Delta I_{Greatest}$ difference of fluorescence intensity at the greatest NO concentration
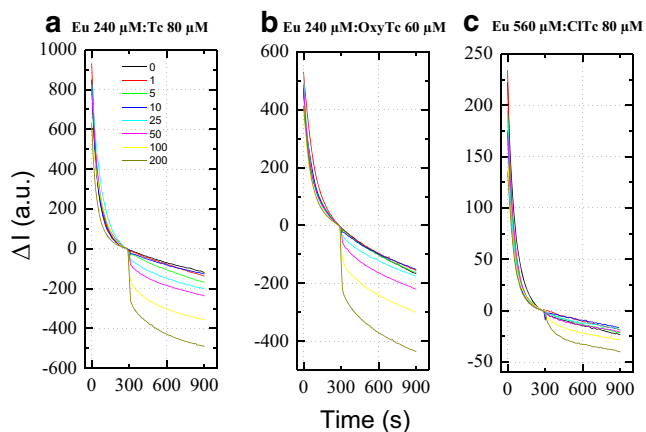
Fig. 5 Typical time profiles of the fluorescence intensity variation by addition of different NO concentrations of the **a** Eu:Tc, **b** Eu:OxyTc and **c** Eu:ClTc complexes in the presence of H_2O_2 200 µM at the more adequate Europium:tetracyclines ratio and concentrations

results were obtained with a H_2O_2 concentration from 100 to 300 µM for the three europium tetracycline complexes and the H_2O_2 concentration of 200 µM was chosen as optimum because it resulted in the highest correlation coefficient and to an adequate linear range. Figure 5 shows typical time profiles of the fluorescence

intensity variation by addition of different NO concentrations to the three europium tetracycline complexes in the presence of H2O2 200 µM.

The previous analysis show that the more adequate analytical conditions for the NO sensing at pH 7 are: H_2O_2 200 µM; and one of the following Tc complexes—Eu:Tc 3:1 (Eu 80 µM:Tc 240 µM); Eu:OxyTc 4:1 (Eu 60 µM:Tc 240 µM); or, Eu:ClTc 6:1 (Eu 80 µM:Tc 560 µM).

NO Quantification

Using the previous optimal conditions DEA/NO 25 µM and a NO 50 µM standard solutions were analysed. The results found are presented in Table 3. Similar results for the quantification of NO were obtained with the Eu:Tc and EuOxyTc complexes and worse results with the Eu:ClTc complex. With the DEA/NO solution the liberation of 1.5 mol of NO per mole of DEA/NO (≈37.5 µM) with a half-life at room temperature (≈10′) similar to the expected and with the standard solution an adequate recovery (97 and 102 %) and an acceptable precision (4 and 1 %) were achieved with the two complexes in the NO quantification assessment. The lowest detection limit, evaluated by the intercept standard deviation ($3.s_a/b$), was found for the Eu:OxyTc complex.

Table 3 NO quantification results in a DE/ANO a) and in a standard solution b) obtained by the europium:tetracyclines complexes in the presence of H_2O_2 200 µM

Eu 240 µM:Tc 80 µM (3:1)		Eu 240 µM:OxyTc 60 µM (4:1)		Eu 560 µM:ClTc 80 µM (7:1)	
a) DEA/NO (25 µM)					
Time (min)	$C_{NO} \pm 4.12$ (µM)	Time (min)	$C_{NO} \pm 6.22$ (µM)	Time (min)	$C_{NO} \pm 6.22$ (µM)
20	34.26	20	34.83	20	98.44
40	20.80	40	41.03	40	109.52
60	23.58	60	31.23	60	96.98
Recovery	92.88 %	Recovery	91.36 %	Recovery	262.50 %
$y = bx + a[\Delta I = f(C_{NO})]$					
$a=-82.03$; $s_a=2.79$		$a=-47.36$; $s_a=5.60$		$a=-4.27$; $s_a=0.49$	
$b=-1.18$; $s_b=0.03$		$b=-1.76$; $s_b=0.06$		$b=-0.12$; $s_b=0.005$	
$s_{y/x}=4.39$; $R=-0.9992$		$s_{y/x}=10.13$; $R=-0.9977$		$s_{y/x}=0.73$; $R=-0.9978$	
$m=5$; 5–200 µM		$m=6$; Lin. range—1–200 µM		$m=5$; Lin. range—10–200 µM	
b) Standard (50 µM)					
C_{NO} (µM)	48.41	C_{NO} (µM)	51.06	C_{NO} (µM)	44.09
Recovery	96.82 %	Recovery	102.12 %	Recovery	88.2 %
n	3	n	3	n	2
s	1.87	s	0.35	s	4.39
RSD (%)	3.86	RSD (%)	0.69	RSD (%)	9.96
$DL_{intercept}$	10.81	$DL_{intercept}$	3.11	$DL_{intercept}$	7.74
$y = bx + a[\Delta I = f(C_{NO})]$					
$a=-50.67$; $s_a=3.71$		$a=3.06$; $s_a=1.12$		$a=4.29$; $s_a=0.49$	
$b=-1.03$; $s_b=0.04$		$b=-1.08$; $s_b=0.01$		$b=-0.19$; $s_b=0.02$	
$s_{y/x}=6.64$; $R=-0.9971$		$s_{y/x}=2.01$; $R=-0.9998$		$s_{y/x}=0.76$; $R=-0.9805$	
$m=6$; Lin. range—1–200 µM		$m=6$; Lin. range 1–200 µM		$m=5$; Lin. range 1–50 µM	

The C_{NO} error present is the interpolated standard deviation

ΔI fluorescence intensity variation; *a* intercept; s_a intercept standard deviation; *b* slope; s_b slope standard deviation; $s_{y/x}$ standard deviation of residuals; *R* linear correlation coefficient; *m* number of calibration points; *Lin. range* linear range; *n* number of determinations; *s* mean standard deviation: *RSD* relative standard deviation; $DL_{intercept}$ detection limit estimated by the standard deviation of the intercept

Conclusions

It was observed an enhancement of fluorescence trend when the complexes Eu:Tc, Eu:OxyTc and Eu:ClTc are mixed with $ONOO^-$, H_2O_2 and $O_2^{\cdot-}$ —the highest enhancement was observed for H_2O_2. On the contrary, a quenching trend was observed for NO. This behaviour was used to develop an analytical strategy for NO sensing based on the quenching of fluorescence of the Tc complexes in the presence of relatively high quantities of H_2O_2 which resulted in an increase of sensitivity and interference elimination (although the same approach can be used for $ONOO^-$ or $O_2^{\cdot-}$).

The NO sensing by the different europium tetracyclines complexes was achieved at 30 °C, pH 7.00 in the presence of H_2O_2 200 μM. The better analytical conditions for the NO sensing—Eu 240 μM:Tc 80 μM (3:1), Eu 240 μM: OxyTc 60 μM (4:1) and Eu 560 μM:Tc 80 μM (7:1) complexes. Good quantification results were possible with the Eu:Tc and with the Eu:OxyTc complexes.

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