Characterization of the Europium Tetracycline Complex as a Biomarker for Atherosclerosis

Lilia Coronato Courrol[a], Mônica Nascimento da Silva[a], Letícia Bonfante Sicchieri[b]
[a]Depto de Ciências Exatas e da Terra, Univ. Federal de São Paulo (Brazil), Rua Arthur Riedel 275, Eldorado, 09972-270 – Diadema - SP Brazil; [b]IPEN-CNEN/SP, Av. Lineu Prestes 2242 - Cidade Universitária, 05508-000 - São Paulo - SP Brazil.

ABSTRACT

Atherosclerosis is a narrowing of the arteries caused by an increase of atheromatous plaque: material formed by macrophage cells containing cholesterol and fatty acids, calcium and a variable amount of fibrous connective tissue. The elation between vulnerable plaques and cardiovascular events can be determined using plaque biomarkers. In this work, atherosclerotic plaques stained with different molar ratios of europium, in a potential plaque biomarker, europium tetracycline complex, were studied by fluorescence microscopy. The tetracycline antibiotic used was chlortetracycline. The growth of atherosclerotic plaque was followed during 60 days in New Zealand rabbits divided in two groups: an experimental group (EG), with nine animals and a control group (CG) with three animals. The animals in the EG received a diet with 1% of cholesterol and the animals of GC received a normal diet. The aortic arch of the animals with 60 days were cut in the vertical plane in 6 μm thick slices, which were mounted on glass slides and stained with hematoxylin an eosin and europium chlortetracycline complex (EuCTc). The fluorescence images were obtained exciting the EuCTc absorption band with a filter cube D (BP 355 – 425) and the emission was collected with a LP 470 suppression filter. Light intensity, detector gain and acquisition time were fixed for comparisons. The 20× magnified images were collected with 12 bit (or 4096 gray tones) resolution. The mean value of gray scale for each molar ratio of EuCTc was different, indicating that the complex interacts with the components of atherosclerotic plaque and the best molar ratio was 1.5 EuCTc. These results indicate the potential use of the EuCTc biomarker for atherosclerotic plaque characterization.

Keywords: Atherosclerosis, EuTc complex, Fluorescence Microscopy

1. INTRODUCTION

Atherosclerosis is a progressive and inflammatory disease. It is characterized by accumulation of lipids and fibrous elements in the arteries[1]. One of the first events to atherosclerotic plaque formation is the accumulation of low density lipoprotein (LDL) in the sub endothelial layer, where it undergoes oxidative modifications, and is internalized by macrophages through scavenger receptors, originating foam cells and inducing an immune inflammatory reaction[2-4].

The relation between vulnerable plaques and cardiovascular events can be determined using plaque biomarkers[5]. A biomarker is an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention[6]. Serum biomarkers have been extensively studied as predictive risk factors, especially in patients with manifest atherosclerosis, but have limited application in clinical settings for risk stratification[7].

Tetracyclines form a complex with europium trivalent ions[8]. Some works shows that this complex can be used to quantify LDL[9], urea hydrogen peroxide[10], hydrogen peroxide[11] and glucose[12]. It was demonstrated that the europium luminescence of the europium tetracycline complex increases in the presence of low density lipoprotein (LDL)[9, 13-16], and can be used as a serum biomarker.

Chlortetracycline is a member of tetracycline family. The Europium-Chlortetracycline complex (EuCTc) has an absorption band centered at 400 nm, emission at ~617 nm and a large Stokes-Shift (approximately 217 nm). This work reports the study of the EuCTc complex potential use as an atherosclerotic tissue biomarker by fluorescence microscopy

Biophotonics: Photonic Solutions for Better Health Care V, edited by Jürgen Popp, Valery V. Tuchin, Dennis L. Matthews, Francesco Saverio Pavone, Proc. of SPIE Vol. 9887, 98872D
 · doi: 10.1117/12.2227958

in cholesterol-fed rabbits. The atherosclerotic plaque stained with different molar ratios of Europium in the complex EuCTc was studied.

2. MATERIALS AND METHODS

2.1 Animal Experimental

A total of 12 adult white male rabbits (New Zealand species Orytolagus cuniculus, 2.3 ± 0.1 kg, and ~3.5 months of age) were divided in two groups, an experimental group (EG) with nine animals, and a control group (CG) with three animals. During 60 days, the animals in the EG received a 1% cholesterol diet, and the animals of GC received a normal one. The animals were individually housed in a controlled environment maintained at 19°C with food and water provided ad libitum. The protocol was approved by the Ethics Committee of UNIFESP (Protocol nº 0327/12).

In the 60th day after the overnight fasting, blood was drawn from the marginal ear vein at the baseline time point. The blood samples were stored on ice for 2 h and centrifuged (3000 rpm, 10 min, 4 °C) to obtain plasma. Plasma samples were processed by the sequential separation ultracentrifuge. A "pool" of samples from the rabbits of the same groups was done to obtain a larger quantity of lipoproteins. For sequential isolation of plasma, density was adjusted to VLDL(very low density protein) d < 1.006 g/mL, LDL d = 1,030-1,063 g/mL and HDL (high density lipoprotein) d = 1,063-1.21 g/mL and then centrifuged at 105, 4° C, for 20 hours, using 75 rotor (Beckman Instruments). Due to the large amount of extracted VLDL in the procedure, the amount of LDL removal was insufficient to be quantified.

The VLDL extracted was diluted in water to obtain different concentrations. Table 1 shows the volumes used in the dilutions and their concentrations measured from the quantification of triglycerides (enzymatic colorimetric assay (Labtest Diagnostic S.A).

Table 1. Different concentrations of VLDL obtained from plasma of the rabbits of the group 60 days (mg/dL).

Dilutions	*VLDL concentration (mg/dL)*
VLDL I (600 μL VLDL)	473.44
VLDL II (500 μL VLDL +100 μL H_2O)	347.13
VLDL III (400 μL VLDL+ 200 μL H_2O)	306.22
VLDL IV (300 μL VLDL+ 300 μL H_2O)	202.87
VLDL V (200 μL VLDL+ 400 μL H_2O)	148.80
VLDL VI (100 μL VLDL +500 μL H_2O)	72.25

The aortic arch of animals from CG and EG groups were cut in the vertical plane in 6 μm thick slices on a cryostat, then mounted on glass slides and stained with EuCTc and hematoxylin an eosin (HE).

2.2 EuCTc preparation

The europium chlortetracycline (EuCTc) complex was prepared starting from inorganic salts with analytical purity. All solutions were prepared in 10 mmol/L 3-(N-Morpholino) propanesulfonic acid (MOPS, from Carl Roth) buffer, at pH = 6.9. The europium(III) chloride hexahydrate and chlortetracycline hydrochlorides used are from Sigma–Aldrich. The EuCTc solutions were prepared by fixing the chlortetracycline chlortetracycline hydrochlorides (21 μmol L^{-1}) and varying the europium(III) chloride hexahydrate from 21.0 to 73.5 μmol L^{-1}.

2.3 Instrumentation

The emission spectra were obtained by exciting the samples at 405 nm using a 1 mm optical path cuvette (Hellma). The sample fluorescence was measured from 570–650 nm using a Horiba Jobin Yvon Fluorolog 3 Fluorimeter.

The images were obtained with a Leica DMI6000 CS fluorescence microscope with Leica DFC360FX (monochrome) and DMC4500 (color) digital video cameras and analyzed by the Leica AF6000 software. The fluorescence images were obtained using excitation at the EuCTc absorption band using a filter cube D (BP 355 – 425) and a suppression filter LP 470. For emission quantification the monochrome camera was used, and the light intensity,

gain and acquisition time were fixed for comparisons. The 20× magnified aortic sections images were collected with 4096 gray tones (12 bit).

3. RESULTS

The morphology of the artery from animals from GC and EG-60 days group was analyzed. Representative images obtained of aortas stained with H&E are shown in the Figure 1. The aortas of the rabbits fed with a normal diet (a) have normal thicknesses and no lipid is present in the intima layer. The aortas from rabbits fed with the high-cholesterol diet (b) present foam cells and some lymphoid cells in the thickened intima.

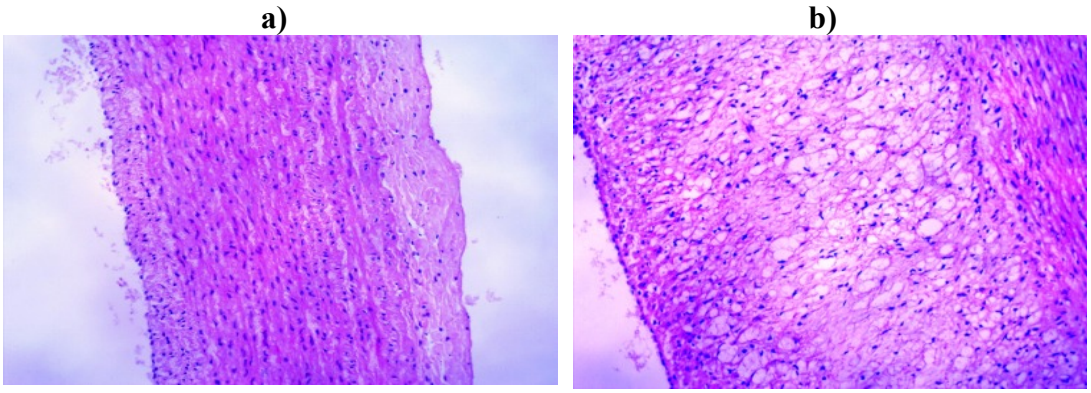

Figure 1 – Cross section of artery of animal of 60 days group stained with HE: CG (a) and EG (b).

To evaluate the potential of use EuCTc as atherosclerosis marker, solutions with different molar ratios were prepared. Previous works[9] demonstrated that solutions with molar ratios of 1.5 Eu:1.0 CT presented increased europium emission band intensity when compared to those with other molar ratios solutions. Following these results, the fluorescence of EuCTc complex prepared with 1.5 Eu:1.0 CT was measured in the presence of different VLDL concentrations, extracted from cholesterol diet fed rabbits, and the results are shown in the Figure 2. The samples were excited at 400 nm and the emission peak was measured at 617 nm. The results indicate an increase in the europium emission with the VLDL concentration increase. In hypercholesterolemic rabbis, a large fraction of the plasma cholesterol present in lipoproteins is of very low density (VLDL) and oxidated VLDL [17], and a positive correlation between the amount of oxidized VLDL/LDL in the lesions and the plasma levels of VLDL/LDL is observed.

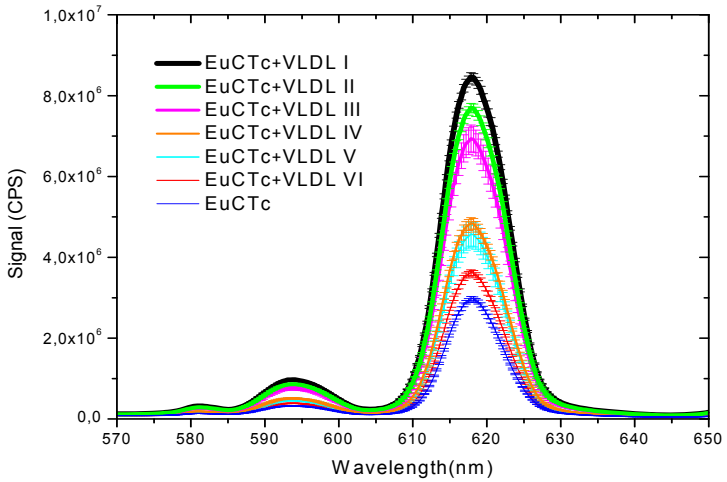

Figure 2. Emission spectra of the complex EuCTc (with VLDL obtained from Rabbit plasma 60 days group). EuCTc+VLDL samples were prepared in triplicate. The EuCTc complex was prepared with 31.5 µmol L−1 of EuCl3.6H2O and: 21 µmol L−1 of CTc in 100 mL Mops buffer.

The aortas stained with EuCTc were studied. Representative color images obtained of the artery from animals EG-60 days group not stained (a) and stained with EuCTc complex (1.5 Eu:1.0 CT) are shown in the Figure 3. In figure 3a, the green autofluorescence of tunica media fluorophores (collagen and elastin) is observed. A dark area in the tunica intima shows that is impossible to observe atheromatous plaque by autofluorescence.

A representative image of the arteries slices stained with EuCTc complex (1.5 Eu:1.0 CT) is shown in the figure 3b. A green fluorescence in the tunica media and a red emission in the tunica intima are observed, both originating from the interaction of oxidized VLDL/LDL in the lesions with EuCTc.

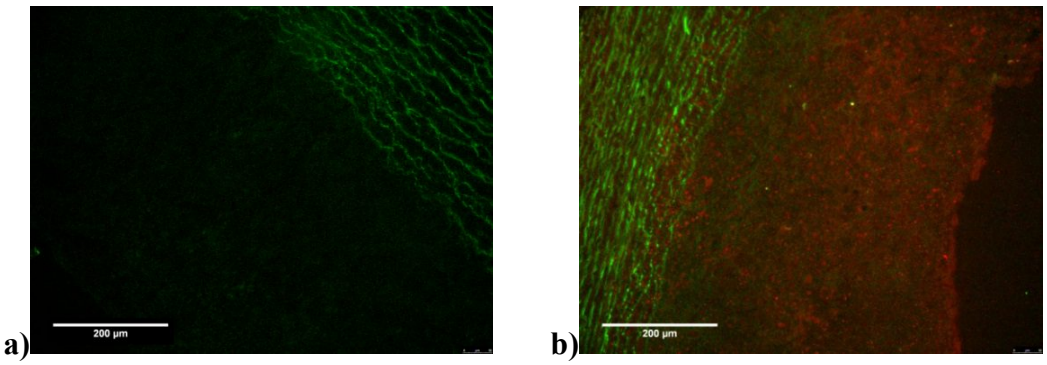

Figure 3 – Cross section of artery of animal of EG not stained (a) and stained with EuCTc (b). The images were obtained with color video camera, Filter cube D (BP 355 – 425) and suppression filter LP 470.

Slides stained with different molar ratios of EuCTc, for animals from EG-60 days, were studied. The images obtained for the molar ratios Eu:CTc, 1:1; 1.5:1; 2:1; 2.5:1, 3:1 and 3.5:1, are shown in the Figure 4. A ROI (Region OF Interest) was selected in the images, and the gray scale histogram was obtained, shown in Figure 5. The distribution with highest values of gray scale came from the slide stained with 1.5 Eu: 1.0 CTc.

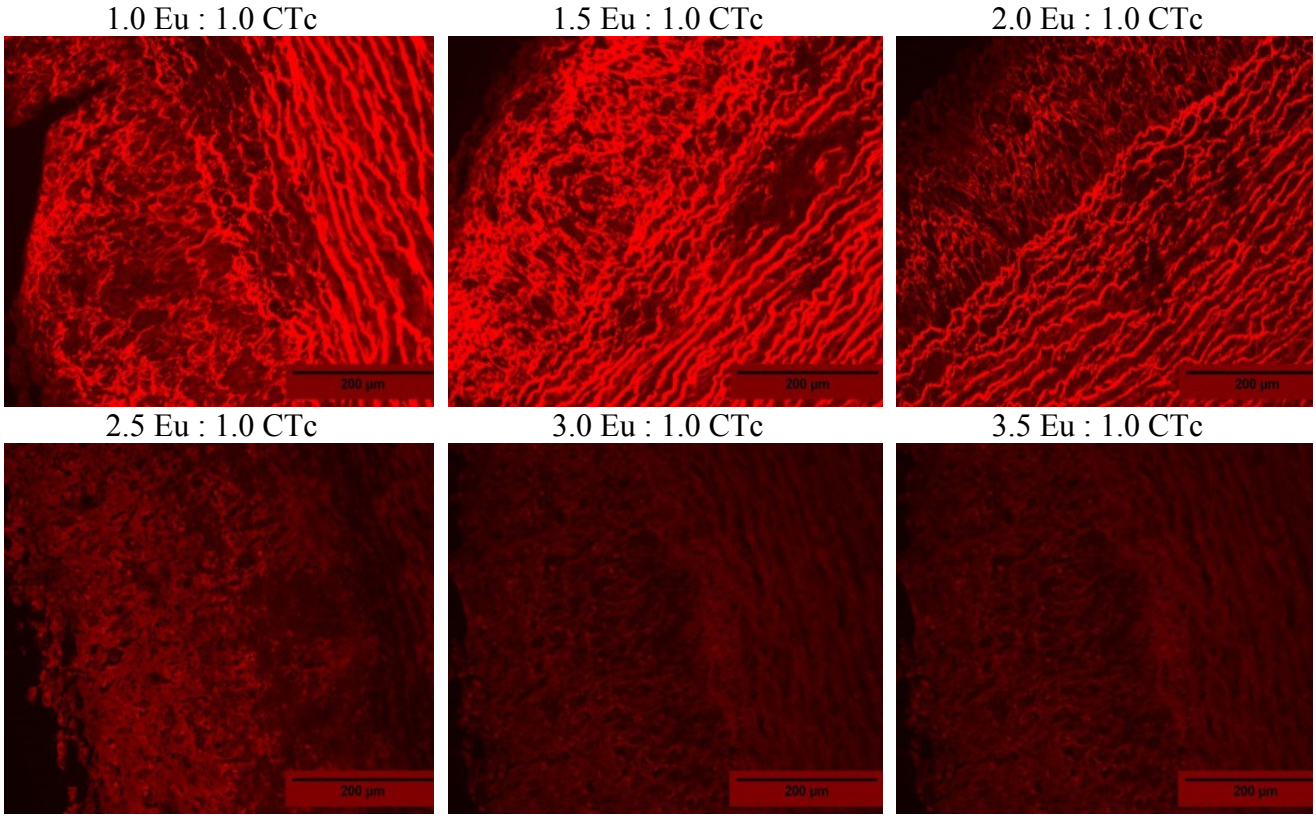

Figure 4 – Cross section of arteries stained with different molar ratios of EuCTc (1.0 Eu: 1.0 CTc to 3.5 Eu: 1.0 CTc). These images were obtained with the high-resolution monochrome camera.

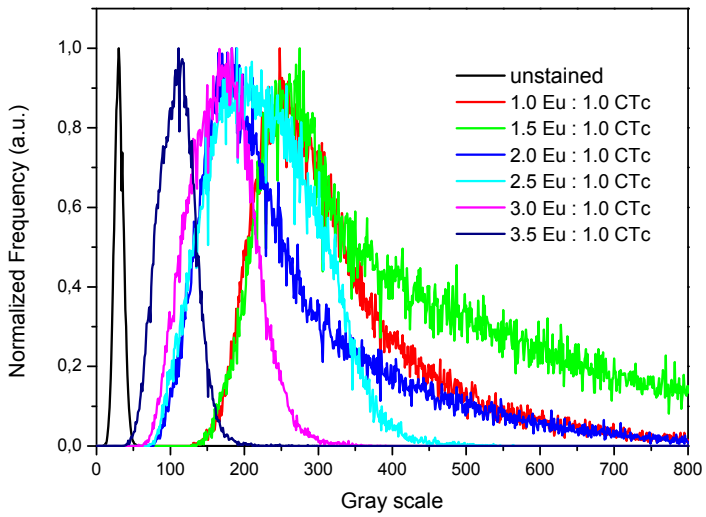

Figure 5 - Distribution of gray scale values for a selected ROI in stained images with different molar ratios of EuTc (1.0 Eu : 1.0 Tc to 3.5 Eu : 1.0 Tc).

DISCUSSION

Lipids (both fatty acids and cholesterol) are typically washed out in the processing for conventional histology from paraffin embedded sections, leaving only empty spaces and making quantitative evaluation difficult. Triglycerides and esterified cholesterol can be imaged in frozen sections stained with, e.g., Oil Red O. It is, however, advantageous to have a fluorescent marker which can generate high contrast images of lipid accumulations.

In this study, aortas stained with EuCTc complex were studied by in an attempt to characterize a new marker for atherosclerosis. By the analyses of the images, it was observed that the best molar ratio to visualize the atherosclerotic plaque is 1.5Eu:1CTc, the same molar ratio determined in the interaction of the complex with VLDL. This result indicates that the europium emission can distinguish regions rich in oxidized VLDL/LDL, providing selective indication of plaque region and the possibility of use of EuCTc complex as fluorescent marker for atherosclerosis.

Another advantage of use EuCTc as biomarker is the interaction of tetracyclines with atherosclerotic tissue. Lindgren and Raekallioll[18] observed that tetracycline binds selectively to atheroma when administered orally in therapeutic concentrations. Moreover, tetracycline was located in the same area of the atheroma as the phospholipid stains[19], suggesting that it is lipophilic. Golub et al.[20] observed that the non-antibacterial properties of Tetracyclines, could have therapeutic potential in cardiovascular diseases. These facts indicate that Europium Chlortetracycline can be retained in the artery walls facilitating the optical diagnosis *in vivo*, by fluorescent angioscopy.

CONCLUSION

This work investigated the potential use of the EuCTc as a marker for atherosclerotic plaque. Atherosclerotic aortas stained with EuCTc showed intense red emission not present in the normal aortas, indicating that this marker can differentiate healthy aortas from atherosclerotic ones, also evidencing that the complex EuTc interacts with the VLDL/LDL. The images and distributions of gray scale that produced greater contrast, indicating the best ratio for plaque visualization, were the ones obtained with 1.5 Eu : 1.0 Tc molar ratio.

Acknowledgment

This work was supported by the "Fundação de Amparo a Pesquisa do Estado de São Paulo" (FAPESP), Grant 2014/06960-9.

REFERENCES

[1] P. Libby, P. M. Ridker, and G. K. Hansson, "Progress and challenges in translating the biology of atherosclerosis," Nature, 473(7347), 317-325 (2011).

[2] G. O. Angheloiu, J. T. Arendt, M. G. Mueller *et al.*, "Intrinsic fluorescence and diffuse reflectance spectroscopy identify superficial foam cells in coronary plaques prone to erosion," Arteriosclerosis Thrombosis and Vascular Biology, 26(7), 1594-1600 (2006).

[3] F. D. Brites, C. D. Bonavita, C. De Geitere *et al.*, "Alterations in the main steps of reverse cholesterol transport in male patients with primary hypertriglyceridemia and low HDL-cholesterol levels," Atherosclerosis, 152(1), 181-192 (2000).

[4] W. P. Castelli, "Lipids, risk factors and ischaemic heart disease," Atherosclerosis, 124, S1-S9 (1996).

[5] M. N. da Silva, L. B. Sicchieri, F. R. D. Silva *et al.*, "Liquid biopsy of atherosclerosis using protoporphyrin IX as a biomarker," Analyst, 139(6), 1383-1388 (2014).
[6] A. J. Atkinson, W. A. Colburn, V. G. DeGruttola *et al.*, "Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework," Clinical Pharmacology & Therapeutics, 69(3), 89-95 (2001).
[7] H. Drexel, F. W. Amann, J. Beran *et al.*, "PLASMA TRIGLYCERIDES AND 3 LIPOPROTEIN CHOLESTEROL FRACTIONS ARE INDEPENDENT PREDICTORS OF THE EXTENT OF CORONARY ATHEROSCLEROSIS," Circulation, 90(5), 2230-2235 (1994).
[8] L. C. Courrol, F. R. d. O. Silva, L. Gomes *et al.*, "Energy transfer study of europium-tetracycline complexes," Journal of Luminescence, 122, 288-290 (2007).
[9] L. d. S. Teixeira, A. N. Grasso, A. M. Monteiro *et al.*, "Enhancement on the Europium emission band of Europium chlortetracycline complex in the presence of LDL," Analytical Biochemistry, 400(1), 19-24 (2010).
[10] L. C. Courrol, F. R. de Oliveira Silva, L. V. G. Tarelho *et al.*, [Enhancement of europium luminescence in tetracycline-europium complex in the presence of urea hydrogen peroxide - art. no. 60970D], (2006).
[11] A. N. Grasso, L. d. S. Teixeira, N. D. Vieira, Jr. *et al.*, "Optical Properties of Metacycline, Oxytetracycline and Chlortetracycline Europium Complexes in the Presence of Hydrogen Peroxide," Journal of Fluorescence, 19(4), 715-721 (2009).
[12] L. C. Courrol, and R. E. Samad, "Applications of Europium Tetracycline Complex: A Review," Current Pharmaceutical Analysis, 4(4), 238-248 (2008).
[13] L. C. Courrol, F. R. de Oliveira Silva, R. E. Samad *et al.*, [Europium tetracycline biosensor for the determination of cholesterol - art. no. 643017], (2007).
[14] L. C. Courrol, A. M. Monteiro, F. R. O. Silva *et al.*, "Novel fluorescent probe for low density lipoprotein, based on the enhancement of europium emission band," Hypertension, 50(4), 816-816 (2007).
[15] F. R. de Oliveira Silva, R. E. Samad, L. Gomes *et al.*, "Enhancement of europium emission band of europium tetracycline complex in the presence of cholesterol," Journal of Fluorescence, 18(1), 169-174 (2008).
[16] F. R. de Oliveira Silva, A. M. Monteiro, A. M. Figueiredo Neto *et al.*, [Optical characterization of europium tetracycline complex in the presence of Low Density Lipoprotein and its applications], (2008).
[17] P. Holvoet, and D. Collen, "beta-VLDL hypercholesterolemia relative to LDL hypercholesterolemia is associated with higher levels of oxidized lipoproteins and a more rapid progression of coronary atherosclerosis in rabbits," Arteriosclerosis Thrombosis and Vascular Biology, 17(11), 2376-2382 (1997).
[18] I. Lindgren, and Raekalli.J, "ACCUMULATION OF TETRACYCLINES IN ATHEROSCLEROTIC LESIONS OF HUMAN AORTA," Acta Pathologica Et Microbiologica Scandinavica, 66(3), 323-& (1966).
[19] G. Caminati, C. Focardi, G. Gabrielli *et al.*, "Spectroscopic investigation of tetracycline interaction with phospholipid Langmuir-Blodgett films," Materials Science & Engineering C-Biomimetic and Supramolecular Systems, 22(2), 301-305 (2002).
[20] L. M. Golub, and R. A. Greenwald, "Clinical applications of non-antibacterial tetracyclines. Part II Preface," Pharmacological Research, 64(6), 549-550 (2011).