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Optical Properties of Europium Tetracycline Complexes in the Presence of High-Density Lipoproteins (HDL) Subfractions

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Abstract

Standard lipoprotein measurements of triglycerides, total cholesterol, low-density lipoproteins (LDL), and high-density lipoproteins (HDL) fail to identify many lipoprotein abnormalities that contribute to cardiovascular heart diseases (CHD). Studies suggested that the presence of CHD is more strongly associated with the HDL subspecies than with total HDL cholesterol levels. The HDL particles can be collected in at least three subfractions, the HDL2b, HDL2a, and HDL3. More specifically, atherosclerosis is associated with low levels of HDL2. In this work, the optical spectroscopic properties of europium tetracycline (EuTc) complex in the presence of different HDL subspecies was studied. The results show that the europium spectroscopic properties in the EuTc complex are influenced by sizes and concentrations of subclasses. Eu^{3+} emission intensity and lifetime can discriminate the subfractions HDL3 and HDL2b.

Keywords

High-density lipoprotein subfractions, HDL, cardiovascular diseases, europium tetracycline, fluorescence lifetime

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Introduction

High-density lipoproteins (HDL) perform various functions which of great clinical interest for the treatment and prevention of atherosclerosis.[1] These include reverse cholesterol transport, antioxidant, anti-inflammatory and anti-thrombotic activities, and protection of endothelial integrity.[2–4] Epidemiological studies have found that low serum HDL concentrations constitute an independent risk factor for coronary heart disease (CHD). In addition, for each decrement of 1 mg/dL in HDL, the CHD risk is elevated by 3% in women and 2% in men.[5]

High-density lipoproteins are macromolecular complexes of lipids and proteins, apoA-I (~70%), apoA-II (~20%), apoC-III, (~2–4%), apoD, and apoE (<2%).[6] High-density lipoprotein particles are highly heterogeneous in terms of their size, density, charge, chemical composition, and functionality.[7] The protein constituents, phospholipid content of the surface monolayer, and the ratio of cholesteryl esters to triglycerides on HDL particles vary depending on the individual's metabolic and inflammatory state.[8,9] High-density lipoprotein particles have several classifications depending on the methodology used, by apolipoprotein content, by size or by surface charge. By size, HDL3 are the small subspecies or subfractions a, b, c (a is larger than c), and HDL2 are the large subfractions a, b (b is larger than a).[10]

Measurement of HDL subclasses has been a useful tool in studies adding important information about association of the lipoprotein metabolism, pathophysiology, and risk of CHD.[11] Some studies suggest that the measurement of HDL size might improve the prediction of risk of CHD,

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where levels of the large HDL frequently have inverse relationship with cardiovascular disease. Concentration of small HDL particles revel positive correlations with the CHD risk.[12]

There are various methods that allow us to classify HDL subpopulations including nuclear magnetic resonance (NMR) (17), gradient gel electrophoresis,[13] non-denaturing polyacrylamide,[14] two-dimensional electrophoresis,[15] ultrafiltration,[16] and size-exclusion chromatography.[17] Optical spectroscopic methods for HDL classification are not described in the literature. The advantages of fluorescence method when compared to conventional methods are that it dispenses the specialized and expensive instrumentations and technicians and instantaneously delivers the results. Also, with this method it is possible to identify and quantify HDL subfractions.

It was demonstrated that the europium luminescence of europium tetracycline complex increases in the presence of low-density lipoprotein (LDL). Europium tetracycline (EuTc) is a complex formed by europium trivalent ions and the antibiotic tetracycline (Tc).[18] The europium tetracycline complex (EuTc) presents an emission around 615 nm and an absorption band centered on 400 nm, with a Stokes shift of 215 nm.[19] This luminescence was observed to increase in the presence of LDL due to this molecule capability of displacing water molecules from the europium neighborhood, minimizing radioactive losses.[20]

In this work, the spectroscopic properties of EuTc in the presence of different HDL subspecies was determined verifying the possibility to use this complex for determining the size and concentration of HDL in human plasma.

Materials and Methods

High-Density Lipoprotein Isolation

Human blood was collected with EDTA (1 g/L solution) and plasma was separated by centrifugation at 4°C. The HDL subspecies were isolated from total HDL by sequential ultracentrifugation method:[21] large HDL or HDL2b (density 1.063–1.100 kg/L), medium HDL or HDL2a (density 1.100–1.125 kg/L), and small HDL or HDL3 (density 1.125–1.21 kg/L). The HDL subspecies were divided into two groups. With the first group, the particle sizes were determined by small-angle X-ray scattering (SAXS) and dynamic light scattering (DLS).[22] With the second group, the analysis was carried out with EuTc. For this second group, the concentration of total cholesterol of each subspecies was measured using a commercial kit.

Total Cholesterol Concentration

The total cholesterol concentration was assessed using an enzymatic colorimetric assay (Labtest Diagnostic SA) with an automatic biochemical analyzer.

The concentration of total cholesterol measured was 80 mg/dL and the concentrations of particles were calculated from the radius of each subspecies.

EuTc Complex

The EuTc complex was prepared in 10 mmol/L 3-(N-Morpholino) propane sulfonic acid (Mops, from Carl Roth) buffer with pH 6.9, in the ratio 3.0 Eu: 1.0 Tc (63 μmol/L of europium chloride ($EuCl_3 \cdot 6H_2O$), 21 μmol/L of tetracycline hydrochloride). All reagents were obtained from Sigma Aldrich.

Instrumentation

High-density lipoprotein SAXS measurements were performed on the laboratory SAXS instrument Nanostar (Bruker, Billerica, MA, USA). The DLS technique was used to evaluate the particle-size distribution on the samples. LDL measurements were performed using a Zetasizer Nano ZS (Malvern, Worcestershire, UK).

The absorption spectra were obtained in a spectrophotometer from Varian Cary 5000 with a cuvette with an optical path of 10 mm.

The europium fluorescence was obtained with a Jobin Yvon fluorimeter Fluorolog 3, from Horiba. The excitation wavelength was fixed at 400 nm and emission was measured from 500 to 750 nm. The emission experiment was performed in a microplate reader from Jovin Yvon. For the spectroscopic measurements of EuTc in the presence of HDL subspecies, 1 mL of EuTc was mixed with 10 μL of HDL subspecies. The fluorescence measurements were made in triplicate and curves presented are the average of the three results.

The europium fluorescence lifetime in the EuTc complex was measured 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 with a pulsed excitation of 4 ns and 10 Hz of repetition rate laser. The samples were excited at 425 nm. It was used a cuvette with 1 cm of optical path.

Results

High-Density Lipoprotein Subspecies Concentrations

The average core diameters of the particles (D, nm) were measured and summarized in Table 1. High-density lipoprotein subspecies concentrations were estimated considering subspecies formed only by total cholesterol. It was considered that HDL particles are distributed in a medium as a body centered cubic structure.[23] On this structure,[24] the cube volume (V_c) is equal to the volume of two particles (V_p) and the particle concentration can be obtained by

Eq. 1, the number of particles divided by the volume of particles:

$$C_{pc} = \frac{2 \text{ particles}}{V_c} \quad (1)$$

where C_{pc} is particle concentration and V_c is cube volume. At the same time:

$$C_{CTc} = \frac{m_{CT}}{V_c} \text{ and } \rho_{col} = \frac{m_{CT}}{V_p} \quad (2)$$

where C_{CT} is total cholesterol concentration (80 mg/dL), m_{CT} is mass of total cholesterol, ρ_{col} is particles cholesterol density (1.067 g/mL), and V_p is particle volume done by:

$$V_p = \left(\frac{4}{3}\right)\pi R^3 \quad (3)$$

where R is the particle radius.

Table 1. High-density lipoprotein subspecies sizes and concentrations.

	Diameter (nm)	Subspecies concentrations (particle number/m^3)
HDL2b	12.0	8.29E + 20
HDL2a	8.6	2.25E + 21
HDL3	7.4	3.53E + 21

Using Eqs. 1, 2, and 3:

$$C_{CTc} = \frac{2m_{CT}}{V_c}$$

$$C_{CTc} = \frac{2\,\rho_{col}\,V_p}{V_c}$$

$$C_{CTc} = \frac{2\,\rho_{col}\left(\frac{4}{3}\right)\pi R^3}{V_c}$$

Since, $C_{pc} = \frac{2 \text{ particles}}{V_c}$,

$$C_{CTc} = C_{pc}\,\rho_{CT}\left(\frac{4}{3}\right)\pi R^3 \quad (4)$$

then particle concentration is done by:

$$C_{pc} = \frac{3C_{CT}}{4\pi R^3 \rho_{CT}} \quad (5)$$

The particles concentrations calculated using Eq. 5 are shown in Table 1.

High-Density Lipid Subspecies Absorption Spectra

The HDL subspecies optical absorption spectra are shown in Figure 1. The peak observed at 279 nm is due to the amino acids, mainly tryptophan, present in the apolipoproteins. The absorption bands in the range of 350–600 nm are mostly due to carotenoids binding proteins.[25] The HDL2b subspecies have more carotenoids and the HDL3 has the lowest concentration of carotenoids in comparisons with others subspecies.

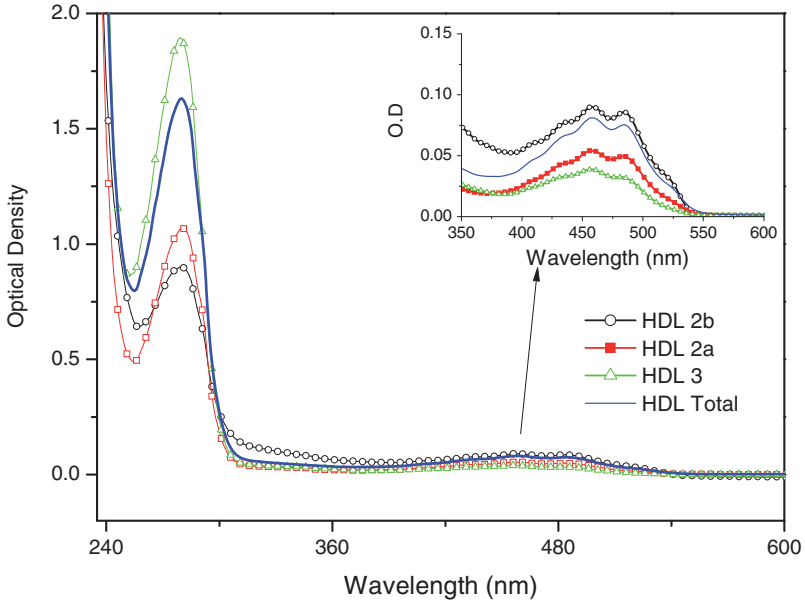

Figure 1. Absorption spectra of HDL 2b, 2a, 3, and total.

The absorption spectra of EuTc complex and EuTc in the presence of HDL subspecies are shown in Figure 2. EuTc presents the maximum absorption at 400 nm, and no significant changes are observed in the presence of HDL subspecies.

Emission Spectra of EuTc in the Presence of High-Density Lipoprotein Subspecies

The EuTc emission spectra obtained with and without the presence of HDL are shown in Figure 3a. In this figure, an increase in the EuTc emission intensity around 617 nm in the presence of HDL can be observed. The EuTc + total HDL solution presents the highest emission intensity and the EuTc + HDL2b presents the lower emission intensity. Since the subspecies concentrations are different, the europium emission spectra were normalized by each HDL subspecies concentration, calculated using the data shown in Table 1. The normalized europium emission intensity is shown in Figure 3b. In this case, EuTc + total HDL fraction presents the highest intensity and EuTc + HDL2b the lowest intensity. Since europium emission intensity changes

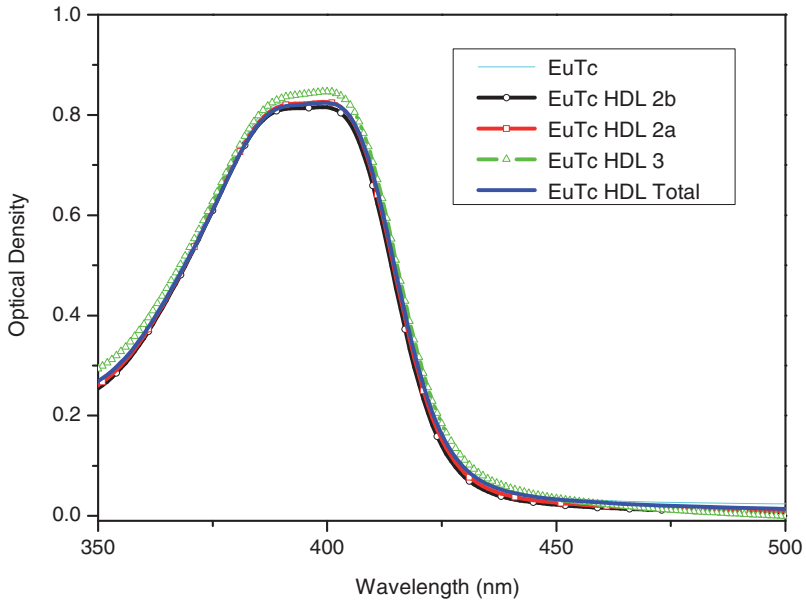

Figure 2. Absorption spectra of EuTc in the presence of subspecies of HDL (HDL2b, HDL2a, HDL3, and Total HDL).

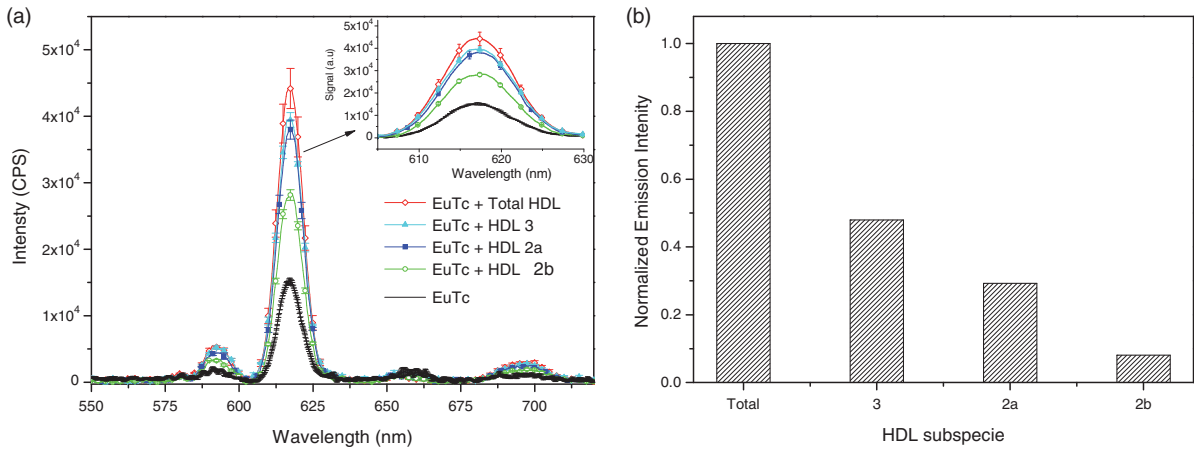

Figure 3. (a) Emission spectra of EuTc in the presence of HDL subspecies (HDL2b, HDL2a, HDL3, and Total HDL). (b) Normalized EuTc emission in the presence of HDL subspecies. The emission spectra of EuTc + HDL subspecies were multiplied by the particle concentration measured in Table 1. The EuTc + Total HDL concentration was considerate the sum of subspecies concentrations. Finally, the results were normalized to 1 (total HDL concentration).

in the presence of each type of HDL subclass, it is possible to determine the subclass concentration directly by measuring the emission spectra of subspecies added to EuTc complex.

Fluorescence Lifetime of EuTc in the Presence of High-Density Lipoprotein Subspecies

The europium emission lifetime of EuTc complex in the presence of 2a, 2b, and 3 subspecies and total HDL were measured and the results are shown in Figure 4.

It is known that the intrinsic decay of a trivalent rare earth ion (TR^{3+}) is given by Eq. 6 that considers the radiative decay rate ($1 / \tau_R$) plus rates of all non-radiative processes present in the ion interaction with the surroundings. This non-radiative process comprehends multiphonon process decays (W_{nR}) and interactions between the TR^{3+} ions and the medium.[26] In this case the main energy transfer process occurs between the Eu^{3+} and the water molecules from the vicinity by the electron–phonon coupling effect induced by the permanent electric dipole of the water molecule (W_{NR} (H_2O)). τ_R is usually obtained by applying the Judd–Ofelt theory[27] that takes into account the interaction of ions in the neighborhood as a weaker interaction and which can be calculated from the ion's absorption spectra in the medium.

$$\frac{1}{t_1} = \frac{1}{\tau_R} + W_{nR} + W_{nR}(H_2O) \tag{6}$$

An excited TR^{3+} ion interacts with other TR^{3+} ions by electric or magnetic dipole, or quadrupole. However, the dominant interactions are always the dipole–dipole electric type for distances up to a few angstroms. In this case, the function that describes the excited state of donor ion (D) decay interacting with all the other ions (acceptor) randomly distributed is described in Eq. 7. This function describes the decay in the interaction volume and without involving the migration of excitation (between D ions).[28]

$$I(t) = I_0 \exp(-\gamma\sqrt{t} - t/t_1) \tag{7}$$

The parameter t_1 has already been set previously by Eq. 6. The parameter γ allows for the direct transfer of the donor ion (D) to the acceptor (A) without migration excitement. In this case, the Inokuti lifetime decay[29] (τ) is obtained integrating the function I(*t*) as described in Eq. 8.

$$\tau_1 = \frac{1}{I_0}\int_0^\infty \exp(-\gamma\sqrt{t} - t/t_1)\, dt \tag{8}$$

EuTc and EuTc + HDL subspecies decay curves were fitted using the function described by Eq. 9, where the Inokuti fit with amplitude A was coupled with an exponential function with time constant (lifetime) t_2 and amplitude B as:

$$I(t) = y_0 + A\exp(-\gamma\sqrt{t} - t/t_1) + B\exp(-t/t_2) \tag{9}$$

In this model, there are two characteristic decays, an Inokuti-type decay whose contribution is given by the ratio A / (A + B) (Type 1), and another of exponential type with contribution B / (A + B) (Type 2). Type 1 corresponds to the Eu^{3+} ions complexed as EuTc and transfers

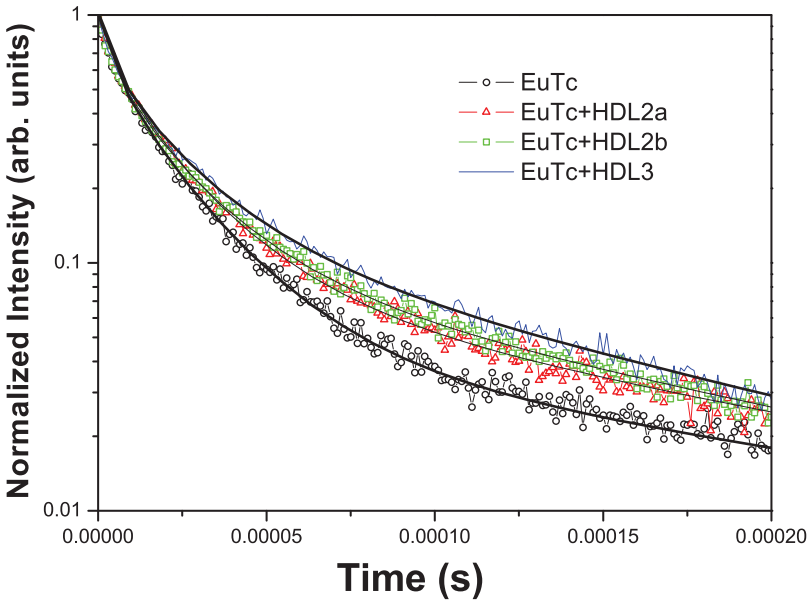

Figure 4. Eu^{3+} emission lifetime for EuTc, and EuTc in the presence of HDL2b, 2a and 3. The curves were fitted using the function described by Eq. 9, were the Inokuti fit with amplitude A was coupled with an exponential function with time constant (lifetime).

Table 2. Eu^{3+} time constants and contribution to the Inokuti + exponential decay.

	τ_1 (integrated) (μs)	$\tau_2 = t_2$ (μs)	A / (A + B) (Inokuti) (%)	B / (A + B) (Exp) (%)
(0) EuTc	14.2 ± 0.7	200 ± 10	95.3	4.7
(1) EuTc + HDL2b	14.1 ± 0.7	167 ± 8	92.2	7.8
(2) EuTc + HDL2a	13.6 ± 0.7	154 ± 7	94.6	5.4
(3) EuTc + HDL3	13.9 ± 0.7	137 ± 6	88.4	11.6
(4) EuTc + Total HDL	13.8 ± 0.7	144 ± 7	96.1	3.9

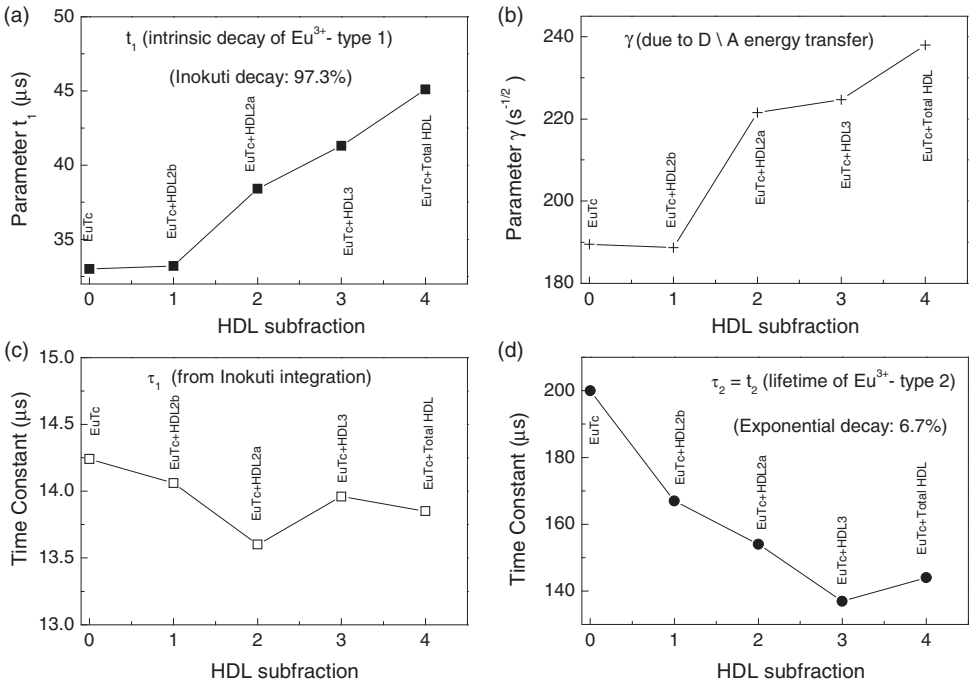

Figure 5. Performance of parameters: t_1 (a), g (b), τ_1 (c), and τ_2 (d) in function of HDL subspecies. The numbers in the *x*-axis represents: (0) EuTc; (1) –EuTc + HDL2b; (2) –EuTc + HDL2a; (3) –EuTc + HDL3; (4) –EuTc + Total HDL.

excitation energy along the lines of Inokuti–Hirayama and corresponds to 97.3% of ions excited according to the data presented in Table 2. The second type corresponds to the isolated Eu^{3+} ions and this exponential decay corresponds to an approximately constant fraction of 6.3% of the initially excited ions (Table 2).

The parameters obtained by the fit of decay curves with the function shown in Eq. 9 are given in Table 2. The fits (Figure 4) made using Eq. 9 show good quality with $R^2 \sim 0.998$, describing well the decay curve, especially around $t = 300\,\mu s$. The same quality is not achieved in the fits using the sum of two exponential functions.

The parameters obtained in these fits, in function of each HDL subspecies, are presented in Figure 5. In Figure 5a, the exponential term (t_1) in Inokuti decay increases with the amount of HDL ranging from 0 (EuTc) to 4 (EuTc + Total HDL). The same occurs with the parameter γ, as shown in Figure 5b. The integrated lifetime (τ_1) decay by Inokuti (type 1 species) is practically constant, as shown in Figure 5c. However, the integrated lifetime (τ_2) decay, obtained by the exponential (type 2 species) decreases for HDL subspecies mainly by HDL3 as shown in Figure 5d. Therefore, the obtained results shown that the Eu^{3+} emission lifetime, in EuTc complex, can discriminate the subfractions HDL3 and HDL2b.

Discussion

For many years, the association between the risk of CHD and measurement of HDL subclasses were ignored. With the advance in techniques for quantification, isolation, and classification in subclasses of HDL, new studies have emerged. Therefore, today, measurement of HDL subspecies has been a useful research tool in studies with

CHD risk.[30] The functionality of HDL is dependent on HDL composition. High-density lipoprotein can be divided into two main subfractions: HDL2 and the smaller HDL3. The HDL2 is composed by HDL2a and HDL2b. Elevated total cholesterol and low HDL cholesterol levels, as well as high triglyceride levels, are associated with low HDL2b levels.[31] Assessment of HDL2b particles may provide a more powerful measure of cardiovascular risk than other HDL2a or HDL3 subfractions, individually or combined.[32] Our results indicated that the HDL2b subspecies have more carotenoids than the HDL3 subspecies. Indeed, HDL2b is the particle most associated with oxidative protection and antioxidant capabilities.[33]

Europium emission intensity changes in the presence of each type of HDL subclass. It is possible to determine the subclass concentration directly by measuring the emission spectra of subfractions added to EuTc complex.

EuTc complex shows very weak fluorescence due to the quenching effect of water molecules.[34] In neutral 1:3 complexes, the coordination number on the lanthanide ion is 6. This means that the coordination of the lanthanide ions is unsaturated and additional ligands can be incorporated. Europium emission intensity changes in the presence of the HDL subspecies allow the subclass concentration determination directly by measuring the emission spectra of subspecies added to the EuTc complex. In the presence of the HDL subspecies, a water ligand, which exerts a quenching effect, is replaced by HDL, resulting in an emission enhancement. Considering only the isolated HDLs subspecies, the smaller subspecies should replace more water molecules and, in this context, HDL3 should increase the Eu^{3+} fluorescence intensity. This was observed in the emission spectra shown in Figure 3.

Eu^{3+} fluorescence lifetime in EuTc + HDL3 is longer than that of EuTc, as observed in Figure 4. A small fraction of ions Eu^{3+} complexed with EuTc (6.7%) (type 2) do not participate in the energy transfer process, showing an exponential decay with a relatively longer lifetime. For EuTc $\tau_2 = 200\,\mu s$ and this value decreases with the addition of HDL ($\tau = 137\,\mu s$ to EuTc + HDL3). In this case, europium ions must be in more isolated sites (shielded from interaction with water), which do not transfer excitation energy to other ions Eu^{3+}. Ghorai et al.[35] showed that the Tc in the complex EuTc resides in the inner part of protein BSA/HSA, so the ions Eu^{3+} are more protected from the interaction with water. Probably the same occurs in the case of proteins present in HDL3. In this context, larger values of τ_2 may indicate reduced risk of CHD risk.

In this study, a new method was described of utilizing the EuTc complex in determining the size (fluorescence lifetime) and concentration (fluorescence steady state) of HDL in human plasma. The advantages of optical spectroscopic methods when compared to conventional methods for the determination of HDL subclasses are the simplicity and facility. Spectroscopic methods do not require specialized and expensive instrumentations and technicians, and take at least a few minutes to deliver results.

In our studies, we prepared the EuTc complex. However, Sigma Aldrich commercializes EuTc complex with high level of purity and stability.

Conclusion

The europium spectroscopic properties in the EuTc complex are influenced by sizes and concentration for the different HDL subclasses. The HDL3 presents 50% of the total HDL emission intensity and HDL2 only 10%. The integrated europium lifetime (τ_2) decay, obtained by the exponential (type 2 species), for EuTc ($\tau_2 = 200\,\mu s$) decreases with the addition of HDL subspecies ($\tau = 147\,\mu s$ for EuTc + HDL2b and $\tau = 137\,\mu s$ for EuTc + HDL3). Therefore, the intensity and lifetime of Eu^{3+}emission can discriminate the subfractions HDL3 and HDL2b.

Conflict of Interest

The authors report there are no conflicts of interest.

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