Novel fluorescent probe for low density lipoprotein, based on the enhancement of Europium emission band

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Abstract: We report here the observation of the enhancement of Europium-tetracycline complex emission in Low Density Lipoprotein (LDL) solutions. Europium emission band of tetracycline solution containing Europium (III) chloride hexahydrate was tested to obtain effective enhancement in the presence of native LDL and oxidized LDL. Europium emission lifetime in the presence of lipoproteins was measured, resulting in a simple method to measure the lipoproteins quantity in an aqueous solution at physiological pH. This method shows that the complex can be used as a sensor to determine the different states of native and oxidized LDL in biological fluids.

OCIS codes: (300.6280) Spectroscopy, fluorescence and luminescence; (170.1470) Blood/tissue constituent monitoring; (170.3650) Lifetime-based sensing

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1. Introduction

Cholesterol is an important molecule in vertebrate metabolism and plays a crucial role building up the plasma membrane and being a precursor for the generation of hormones participating in the human reproduction. Isolated cholesterol molecules are poorly soluble in polar solvents (like e.g. water) and, as a consequence of this, in the human plasma. To overcome this, cholesterol is carried in the context of a large-sized particle (~ 27 *nm*) mainly consisting of cholesterol, cholesteryl ester, triglycerides fatty acids and a powerful anti-oxidant, alpha-tocopherol (vitamin-E) to prevents it from oxidation [1, 2]. This particle also contains an apo-lipoprotein (apoB) moiety that both circumcise and protrude the particle. Different particles of this type

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exist in the plasma and are classified according to their mass density. One of them is the Low-Density Lipoprotein, usually named LDL, which is the main carrier of cholesterol [3] in the plasma. The structure of the LDL particles has two well-defined regions, a core and a surface layer. The outer shell is composed by phospholipids, unesterified cholesterol and the apoB. The inner core is composed mainly by cholesteryl esters and triglycerides [1], which are water insoluble. The excess of cholesterol, associated with LDL, in the blood is one of the main risk factors for the development of cardiovascular diseases, including atherosclerosis and hypertension, among others [4, 5]. Recently, research in this field has focused on the role of modified lipoproteins, primarily oxidized Low-Density Lipoprotein (OxLDL). Several lines of evidence support the concept that OxLDL may be a key antigen in atherosclerosis [6]. Oxidized lipoproteins are also taken up by macrophage cells that lead to the appearance of foam cells, the hallmark of atherosclerotic plaques in the vascular [7, 8]. Oxidative stress induces the formation of aggregates from initial nucleation units through linear to fractals and vesicles [9]. LDL can be modified in vitro by acetylation, acetoacetylation, malondialdehyde, derivazation, and oxidation. The most used process of oxidation in vitro essays is that which uses copper oxides. The origin of this oxidative process could be a change in the surface charge density of the oxidized particles. According to the American Heart Association, normal levels of the sanguineous total cholesterol are below of 200 mg/dL, while concentrations above of 240 mg/dL are high risk factors for coronaries illnesses [10]. For these reasons, cholesterol has become one of the main parameters to be determined in routine clinical diagnosis. A number of tools are available to assess LDL particles. The methods in most common use include gradient gel electrophoresis (GGE) [11] and density gradient ultracentrifugation [12]. However, only few and heavy techniques exist to quantify the proportion of native LDL and OxLDL in the plasma. To the best of our knowledge, except for the recently proposed Z-scan technique [13], no other optical-based method is used to investigate both the LDL and OxLDL particles in aqueous solutions.

In aqueous solution, Europium trivalent ions exhibits weak luminescence due to its small absorption cross section and strong energy transfer to surrounding water molecules [14, 15]. However, when the Europium form a complex with tetracycline [16], the ion luminescence can be increased. This increase is due to the ligand large absorption and an antenna-effect [17] that transfers the absorbed energy to the Europium through an intramolecular process [18], which efficiency depends on the chemical nature of the ligand. The ion luminescence is also enhanced by the isolation that the ligand provides from the water molecules, preventing energy transfer to them. The Europium-tetracycline complex (EuTc) has an absorption band centered around 400 *nm*, presenting a large Stokes-Shift (approximately 210 *nm*) due to an efficient antenna-effect, strongly emitting around 615 *nm* [19], in the red region of the visible spectrum. This luminescence was observed to increase up to 15 times in the presence of H_2O_2 due to this molecule capability of displacing water molecules from the Eu neighborhood [20]. These characteristics make the complex highly sensitive and specific for H_2O_2 detection [21]. As an additional advantage, the EuTc complex can be excited by commercial LEDs and lasers, and works in neutral pH (~ 7.0) [22]. The coordination chemistry of the tetracycline involves the existence of three or more binding sites and four sites of prototropic dissociation on each molecule [23]. The possible complexation sites include the β-diketone, the enolic groups and the carboxamide of the tetracycline molecule rings [24].

Time-resolved fluorescence spectroscopy is a technique for studying the emission dynamics of fluorescent molecules [25]. The temporal extent of this distribution is referred to as the fluorescence lifetime of the molecule and the lifetime measurements can yield information on the molecular microenvironment of a fluorescent molecule. Different factors can all modify the lifetime of a fluorophore, such as: ionic strength, hydrophobicity, oxygen concentration, binding to macromolecules and the proximity of molecules that can deplete the excited state by

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resonance energy transfer. An additional advantage of this technique is that these measurements are generally absolute, being independent of the concentration of the fluorophore.

Due to the different structural characteristics of LDL and OxLDL in the plasma (or, *in vitro*, in aqueous solutions), it is expected that their environments with respect to the neighboring water molecules (or, more generally, polar molecules) are different. In this scheme, the EuTc luminescence in the presence of aqueous solutions of LDL and OxLDL may be different and a fingerprint of the state of the lipoprotein. In this paper we investigate the EuTc luminescence present in aqueous solutions of LDL and OxLDL and propose a new optical method for the LDL and OxLDL quantification, based on the enhancement of the EuTc complex emission. The time-resolved fluorescence spectroscopy is also used to investigate the environment of the EuTc aqueous solutions in the presence of LDL and OxLDL.

2. Materials and methods

2.1. Isolation of LDL

Blood was drawn from healthy fasting (12 *h*) normolipidemic blood-donor volunteers and plasma was obtained after centrifugation at $10^3 g$ and at $4^o C$, during 15 *min*. Thereafter, benzamidine (2 *mM*), gentamicin (0.5%), chloramphenicol (0.25%), PMSF (phenyl-methyl-sulfonyl-fluoride) (0.5 *mM*), and aprotinin (0.1 $unit/mL$) were added. LDL was isolated by sequential ultracentrifugation at $10^5 g$, at $4^o C$, using a 75 Ti rotor (Beckman Instruments) and, thereafter, dialyzed at $4^o C$ against PBS (phosphate buffered saline) pH = 7.4, with 0.01% EDTA (ethylene-diaminetetraacetic acid). The LDL was sterilized via filtration through a 0.22 μm-pore filter.

2.2. Oxidation of LDL by copper ions

To obtain LDL oxidized by copper ions (OxLDL), LDL was dialyzed overnight against PBS without EDTA followed by incubation with $CuSO_4$, 20 $\mu M/mg$ of LDL protein, at $37^o C$ overnight. This experimental procedure was standardized in our laboratory and results in a completely oxidized LDL as defined by the plateau phase using TBARS assay [26] in addition to further increase in negative charges. The oxidation process was terminated by the addition of 1 *mM* EDTA.

2.3. Determination of lipid peroxidation

Lipid peroxides formation was determined by TBARS assays [27]. To quantify the degree of oxidation by the TBARS assay, we have incubated the sample (50 μL) with the TBARS reagent (200 μL, 1% thiobarbituric acid, 562.5 μM HCl and 15% Trichloroacetic acid (Merck-Germany)). The mixture was boiled during 15 *min*, centrifuged ($10^3 g$, 10 *min*) and the absorbance of the supernatant at $\lambda = 540$ *nm* was measured using a spectrophotometer (GENIOS TECAN, Austria) and compared with a malondialdehyde (MDA) standard.

2.4. Preparation of EuTc solutions

The EuTc complex was prepared starting from inorganic salts with analytical purity, obtained from Sigma Aldrich and Molecular Probe. All solutions were prepared in 10 $mmol L^{-1}$ 3-(N-Morpholino) propanesulfonic acid (Mops, from Carl Roth) buffer with pH = 6.9. The tetracycline-HCl solution used is a secondary pattern gently provided by Bunker Indústria Farmacêutica Ltda. Cholesterol solution (CAT N^o 01401; 200 mg/dL) used in this work was obtained from Laborlab Produtos para Laboratórios LTDA. The prepared solutions were:

Solution I: Mops buffer – 544.1 *mg* of Mops salt in 200 *mL* of distilled water (pH = 6.9).

Solution II: 63 $\mu mol L^{-1}$ solution of Eu^{3+} – 2.3 *mg* of $EuCl_3 \cdot 6H_2O$ in 10 *mL* solution I.

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Solution III: 21 $\mu molL^{-1}$ solution of tetracycline – 1.0 *mg* of tetracycline in 10 *mL* of solution I.

Solution IV: EuTc solution – Mix 10 *mL* of solution II and 10 *mL* of solution III in 80 *mL* solution I.

Solution V: EuTc:LDL and EuTc:OxLDL solutions – Mix of 1000 μL of solution IV with 10 μL LDL (0.6; 0.7; 1.0; 1.5; 3.0; 5.6 and 7.7 mg/mL) or OxLDL (0.7; 1.0; 1.5 and 3.0 mg/mL).

These ranges of concentration of LDL and OxLDL were chosen because they are those typically found in the human plasma.

2.5. Optical characterization

The absorption spectra of all samples were measured in the range 200 *nm*-500 *nm* at room temperature using a Varian Cary 17D Spectrometer. The emission spectra were obtained by exciting the samples, inside a 1 *mm* optical path cuvette, with a 150 *W* Xenon lamp. The emissions of the samples were analyzed with a 0.5 *m* Spex monochromator and a PMT detector. The signal was amplified with an EG&G 7220 lock-in and processed by a computer.

Emission lifetime was obtained with an excitation system which consists of a tunable optical parametric oscillator OPO from OPOTEK pumped by the second harmonic of a Q-switched Quantel Nd-YAG laser. Laser pump at 410 *nm* was used to excite the EuTc complex. The time-dependence luminescence of the Europium was detected by a 0.25 *m* Kratos monochromator and a S-20 photomultiplier and analyzed using a digital 200MHz Tektronix TDS 410 oscilloscope. The relative errors in the emission and lifetime measurements are estimated to be $< 10\%$.

3. Results

Figure 1(a) shows the absorption spectra of LDL and OxLDL aqueous solutions indicating that the most important absorption band of both LDL and OxLDL occurs around 275 *nm*. Figure 1(b) shows the absorption spectra of the pure EuTc and EuTc:LDL and EuTc:OxLDL aqueous solutions. In this figure it can be seen that EuTc has a large absorption band centered at 395 *nm*. This absorption band is due to the presence of the tetracycline ligand which, in its uncomplexed form, has a slightly blue shifted absorption spectrum. The addition of LDL or OxLDL does not significantly change the shape or position of this band.

The fluorescence spectroscopy study of the EuTc aqueous solutions with, separately, different concentrations of LDL and OxLDL was performed. Samples were excited with light at 405 *nm*. 1 *mL* of EuTc aqueous solution was added to a fixed volume (10 μL) of LDL (or OxLDL) aqueous solutions with 0.6; 0.7; 1.0; 1.5; 3.0; 5.6 and 7.7 mg/mL of LDL (or 0.7; 1.0; 1.5 and 3.0 mg/mL of OxLDL). As stressed above, these ranges of concentration of LDL and OxLDL were chosen because they are those typically found in the human plasma. The fluorescence spectroscopy results are shown in the Fig. 2(a) and Fig. 3(a). In both cases there is an enhancement in the Europium emission band with the increase of the lipoproteins concentrations.

Interestingly, the rate of this increase for the LDL samples differs from that of the OxLDL samples. To quantitatively investigate this aspect, the integrated areas below the fluorescence spectra were calculated and plotted in Fig. 2(b) and Fig. 3(b) as a function of the LDL and OxLDL concentrations. The resulting plots are reasonably well described by a linear function ($S = a\ c + b$) between 0 and 3 mg/mL for both the LDL and OxLDL samples, where c is the concentration of the lipoprotein. The experimental values of a and b in both (LDL and OxLDL) samples are: $a_{LDL} = (4.85 \pm 0.27) \times 10^{-4}$ and $b_{LDL} = (1.98 \pm 0.15) \times 10^{-4}\ (mL/mg)$; $a_{ox} = (4.94 \pm 0.30) \times 10^{-4}$ and $b_{ox} = (2.31 \pm 0.18) \times 10^{-4}\ (mL/mg)$, respectively. The limit of

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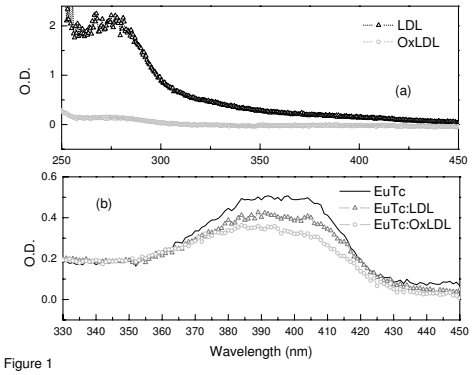

Figure 1

Fig. 1. Absorption spectra of aqueous solutions. O.D. is the optical density (the optical path of the cuvette is 1 *mm*). a) LDL (7.7 mg/mL) and OxLDL (~ 6 mg/mL);b) EuTc, EuTc:LDL; EuTc:OxLDL.

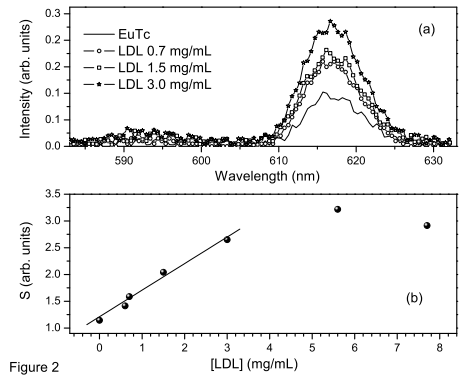

Figure 2

Fig. 2. a) Fluorescence emission spectra of EuTc and EuTc:LDL aqueous solutions at different lipoprotein concentrations;b) Integrated area (S) below the emission spectra as a function of the LDL concentration. The solid line corresponds to a linear fit to the experimental data.

detection in the both cases (defined as 3σ/slope, where σ is the incertitude in the measurement of the parameter *b*) is 0.23 mg/mL. It is important to note that the emission enhancement is not instantaneous, taking approximately 10^3 *s* to saturate and that both the calibration solutions and the samples also can be measured after a rather long incubation time (up to 24 *h* tested) without loss of the linearity observed in Fig. 2(b) and Fig. 3(b).

The time-resolved fluorescence spectroscopy experimental results of the pure EuTc sample and samples with LDL (EuTc:LDL) and with OxLDL (EuTc:OxLDL) are shown in Fig. 4. These results reveal the changes in the Eu lifetime in the two lipoprotein-doped samples with respect to the pure EuTc sample. Generally, complex systems may have multiple fluorescent species, and hence the fluorescence intensity decay cannot be fitted by a single exponential function. A multi-exponential function is used in that case [28], and the fluorescence intensity

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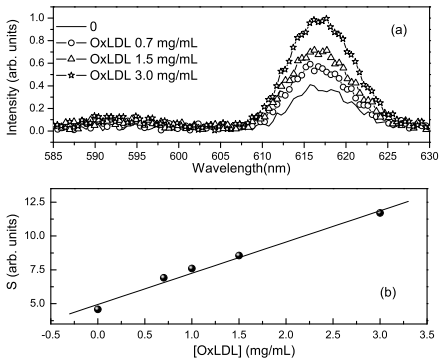

Figure 3

Fig. 3. a) Fluorescence emission spectra of EuTc and EuTc:OxLDL aqueous solutions at different lipoprotein concentrations;b) Integrated area (S) below the emission spectra as a function of the OxLDL concentration. The solid line corresponds to a linear fit to the experimental data.

decay may be described by the function:

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

where A_i and τ_i are i^{th} pre-exponential factor (amplitude) and the lifetime in the multiexponential decay, respectively. In the case of multiexponential decays the average lifetime (τ_{av}), which is proportional to the total area under the fluorescence decay curve, is defined by [28]:

$$\tau_{av} = \frac{i\sum A_i \tau_i^2}{i\sum A_i \tau_i}. \tag{2}$$

The parameters obtained by fitting Eq. 1 to the decay experimental results are shown in Table 1 and the functions are plotted in Fig. 4. The average lifetimes of the samples are 37 μs, 89 μs and 34 μs for EuTc, EuTc:LDL and EuTc:OxLDL complexes, respectively. The results show that the fluorescence of the EuTC:LDL systems is more slowly quenched by molecular oxygen than EuTc and EuTc:OxLDL complexes. The small decrease of τ_{av} of the EuTc:OxLDL with respect to the pure EuTc sample could be due to an energy transfer process occurring between the Eu and the Cu ions, or even the apoB protein modified during the oxidation process.

To explain the Europium emission enhancement of EuTc and the increase in τ_{av} in the presence of (native) LDL we suggest that the key point is the role of water molecules in the different systems. Due to the hydrophilic characteristics of the outer shell of the lipoprotein particles we expect changes in the state and number of the hydration water around the polar hydroxyl groups of the lipoprotein particles. This hydration process strongly depends on the state of the particles, i.e., if it is native (LDL) or oxidized (OxLDL). The LDL can be sketched as an almost spherical particle, having cholesteryl esters and triglycerides contained in a spherical core covered by the closely packed hydrophobic ends of phospholipids and unesterified cholesterol, while the head groups of the phospholipids, together with protein, occupy the surface (hydrophilic shell). On the other hand, OxLDL cannot be trivially sketched because the oxidation process is very complex. Results based upon anion-exchange chromatography, fast protein liquid chromatography (FPLC), and thiobarbituric reactive substance test (TBARS) [29] indicate that LDL particles,

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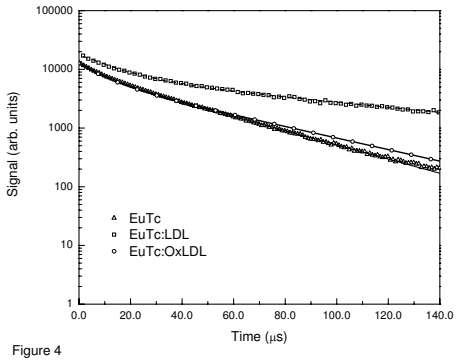

Fig. 4. Profile of the Eu emission lifetime in EuTc aqueous solution and in the presence of LDL (500 μL of aqueous solution of EuTc added to 5 μL of LDL at the concentration of 2.84 mg/mL) and OxLDL (500 μL of aqueous solution of EuTc added to 5 μL of OxLDL at the concentration of 2.84 mg/mL). The solid lines are fits of Eq. 1 to the experimental data, whose parameters are given in Table 1.

Table 1. Fitting parameters of Eq. 1 to the experimental data shown in Fig. 4.

EuTc complexes	Parameters	$\tau_{av}(\mu s)$
EuTc	$y_0 = 0$	37
	$A_1 = 4693 \pm 53$	
	$\tau_1 = (8.41 \pm 0.13) \times 10^{-6}$	
	$A_2 = 8390 \pm 56$	
	$\tau_2 = (40.0 \pm 0.2) \times 10^{-6}$	
EuTc:LDL (5 μL)	$y_0 = 0$	89
	$A_1 = 10463 \pm 71$	
	$\tau_1 = (10.0 \pm 0.2) \times 10^{-6}$	
	$A_2 = 7369 \pm 63$	
	$\tau_2 = (100.0 \pm 0.7) \times 10^{-6}$	
EuTc:oxLDL	$y_0 = 0$	34
	$A_1 = 6018.1 \pm 0.1$	
	$\tau_1 = (10.0 \pm 0.1) \times 10^{-6}$	
	$A_2 = 6490.8 \pm 0.1$	
	$\tau_2 = (40.0 \pm 0.1) \times 10^{-6}$	

under $CuSO_4$ oxidation, show an increase in its negative charge as a function of the oxidation time. This negatively charged LDL particles are mainly characterized by an increase in the lipid peroxidation product content, a depletion of vitamin E and an alteration of apoB. As a function of time, the particles became more electronegatively charged.

In the case of the EuTc:LDL solutions, water molecules participate of the LDL particles hydration and less water molecules are available to the Europium ions, connected to tetracycline molecules, and there is an increase of the Eu average lifetime with respect to the pure EuTc solution. Moreover, the higher the LDL concentration in the solution, the bigger the EuTc fluorescence since more and more water molecules are needed to hydrate the LDL particles. With less water molecules in the vicinity of Europium ions, the energy transfer to water molecules

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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.

In the case of the EuTc:OxLDL, differently from the previous case, the oxidation of the LDL particles strongly modifies the outer hydrophilic shell of the particle and less water molecules participate in the hydration layer of the particles. So, τ_{av} in this solution approaches the value of the pure EuTc solution, but is still a little bit different, indicating that some water molecules still remains in the vicinity of the oxidized particles. Likely the previous case, the higher the OxLDL concentration, the bigger the EuTc fluorescence since more and more water molecules are needed to partially hydrate the OxLDL particles.

4. Conclusions

A characteristic increase of the Europium emission in Europium-tetracycline complex aqueous solution was observed in the presence of LDL and OxLDL particles. The average fluorescence lifetime of the EuTc depends on the presence of LDL particles in the solution. On the other hand, this lifetime in the EuTc:OxLDL is practically the same of the pure EuTc solution. The increase of the EuTc fluorescence depends on the concentration of LDL and OxLDL in the solution. Moreover, the characteristic lifetimes of the fluorescence decay in EuTc solutions with LDL and OxLDL are different and can be used as a fingerprint of the native and oxidized state of the lipoproteins. The data presented herein describes a methodology to distinguish and measure the LDL and OxLDL concentrations in an aqueous solution of these lipoproteins, using a minute amount of lipoprotein at a physiological pH (since the pH of the EuTc complex is $\sim$ 7.0). Our proposal can be used as a complementary technique to measure the amount of native and oxidized LDL from a previously total LDL sample isolated from a patient by using the conventional techniques. One of the endpoints of this methodology could be the direct evaluation of the OxLDL content in the plasma/serum, being a marker for progressive atherosclerosis. The methodology is simple and low cost, with a limit of detection of $0.23\ mg/mL$.

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