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


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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), 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 of 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 of 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 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 a 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...
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 and an antenna-effect [18] that transfers the absorbed energy to the Europium through an intramolecular process. 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 distilled water (pH was adjusted to 6.9).

Solution II: To prepare different molar-rate solutions were necessary:
- 21 μmol L⁻¹ Eu³⁺ solution; 0.0079 g of EuCl₃·6H₂O in 10 mL of solution I.
- 31.5 μmol L⁻¹ Eu³⁺ solution; 0.0115 g of EuCl₃·6H₂O in 10 mL of solution I.
- 42 μmol L⁻¹ Eu³⁺ solution; 0.0154 g of EuCl₃·6H₂O in 10 mL of solution I.
- 52.5 μmol L⁻¹ Eu³⁺ solution; 0.0192 g of EuCl₃·6H₂O in 10 mL of solution I.
- 63 μmol L⁻¹ Eu³⁺ solution; 0.0231 g of EuCl₃·6H₂O in 10 mL of solution I.
- 73.5 μmol L⁻¹ Eu³⁺ solution; 0.0269 g of EuCl₃·6H₂O in 10 mL of solution I.
- 84 μmol L⁻¹ Eu³⁺ solution; 0.0307 g of EuCl₃·6H₂O 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

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**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 1000g 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 albumin (BSA) as standard.

**Europium chlortetracycline solutions**

The Europium chlortetracycline (CTEu) complexes were prepared 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:

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**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 chlortetracyline. 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 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 – cLDL) showed a linear dependence $S = a + b \times c_{DL}$. 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 (cLDL 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
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 Cu²⁺, Fe³⁺, Zn²⁺ and Co²⁺. The luminescence of the CTEu:LDL complex is not affected by the presence of Mn²⁺ in concentrations up to 0.2 μM, while Mg²⁺ and Al³⁺ 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:

\[ y = y_0 + \frac{A_1}{t_1} e^{\frac{-t}{t_1}} + \frac{A_2}{t_2} e^{\frac{-t}{t_2}} \]

where \( A_i \) and \( t_i \) are the \( i \)th 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:

\[ \tau_{av} = \frac{\sum A_i t_i^2}{\sum A_i t_i} \]

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).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>CTEu</th>
<th>CTEu:LDL (1.5 mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Standard error</td>
</tr>
<tr>
<td>( y_0 )</td>
<td>86</td>
<td>10</td>
</tr>
<tr>
<td>( A_1 )</td>
<td>2270</td>
<td>206</td>
</tr>
<tr>
<td>( t_1 ) (ms)</td>
<td>0.050</td>
<td>0.003</td>
</tr>
<tr>
<td>( A_2 )</td>
<td>84082</td>
<td>254</td>
</tr>
<tr>
<td>( t_2 ) (ms)</td>
<td>1000 E–5</td>
<td>7 E–5</td>
</tr>
<tr>
<td>Lifetime ( \tau_{av} )</td>
<td>( \sum \frac{A_i t_i^2}{\sum A_i t_i} = 15 , \mu s )</td>
<td></td>
</tr>
</tbody>
</table>
respectively. These results show that the fluorescence of the CTEu:LDL system 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 ($A_2$) and a decrease of the amplitude of the faster component ($A_1$). 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 luminescence intensity, resulting in a luminescence quantum yield enhancement [32].

Acknowledgments

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Table 2

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Emission intensity at 617 nm</th>
<th>$I_{(EuCTc \times LDL)<em>{int.}}/I</em>{EuCTc}$</th>
</tr>
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<tbody>
<tr>
<td>EuCTc:LDL + (0.1 μM) Ni</td>
<td>7.80 E6</td>
<td>1.11</td>
</tr>
<tr>
<td>EuCTc:LDL + (10 μg/mL) Ag</td>
<td>7.70 E6</td>
<td>1.09</td>
</tr>
<tr>
<td>EuCTc:LDL + (12 μM) Al</td>
<td>7.63 E6</td>
<td>1.07</td>
</tr>
<tr>
<td>EuCTc:LDL (1.12 μM) Ca</td>
<td>7.35 E6</td>
<td>1.05</td>
</tr>
<tr>
<td>EuCTc:LDL (1.5–2 mg/dL) Mg</td>
<td>7.15 E6</td>
<td>1.02</td>
</tr>
<tr>
<td>EuCTc:LDL</td>
<td>7.00 E6</td>
<td>1.00</td>
</tr>
<tr>
<td>EuCTc:LDL + (0.2 μM) Mn</td>
<td>7.01 E6</td>
<td>1.00</td>
</tr>
<tr>
<td>EuCTc:LDL + (0.1 μM) Co</td>
<td>7.06 E6</td>
<td>1.00</td>
</tr>
<tr>
<td>EuCTc:LDL + (20 μM) Zn</td>
<td>6.93 E6</td>
<td>0.99</td>
</tr>
<tr>
<td>EuCTc:LDL (60 μg/dL) Fe</td>
<td>6.57 E6</td>
<td>0.93</td>
</tr>
<tr>
<td>EuCTc:LDL + (70 μg/dL) Cu</td>
<td>4.32 E6</td>
<td>0.62</td>
</tr>
</tbody>
</table>

References

[12] H. Welch, About the chemically descriptive generic terms for aureomycin (chlortetracycline) and terramycin (oxytetracycline), Antibiotics and Chemotherapy 3 (1953) 659–662.


