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Enhancement on the Europium emission band of Europium chlortetracycline complex in the presence of LDL

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ABSTRACT

Low-density lipoprotein (LDL) particles are the major cholesterol-carrying lipoprotein in the human circulation from the liver to peripheral tissues. High levels of LDL-Cholesterol (LDL-C) are known risk factor for the development of coronary artery disease (CAD). The most common approach to determine the LDL-C in the clinical laboratory involves the Friedewald formula. However, in certain situations, this approach is inadequate. In this paper we report on the enhancement on the Europium emission band of Europium chlortetracycline complex (CTEu) in the presence of LDL. The emission intensity at 615 nm of the CTEu increases with increasing amounts of LDL. This phenomenon allowed us to propose a method to determine the LDL concentration in a sample composed by an aqueous solution of LDL. With this result we obtained LDL calibration curve, LOD (limit of detection) of 0.49 mg/mL and SD (standard deviation) of 0.003. We observed that CTEu complex provides a wider dynamic concentration-range for LDL determination than that from Eu–tetracycline previously. The averaged emission lifetimes of the CTEu and CTEu with LDL (1.5 mg/mL) complexes were measured as 15 and 46 μs, respectively. Study with some metallic interferents is presented.

Introduction

Due to the hydrophobic/hydrophilic characteristics of some molecules like lipids and sterols, essential to the cellular metabolism, they are not directly transported by blood in the human circulatory system. To overcome this difficulty the organism developed nanoparticles, known as lipoproteins. These particles can be characterized and classified by their size, density, flotation constant and electrophoretic mobility. The low-density lipoprotein (LDL)[1], which comprises almost spherical particles of about 20 nm diameter, is the main carrier of cholesterol from the liver to the body tissues. The structure of the LDL has two well-defined regions, a core and a surface layer [1]. The outer shell is composed by phospholipids, unesterified cholesterol and the apolipoprotein B-100, which acts as a ligand for cell membrane receptors. The inner core is composed, mainly, by cholesterol esters and triglycerides [2]. Studies have demonstrated that the LDL retention in the extracellular matrix of the arterial wall triggers a cascade the pro-atherogenic events, such as LDL oxidation [3,4]. In addition, these studies have shown that oxidation of human LDL is involved in the development and progression of atherosclerosis plaque. The rupture the atherosclerotic plaque causes exposure of blood to procoagulant material and, consequently, immediate and massive thrombosis. This process leads to serious or fatal cardiovascular events, including myocardial infarction and ischemic strokes [5].

According to the American Heart Association, normal levels of total cholesterol are below of 200 mg/dL, while concentrations above of 240 mg/dL are high-risk factors for coronary artery disease (CAD) [6]. A high level of LDL-Cholesterol (LDL-C) (130 mg/dL and above) reflects an increased risk of CAD. Therefore, the concentration of LDL-C has become one of the main parameters to be determined in routine clinical diagnosis for therapeutic goals.

A number of tools are available to assess the concentration of LDL-C in the human blood [7]. Measurement of LDL-C requires separating LDL particles in serum from other lipoprotein, followed by measuring the cholesterol amount in the LDL fraction. Because the complexity of the method, however, it is not suitable for use in the

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[1] *Abbreviations used*: CAD, coronary artery disease; CTEu, europium chlorotetracycline complex; EuTc, europium tetracycline; LDL, low-density lipoprotein; LDL-C, LDL cholesterol; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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routine clinical laboratory or even in most research laboratory. Thus, the most used lipid profile assay does not quantify the LDL-C concentration directly but, instead, estimates it by using the Friedewald formula [5,8]. This is done, indirectly, through the measurement of the amount of total cholesterol, triglycerides and the high-density lipoprotein (HDL) in the plasma. Despite the Friedewald formula provides a reasonable quantification of the LDL-C in most situations, there are several limitations to this method [8]. Most notably samples must be obtained after 12–14 h fast. Moreover, LDL levels cannot be calculated if the concentration of triglyceride in the plasma is higher than 4.52 mmol/L (equivalent to 400 mg/dL). Still, for LDL concentration levels from 2.5 to 4.5 mmol/L, this formula is considered to be inaccurate [2,9]. Moreover, some authors have demonstrated that this formula cannot be used in patients with diabetes mellitus, and patients with liver or kidney disease [10].

Chlortetracycline is a member of the tetracycline group of broad-spectrum antimicrobial drugs used to treat bacterial infections in animals, and was the first tetracycline to be discovered [11–13]. Tetracycline and Chlortetracycline molecules, shown in Fig. 1, comprise a linear fused tetracyclic nucleus (rings designated by A–D). The tetracyclines are strong chelating agents. Chelation sites include the β-diketone (positions 11 and 12) and the enol (positions 1 and 3) and carboxamide (position 2) groups of the A ring [14]. Europium trivalent ions can form complexes with tetracyclines [15–21]. When Europium tetracycline is excited in a wavelength resonant with the tetracyclines absorption band, Europium ion luminescence is observed. This is due to the ligand broad absorption and an antenna-effect [18] that transfers the absorbed energy to the Europium through an intramolecular process. The ion luminescence may be enhanced by the isolation that the ligand provides from the water molecules, preventing energy transfer to them. The Europium–tetracycline complex (TcEu) has an absorption band at 400 nm, emission around 615 nm and a large Stokes-Shift (approximately 210 nm) [22–24]. It was shown that the Europium luminescence of Europium–tetracycline (EuTc) complex increases in the presence of LDL [18,25–27]. This increase was associated to the capability of EuTc to displace water molecules from the Europium neighborhood. These characteristics make the complex highly sensitive and specific for determining the LDL concentration in a sample. In this paper we study another member of tetracyclines family, the chlortetracycline, complexed with Europium, verifying the possibility to use this complex to quantify the LDL in a sample. The presence of some metallic species in the samples, sometimes present in the plasma, was also investigated.

Materials and methods

Isolation of LDL

Blood was drawn from healthy fasting (12 h) normolipidemic blood-donor volunteers, and plasma was obtained after centrifugation at 1000*g* and at 4 °C, during 15 min. Thereafter were added benzamidine (2 mM), gentamicin (0.5%), chloramphenicol (0.25%), phenyl-methyl-sulfonyl-fluoride (PMSF) (0.5 mM), and aprotinin (0.1 unit/mL). LDL ($1.006 < d < 1.063$ mg/mL) was isolated by sequential ultracentrifugation, as described by Havel et al. [28] at 100,000*g*, at 4 °C, using a 75 Ti rotor (Beckman Instruments), and thereafter dialyzed at 4 °C against phosphate buffered saline (PBS) pH 7.4, with 0.01% ethylenediaminetetraacetic acid (EDTA). The LDL was sterilized via filtration through a 0.22 μm-pore filter (Milipore – German). The lipoprotein concentration was quantified using the BCA kit (PIERCE) using bovine serum (BSA) as standard.

Europium chlortetracycline solutions

The Europium chlortetracycline (CTEu) complexes were prepared starting from inorganic salts with analytical purity, from Sigma Aldrich and Molecular Probe. All solutions were prepared in 10 mmol/L 3-(*N*-Morpholino) propanesulfonic acid (MOPS, from Carl Roth) buffer, at pH = 6.9. The tetracycline hydrochlorides used are from Sigma–Aldrich. The prepared solutions were:

Solution I: Buffer solution: 2.09 g of MOPS in 1000 mL distillated water (pH was adjusted to 6.9).

Solution II: To prepare different molar-rate solutions were necessary:

- 21 μmol L^{-1} Eu^{3+} solution; 0.0079 g of $EuCl_3{\cdot}6H_2O$ in10 mL of solution I.
- 31.5 μmol L^{-1} Eu^{3+} solution; 0.0115 g of $EuCl_3{\cdot}6H_2O$ in 10 mL of solution I.
- 42 μmol L^{-1} Eu^{3+} solution; 0.0154 g of $EuCl_3{\cdot}6H_2O$ in 10 mL of solution I.
- 52.5 μmol L^{-1} Eu^{3+} solution; 0.0192 g of $EuCl_3{\cdot}6H_2O$ in 10 mL of solution I.
- 63 μmol L^{-1} Eu^{3+} solution; 0.0231 g of $EuCl_3{\cdot}6H_2O$ in 10 mL of solution I.
- 73.5 μmol L^{-1} Eu^{3+} solution; 0.0269 g of $EuCl_3{\cdot}6H_2O$ in 10 mL of solution I.
- 84 μmol L^{-1} Eu^{3+} solution; 0.0307 g of $EuCl_3{\cdot}6H_2O$ in 10 mL of solution I.

Solution III: 21 μmol L^{-1} of chlortetracycline in 10 mL of solution I.

Solution IV: CTEu: Mix of 10 mL of Solution II with 10 mL of solution III, and MOPS to complete 100 mL.

Solution VI: CTEu-LDL: 1 mL of solution IV and 10 μL of LDL.

Optical characterization

The absorption spectra of all samples were measured in the wavelength range 200–500 nm, at room temperature, using a Varian Cary 17D Spectrometer. The emission spectra were obtained by exciting the samples, placed inside a 1 mm optical-path cuvette. The emission spectra were obtained by exciting the samples (placed in a 1 mm optical-path cuvette) with a 150 W Xe lamp, at 405 nm. The emission spectra of the samples were analyzed with a 0.5 m monochromator (Spex) and a PMT detector. The signal

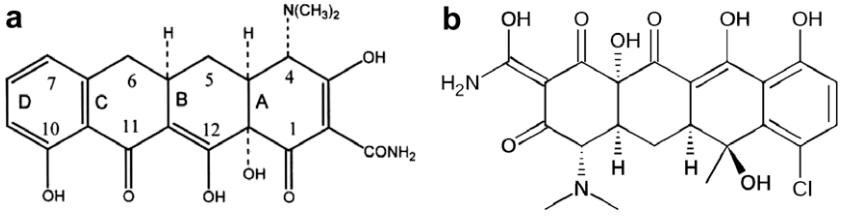

Fig. 1. Tetracycline (a) and chlortetracycline (b) molecules.

was amplified with an EG&G 7220 lock-in and processed by a computer. The relative errors in the measurements are estimated to be <5%. The emission lifetime of the samples were analyzed with a Jobin Yvon, Fluorolog 3, from Horiba. All measurements were made at room temperature.

To study the effect of the presence of metallic-species in the samples, CTEu:LDL(2 mg/dL) solutions were mixed with aqueous standards of Cu, Co, Mn,Zn, Mg, Fe, Ca, Ni, Al and Ag, at concentrations usually found in the plasma [29–31]. The light intensities measured were normalized as a function of the CTEu:LDL emission light intensity.

Results and discussions

The absorption spectra results obtained with solutions I–IV are shown in Fig. 2. It can be observed that solutions I and II do not present absorption bands in the visible region. Solution III (i.e., chlortetracycline solution) presents absorption bands at 230, 276 and 370 nm. In the case of solutions IV (i.e., chlortetracycline Europium–CTEu solutions) prepared by fixing the chlortetracycline concentration (21 μmol L^{-1}) and varying the Europium concentration from 21 to 84 μmol L^{-1}, we verified a shift of the chlortetracycline absorption bands to 239 and 401 nm, indicating the complexation of Europium ions by chlortetracycline. Solutions with molar ratios of 2.0 Eu:1.0 CT and 1.5 Eu:1.0 CT presented an increase of the absorption band intensity when compared to those from other molar-ratio solutions.

The Europium emission spectra (sample excited with light of 401 nm) showed that solutions with 1.0 Eu:1.0 CT molar ratios presented the highest emission intensities (Fig. 3). Probably, higher Europium-concentrated samples present strong auto-absorption process, which masks the emission intensity profile. So, it is more convenient to use the molar ratio 1.0 Eu:1.0 CT.

The same study was performed with solutions where 1.75 mg/mL of LDL were added. A remarkable increase in the emission intensity was observed with all the solutions (Fig. 4). However, solution with molar ratio 1.5 Eu:1.0 CT presented the higher emission intensity and this molar ratio was used in our next experiments.

Fig. 5 shows the Europium emission spectra of CTEu complex in the presence of different concentrations of LDL. The integrated areas below the fluorescence spectra bands were calculated and

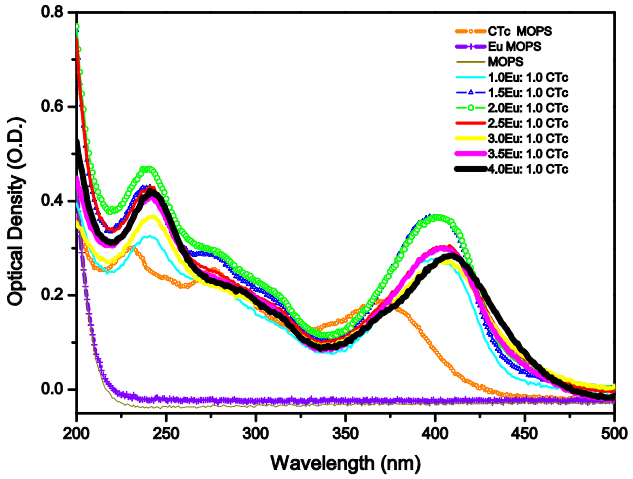

Fig. 2. Absorption spectra of Europium solution (dashed curve), MOPS solution (thin continuous line), chlortetracycline solution (white circles) and CTEu solutions in different molar, 1 Eu:1 CT; 1.5 Eu:1.0 CT; 2 Eu:1.0 CT; 2.5 Eu:1.0 CT; 3 Eu:1.0 CT; 3.5 Eu:1.0 CT; 4 Eu:1.0 CT ratios (thick continuous lines).

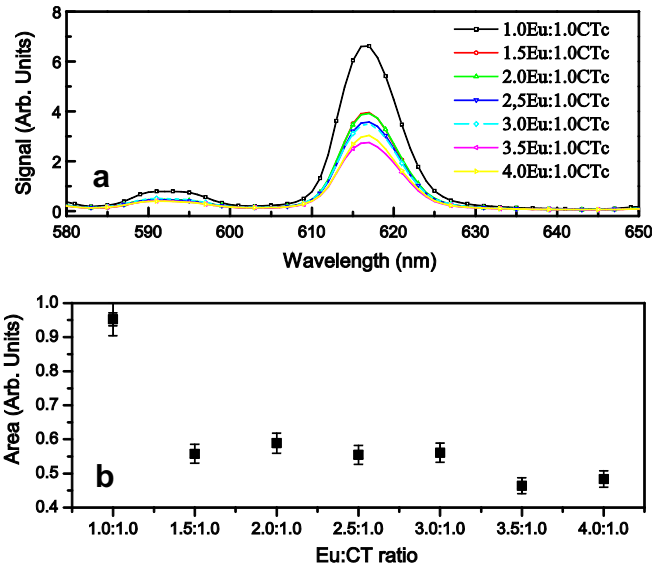

Fig. 3. (a) Europium emission spectra for different molar ratios of CTEu complex. (b) Europium emission intensity as a function of Eu:CT molar ratio.

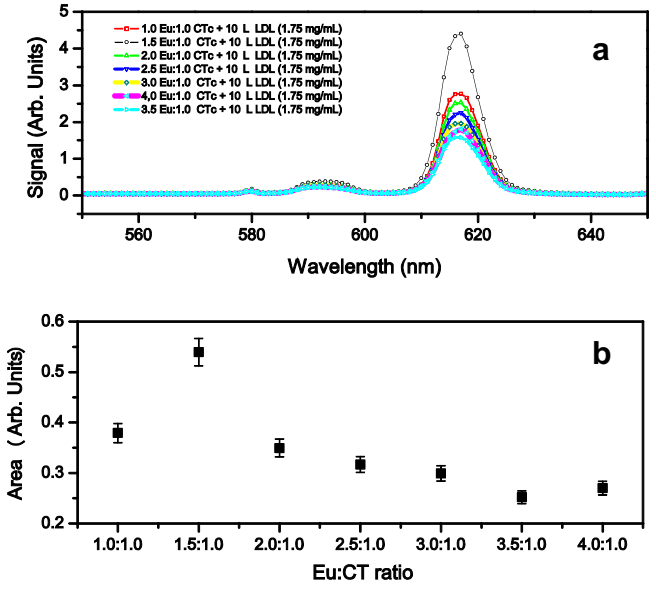

Fig. 4. (a) Europium emission spectra for different molar ratios of CTEu complex, with LDL (1.75 mg/mL). (b) Europium emission intensity as a function of Eu:CT molar ratio.

plotted as a function of the LDL concentrations, resulting in the calibration curve shown in Fig. 6. The data (the integrated area – S – as a function of the LDL concentration – c_{LDL}) showed a linear dependence $S = a + b \times c_{LDL}$. The linear coefficient ($a = 0.017 \pm 0.002$) represents the *background* of the experiment. The angular coefficient b was found to be (0.0184 ± 0.0006 mL/mg). The LDL concentration (c_{LDL} in mg/mL) of unknown samples may be calculated from this equation since the value of the integrated area is experimentally determined. For these results it was possible to obtain the LOD (limit of detection) of 0.49 mg/mL and the SD (standard deviation) of 0.003.

In a previous work [32] we reported on the enhancement of the emission band in EuTc + LDL samples. Comparing those results with our present results, where the EuTc is substituted by the CTEu, we note that, despite the CTEu presents a less intense emission signal, the differences between the emission spectra with and

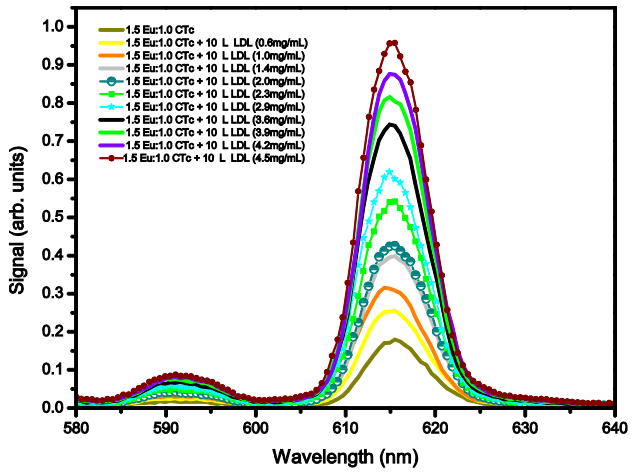

Fig. 5. CTEu:LDL emission spectra as a function of the LDL concentration.

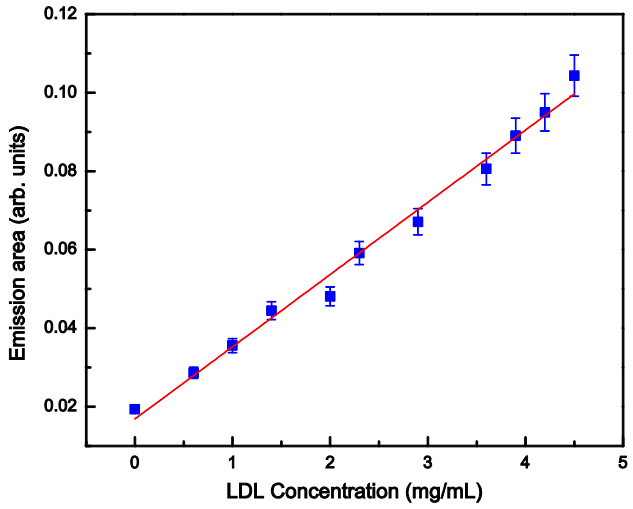

Fig. 6. CTEu:LDL calibration curve. Integrated area of the emission spectra as a function of the LDL concentration. The solid line corresponds to a linear fit to the experimental data.

without the LDL are more evident. Moreover, the dynamic range in the case of CTEu complex is bigger than that for EuTc. In the BCA kit, typically used to quantify LDL, individual proteins differ in their color responses, and this factor must be considered during protein analysis. In addition temperature, detergents, salts and various buffer components may affect the assay. For this reason we can consider the CTEu:LDL method an alternative way to quantify LDL-C.

A study with some possible interferents, eventually present in actual solutions (e.g., contaminants) was also done and the results are shown in Table 1. We observe the decrease in the luminescence intensity of the CTEu:LDL complex in aqueous solution with the presence of Cu^{2+}, Fe^{3+}, Zn^{2+} and Co^{2}. The luminescence of the CTEu:LDL complex is not affected by the presence of Mn^{2+} in concentrations up to 0.2 μM, while Mg^{2+} and Al^{3+} have a weak effect on it.

Fig. 7 shows the Europium emission lifetime with and without the presence of LDL in CTEu solutions. Generally, complex systems have multiple fluorescent species, and hence, the fluorescence intensity decay cannot be fitted to a single exponential function. This is the case of CTEu complex with and without the LDL. One needs to use a multi-exponential function and, in that case, the fluorescence intensity decay equation becomes:

$$y = y_0 + A_1 e^{(-t/\tau_1)} + A_2 e^{(-t/\tau_2)}, \quad (1)$$

where A_i and τ_i are the ith pre-exponential factor (amplitude) and the lifetime in the multi-exponential decay, respectively. In the case of multi-exponential decays, the average lifetime, which is proportional to the total area under the fluorescence decay curve, is defined by:

$$\tau_{av} = \frac{\sum_i A_i \tau_i^2}{\sum_i A_i \tau_i} \quad (2)$$

The parameters obtained by fitting Eq. (1) to the decay curve of CTEu:LDL solution are given in Table 2. The average lifetimes of the samples are 15 and 46 μs for CTEu and CTEu with LDL (1.5 mg/mL),

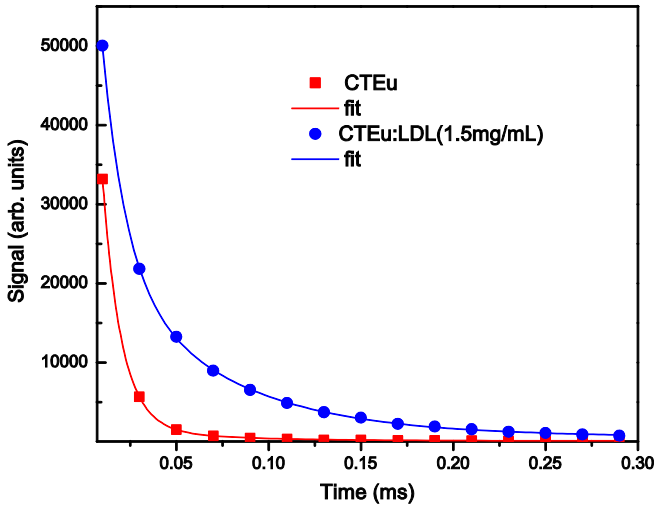

Fig. 7. Europium emission lifetime measurement with and without the presence of LDL.

Table 1
Europium lifetime emission parameters obtained with the CTEu and CTEu:LDL solutions.

CTEu $y = A_1 * \exp(-x/t_1) + A_2 * \exp(-x/t_2) + y_0$ Adj. R-square 1			CTEu:LDL (1.5 mg/mL) $y = A_1 * \exp(-x/t_1) + A_2 * \exp(-x/t_2) + y_0$ Adj. R-square 0.99994		
	Value	Standard error		Value	Standard error
y_0	86	10	y_0	634	79
A_1	2270	206	A_1	25174	997
t_1 (ms)	0.050	0.003	t_1(ms)	0.06	0.002
A_2	84082	254	A_2	61890	706
t_2 (ms)	1000 E−5	7 E−5	t_2(ms)	100 E−4	4 E−4
Lifetime $\tau_{av} = \frac{\sum_i A_i \tau_i^2}{\sum_i A_i \tau_i} = 15$ μ s			Lifetime $\tau_{av} = \frac{\sum_i A_i \tau_i^2}{\sum_i A_i \tau_i} = 46$ μ s		

Table 2
The luminescence of the CTEu:LDL(2 mg/mL) complex in the presence of interferents.

Complexes	Emission intensity at 617 nm	$I_{(EuCTc + LDL\ int.)}/I_{(EuCTc + LDL)}$
EuCTc:LDL + (0.1 μM) Ni	7.80 E6	1.11
EuCTc:LDL + (10 μg/L) Ag	7.70 E6	1.09
EuCTc:LDL + (12 μM) Al	7.63 E6	1.09
EuCTc:LDL (1.12 μM) Ca	7.35 E6	1.05
EuCTc:LDL(1.5–2 mEq/L) Mg	7.15 E6	1.02
EuCTc:LDL	*7.00 E6*	*1.00*
EuCTc:LDL + (0.2 μM) Mn	7.01 E6	1.00
EuCTc:LDL + (0.1 μM) Co	7.06 E6	1.00
EuCTc:LDL + (20 μM) Zn	6.93 E6	0.99
EuCTc:LDL + (60 μg/dL) Fe	6.57 E6	0.93
EuCTc:LDL + (70 μg/dL) Cu	4.32 E6	0.62

respectively. These results show that the fluorescence of the CTEu:LDL systems is less quenched by molecular oxygen than the CTEu complex.

Europium ions can occupy different sites in the chlortetracycline molecule. Europium emission present a long decay ratio when is located in the sites better blinded from water molecules. If Europium ions are in sites more exposed to energy transfer to water molecules, they have smaller decay time. For this reason CTEu complex lifetime is well described by a two-exponential fitting-function. It is reasonable to assume that, when the complex is in the presence of LDL, some water molecules in the neighborhood of them are removed to form the hydration layer around the lipoproteins. This process causes an increase of the amplitude of the slower component (A_1), and a decrease of the amplitude of the faster component (A_2). This result may indicate that the site that initially has more probability to transfer energy to water molecules was blinded by the LDL.

To explain the Europium emission enhancement of CTEu in the presence of LDL, we suggest that water molecules in the solution containing LDL are grouped around the polar hydroxyl groups of the LDL particles, isolating Europium ions connected to tetracycline molecules. With less water molecules in the vicinity of Europium ions, the energy transfer to water molecules is minimized, and the energy is mainly kept in the Eu ions, increasing its lifetime and luminescence intensity, resulting in a luminescence quantum yield enhancement [31].

Conclusions

A characteristic increase of the Europium emission in CTEu complexes aqueous solution was observed in the presence of different concentrations of LDL. A Job's plot based determination of CT:Eu stoichiometry was done and it was found that the solution with molar ratio 1.0 CT:1.5 Eu presented the higher emission and this molar ratio was used in our experiments.

We verified that the CTEu complex emission intensity is smaller than that of the EuTc complex previously studied. However, the dynamic range in the case of CTEu complex is bigger than that of the EuTc. From the calibration curve obtained it was possible to calculate the LOD (limit of detection) of 0.49 mg/mL and the SD (standard deviation) of 0.003.

The average lifetimes of the CTEu and CTEu with LDL (1.5 mg/mL) complexes are 15 and 46 μs, respectively. These results show that the fluorescence of the CTEu:LDL systems is less quenched by molecular oxygen than that from the CTEu complex.

We observed a decrease in the luminescence intensity of the CTEu:LDL complex in aqueous solution with the presence of Cu^{2+}, Fe^{3+}, Zn^{2+} and Co^{2}. The luminescence of the CTEu:LDL complex is not affected by the presence of Mn^{2+}, while Mg^{2+} and Al^{3+} have a weak effect on it.

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