Atherosclerosis staging: imaging using FLIM technique

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ABSTRACT

In this work it was used fluorescence lifetime imaging (FLIM) to analyze biochemical composition of atherosclerotic plaque. For this purpose an animal experimentation was done with New Zealand rabbits divided into two groups: a control group of 4 rabbits that received a regular diet for 0, 20, 40 and 60 days; and an experimental group of 9 rabbits, divided in 3 subgroups, that were fed with 1% cholesterol diet for 20, 40 and 60 days respectively. The aortas slices stained with europium chlortetracycline were analyzed by FLIM exciting samples at 440 nm. The results shown an increase in the lifetime imaging of rabbits fed with cholesterol. It was observed that is possible to detect the metabolic changes associated with atherosclerosis at an early stage using FLIM technique exciting the tissue around 440 nm and observing autofluorescence lifetime. Lifetimes longer than 1.75 ns suggest the presence of porphyrins in the tissue and consequently, inflammation and the presence of macrophages.

Keywords: Aorta, Atherosclerosis, Europium, Biomarker, Fluorescence, Microscopy, FLIM

1. INTRODUCTION

Atherosclerosis is a progressive disease process that generally begins in childhood and manifests clinically in middle to late adulthood. Atherosclerotic plaque formation occurs due to the accumulation of lipids, and is the main cause of coronary artery disease [1]. It initiates through uptake of low-density lipoprotein, LDL, from blood by endothelial cells, and oxidation of LDL by reactive oxygen species within the vessel wall. LDL in its modified form contributes to inflammation as well as to macrophages evolution to foam cells [2].

Recent studies have demonstrated that fluorescence spectra can be used to distinguish fibrous plaque from healthy arterial wall. For atherosclerotic samples with calcified plaques have been observed three characteristic bands (424-460nm, 510-560nm, and around 600nm) and only one for the lipid plaques around 460mn [3].

Fluorescence lifetime imaging (FLIM) is a powerful tool to study atherosclerosis based on its ability to assess biochemical composition. Park et al.[4] classified plaques as either "High-Collagen", "High-Lipids" or "Low-Collagen/Lipids" based on the endogenous multispectral FLIM. Phipps et al.[5] described a novel FLIM classification method to determine the ratio of collagen to lipid content in the fibrous cap of atherosclerotic plaques. Both works used ultraviolet laser for the excitation.

Although time-resolved spectroscopy it is a robust and a sensitive technique, it is more difficult to implement than steady-state fluorescence spectroscopy. The majority of the fluorescent markers for lipids, especially those emitting in the visible light region, are hydrophilic[6]. The dye generally changes the hydrophilic/hydrophobic balance of the lipid molecule when attached in the chain region modifying the lipid's ability to integrate into membranes [7]. Studies show that europium-tetracycline complex (EuTc) can be used as lipoprotein fluorescent marker[8-11]. In a recent work, it was shown that Europium Chlorotetracycline (EuCTc) can be used to observe the formation of foam cells with fluorescence microscopy[10]. This complex presents maximum absorption and emission around 400 nm and 615 nm, respectively, and a fluorescence lifetime of a few microseconds, which differs from the lifetimes of biological tissues. The lipoproteins

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replace water molecules around europium ions diminishing quenching and consequently increase europium emission. The EuCTc complex is easily synthesized, operates at neutral pH, has high stability and low cost.

This paper presents the results obtained with FLIM technique of microscopic slices at different stages of formation of atheroma plaque in order to elucidate the biochemical composition of the tissue. For this purpose, it was used EuCTc as dye and excitation at 440 nm.

2. MATERIALS AND METHODS

2.1 Materials

The Europium Chlortetracycline (EuCTc) 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 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: 31.5 μmol.L^{-1} of $EuCl_3.6H_2O$ in 10 mL solution I.

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

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

Solution V: 0.75 mmol.L^{-1} of $MgSO_4.7H_2O$ in 100 mL in water bi-deionizate.

Solution VI: EuCTcMg: Mix of EuCTc and $MgSO_4.7H_2O$ in the ratio 1:1.

2.2 Animal experimentation

New Zealand rabbits were divided into two groups: CG (control group) of 4 rabbits received a regular diet for 0, 20, 40 and 60 days; EG (experimental group) 9 rabbits, divided in 3 subgroups, were fed with 1% cholesterol diet (Sigma-Aldrich) for 20, 40 and 60 days respectively. The animals were individually housed in a controlled environment maintained at 19°C with food and water provided ad libitum. For this study, the protocol was approved by the Ethics Committee of UNIFESP (Protocol nº 0327/12).

Rabbits were anaesthetized by injection of Ketamine and Xylazine then euthanized by exsanguination. Cryosections of the aortic specimens were cut in the vertical plane at 10 μm thickness on a cryostat, then mounted on glass slides and stained with europium chlortetracycline and oil red.

2.3 Biochemical analyses of plasma

After overnight fasting, blood was drawn from the marginal ear vein at the baseline time point and at the end of 20, 40 and 60 days after begins high cholesterol diet protocol. The blood samples were stored on ice for 2 h and centrifuged (1200 rpm, 15 min, 4 °C) to obtain plasma and serum. The total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL-C), low-density cholesterol (LDL-C) and glucose levels in the serum samples were assessed using an enzymatic colorimetric assay.

2.4 Instrumentation

Fluorescence Inverted Microscope

The images were obtained with Leica DMI6000 CS fluorescence microscope, Leica DFC360FX digital video Camera and Leica AF6000 software. The aortic sections were magnified (40x).The fluorescence images were obtained using excitation at the europium absorption using filter cube D (BP 355 – 425) and suppression filter LP 470.

FLIM

FLIM microscopy images (Fluorescence Lifetime Imaging Microscopy) were obtained with Microtime 200, from PicoQuant. The microscope unit was an inverted microscope (Olympus IX-71) with water immersion objective UPlanSApo 60X, NA 1.2 with laser diode excitation (440 nm) directed by a dichroic mirror (z440bcm AHF / Chroma) toward the objective. The laser at 440 nm features pulse widths <70ps and an average output power up to 5 mW at 40 MHz repetition rate. This system allows EuCTc excitation but cannot measure europium emission lifetime.

The fluorescent emission was collected by the same objective and transmitted by the dichroic mirror passing through a tube lens that focuses the fluorescence emission collected on a pinhole 50 um (micrometer), which selects the image plane and rejects light originated outside focus. The beam was then directed by mirrors to a detector type SPAD (Single Photon Avalanche Diodes, or photon detector single avalanche photodiode) before passing through a filter which blocks any reflection of the excitation (emission filter and HQ460LP Chroma). The acquisition and analysis of data was done with SymPhoTime v. 5.2.4 software. To measure the FLIM, the sample was scanned with monodirectional movement of the objective in an area of 80 × 80 μm^2, with a nominal positioning accuracy of 1 nm.

2.5 Statistical Analysis

The data were expressed as mean ± standard error of the mean (S.E.M.). The statistical comparisons among groups were performed using a one-way analysis of variance (ANOVA). Statistical significance was considered for probability values of $p < 0.05$. All of the experiments were performed independently and repeated a minimum of three times.

3. RESULTS

3.1 Effect of high cholesterol diet on growth and body weight

At baseline, the body weight of all experimental rabbits ranged from 2.2 to 2.4 kg. Over the course of the study, all of the animals gained weight, but no significant difference was observed at any time point. The final weight ranged from 2.4 to 3.4 kg. A comparison of serum lipids in the control and experimental groups is shown in the figure 1. All of the rabbits completed the experimental process.

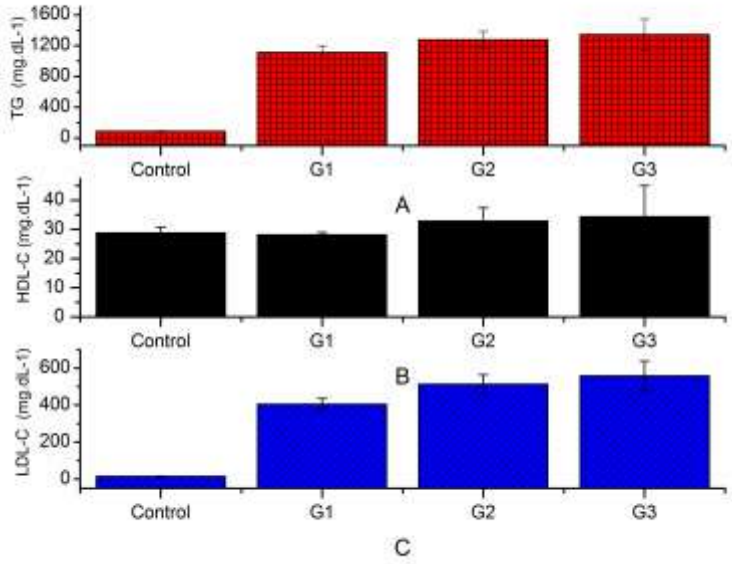

Figure 1. Serum lipids. Normal control group: rabbits were fed a normal diet; experimental group: rabbits were fed a high-cholesterol-diet for 20 days (EG1), 40 days (EG2) and 60 days (EG3). Data are presented as the mean ± SEM, n = 4 for control group and 3 for experimental groups. A) TC: total cholesterol; B) HDL-C: high-density lipoprotein cholesterol and C) LDL-C: low density lipoprotein cholesterol.

3.2 Aorta

The figure 2a shows the images obtained from aortas stained with oil red for the control and experimental groups. It was shown the control group and the experimental group with 60 days of diet. It can be seen in this figure that the aorta of rabbit fed with normal diet (control group) has normal thickness, and no lipid is present in the intima. For rabbits fed with high cholesterol diets (Experimental group) the intima layer is thickened.

The figure 2b shows the images obtained for aortas stained with EuCTc complex for control and experimental groups with 60 days. It can be observed a green emission, characteristic from tissue autofluorescence in the media layer and a red fluorescence, characteristic for europium emission, in the intima layer thickness.

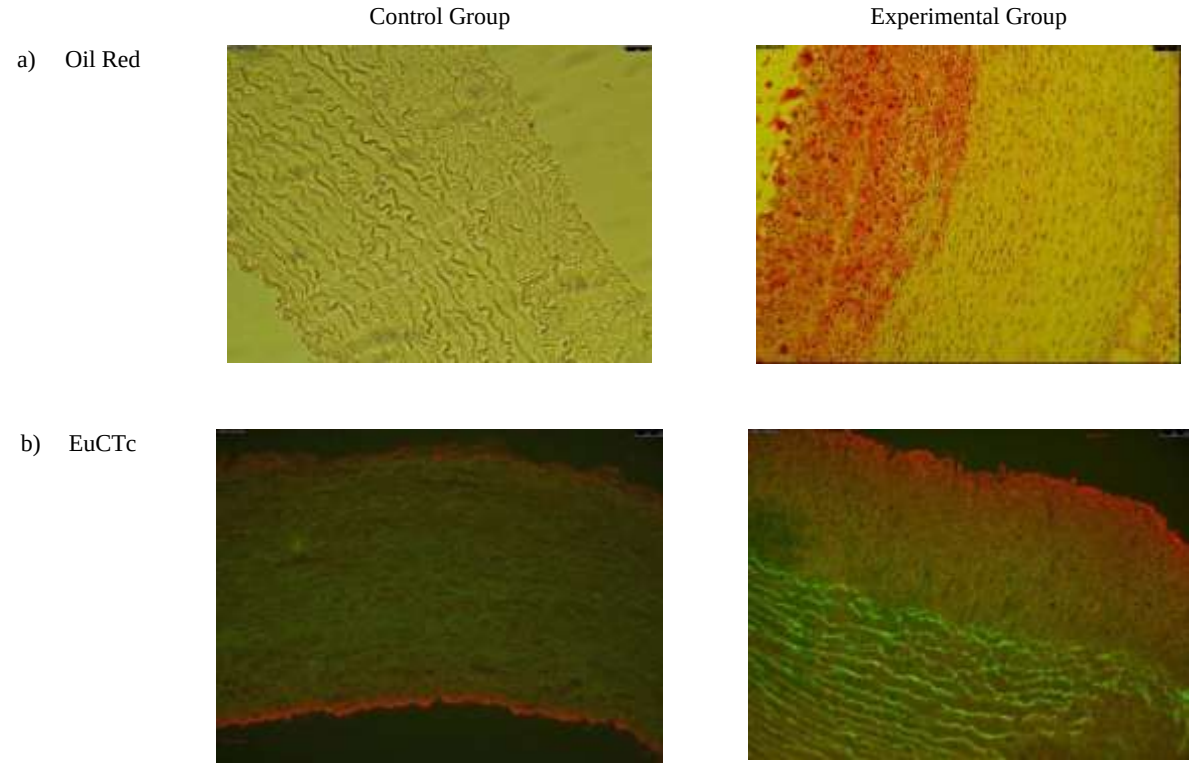

Figure 2. Cross section of thoracic aortas from control and experimental groups stained with a) Oil red and b) Europium Chlortetracycline. Control group and Experimental group with 60 days of diet. Magnification 40x.

In order to obtain more information about the fluorescent species found in EuCTc stained slides, we performed fluorescence lifetime imaging microscopy (FLIM) exciting samples at 440 nm. The results are shown in the figures 3 and 4.

Figure 3 shows the aorta lifetime autofluorescence quantified in terms of the relative fluorescence intensity and the average lifetime at each pixel of the image. The intrinsic fluorescence decays considering three emission lifetimes (probably, lipids, flavins and porphyrins) for each pixel of the image were estimated using a computationally efficient time-deconvolution algorithm [12].

Continuing the analysis of control versus experimental groups, it was obtained the histogram of lifetime's values shown in figure 4. This figure shown an enhancement in the lifetimes measured in the rabbits aortas with the increase in the aortic lesion indicating change in the biochemical composition of the lesion. The FLIM measurements showed that the atherosclerotic aorta (cholesterol group, 60 days) was on average 30 % brighter than the normal aorta (control group, 20 days), suggesting that the atheroma plaque contained more than one fluorophore emitting light with wavelengths higher than 460nm. Atherosclerosis has radically altered metabolic pathways. It is known that the lifetime of a fluorophore is independent of fluorophore concentration, laser power, and other imaging variations. On the other hand the fluorescence lifetime is influenced by a number of factors such as pH, oxygen levels, and refractive index.

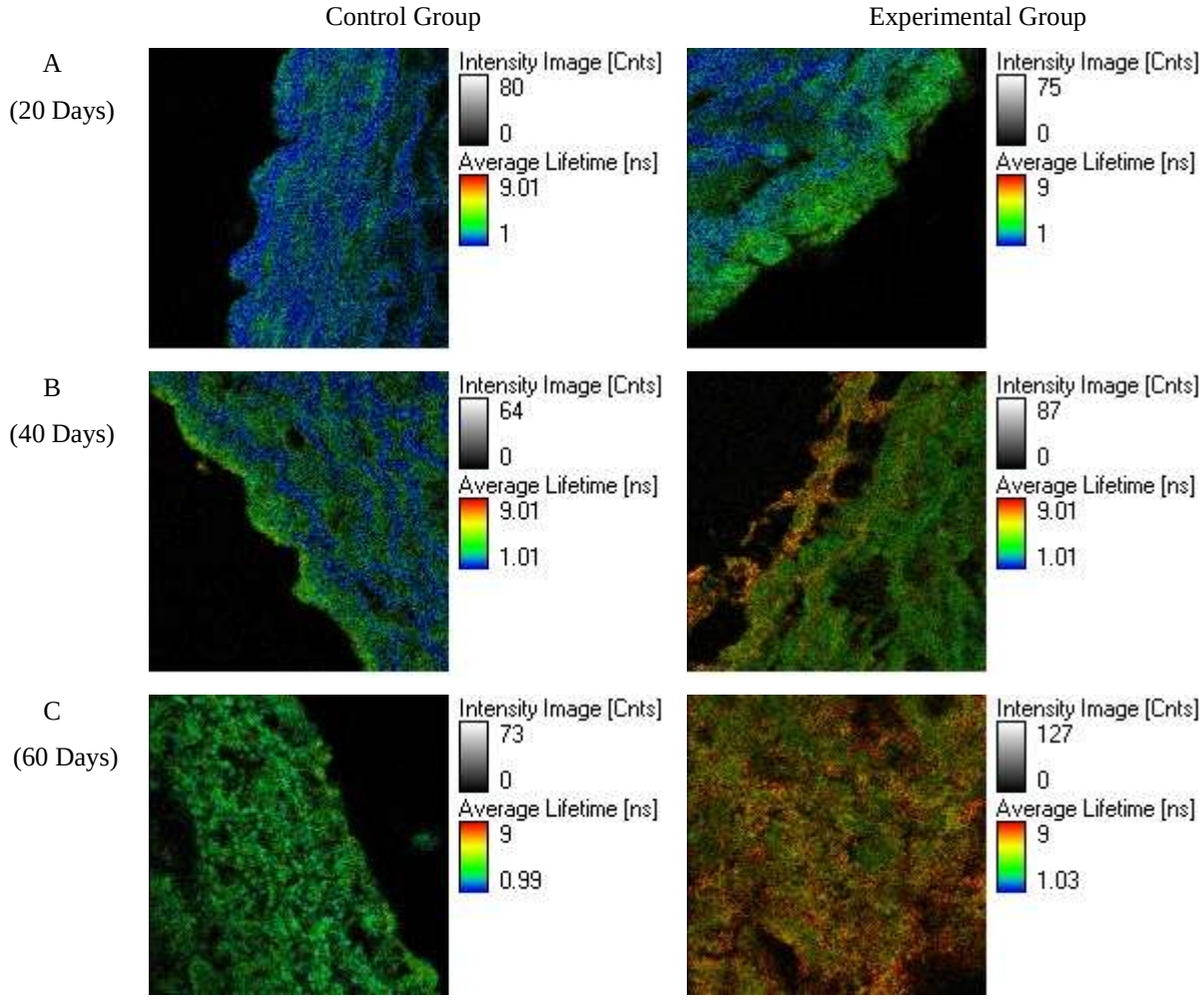

Figure 3. Cross section of thoracic aortas from control and experimental groups analyzed by using fluorescence lifetime imaging microscopy (FLIM). (Ex: 440 nm, Em: >460 nm). Images have 80 μm..A) 20 days, B) 40 days and C) 60 days. Magnification 60x.

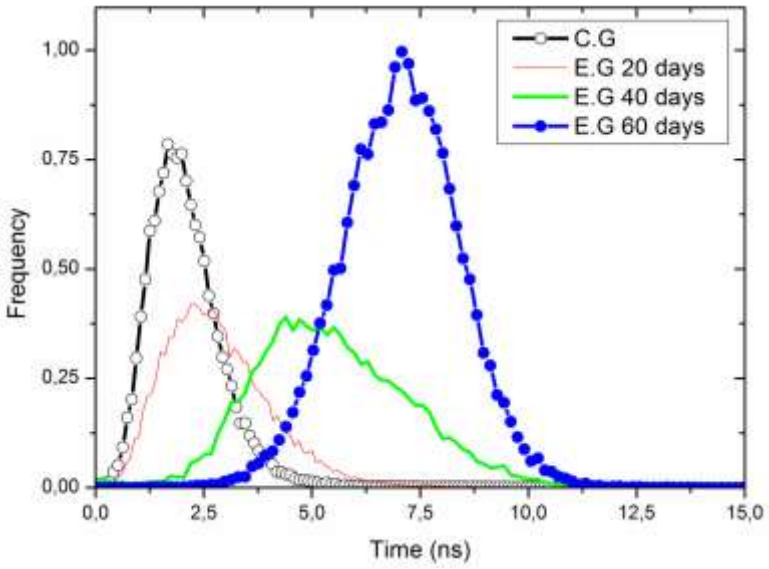

Figure 4. Histogram analysis indicating the range of lifetime values of the different images. The black line was the control group, the red line the EG/20 days, the green line the EG/40 days and the blue line the EG-60 days. These histograms reveal longer lifetime with the increase of plaque formation.

4. DISCUSSION

Different laser light sources can be used to selectively excite the various natural fluorophores present in the tissue. The most important endogenous fluorophores in normal and atherosclerotic arteries are elastin, collagen and lipids. But there are other important fluorophores as flavins and porphyrins as shown in the table 1.

Table 1. Spectral properties of aortic tissue fluorophores.

Fluorophores	Absorption peak (nm)	Emission peak (nm)	Emission lifetime
Collagen	325	380	1.9 ns[4]
Elastin	290, 325	390	5.2 ns[14]
Phospolipids	436	540,560	1.5 ns[21]
Flavins	450	535	2.7 ns[22]
Porphyrins (PpIX)	400-450	635, 705	17 ns[23]
Europium	400	615	20μs[10]
Chlortetracycline	370	590	~10ps[24]

Elastin is reported to have an average fluorescence lifetime of 1.9 ns at 380 nm [14]; collagen I is the predominant collagen molecule in atherosclerosis and is reported to have longer lifetimes 5.2 ns at its emission peak 390 nm [4].The time-resolved fluorescence properties of lipids have short lifetimes (<1.5 ns) across the emission spectrum. The lipid-laden macrophages (foam cells) exhibit short average fluorescence lifetimes according to their lipid content. It is difficult to distinguish lipids from elastin since both have short average lifetimes around 1.8 ns.

Existing methods of interpreting fluorescence lifetime imaging microscopy (FLIM) images are based on comparing the intensity and lifetime values at each pixel with those of known fluorophores and normally results are obtained under ultraviolet laser excitation.

Flavins and pyridine nucleotides are strong electron acceptors and play an important role in cellular energy metabolism. Flavin adenine dinucleotide (FAD) is the major flavin-related electron carrier. Its oxidized form (FAD) is fluorescent, while its reduced form (FADH2) is not. The fluorescence excitation maximum of FAD can be observed around 450 nm and the emission maximum around 530 nm.

Protoporphyrin IX (PPIX) is an important endogenous fluorophore. Its absorption spectrum has five bands: the Soret band (in the region of 400 nm) and four more bands, known as Q bands, which comprise the region between 450 and 750 nm [15]. PPIX emission is observed in its two characteristic bands at approximately 635 nm and 705 nm. Analyzing these PPIX spectroscopic properties it is possible to monitor its concentration in tissues and biological fluids [15, 16]. Oh-Choon Kwon et al. reported that PPIX accumulated in atherosclerotic plaque [17]. Peng et al. detected the accumulation of 5-aminolevulinic acid (ALA)- PPIX derived in inflamed atherosclerotic plaque in rabbit model and evaluate the efficacy of PDT [18]. Murayama et al [19] observed the fluorescence lifetime of the metastatic lesion of lymph nodes was longer than that of the nonmetastatic lesion in mouse rectal cancer by using 5-aminolevulinic acid - PPIX FLIM imaging.

Reyftmann et al.[20] observed a complexation of hydrophobic porphyrins in LDL. The PPIX synthesis is affected by physiological conditions of the atheroma plaque, the cell type and the cell proliferation rate. Local accumulation of cells, or hypercellularity, in the intima of the arterial wall as a result of cell proliferation is recognized as one of the major manifestations of human atherosclerosis. Intima cells require high PPIX production rate due to the high metabolic activity because of the high rate of cell proliferation. Furthermore, intima cells tend to retain more PPIX produced due the presence of LDL and therefore accumulate PPIX.

The serum lipids analysis shown the increase of total cholesterol, LDL cholesterol, but HDL cholesterol remained practically unchanged with the time of diet. Microscopic images obtained using the EuCTc probe show the formation of the plaque with different times of diet, and the intima layer presents an increase in thickness, this result is also in according with previous results of the group[13].

When the diode laser excitation at 440 nm reaches the aortic tissue, some processes can occur: a) Europium chloride can weakly absorb light. (Europium emission lifetime cannot be observed for the used experimental setup since Europium emission is longer than 10 μs.) b) Free chlortetracycline can absorb light. (Emission in this case occurs around 590 nm and lifetime range is expected to be shorter than 1 ns). Chlortetracycline lifetime can be changed in the presence of calcium[12]. c) The excitation at 440 nm promotes autofluorescence of flavins and porphyrins.

The increase in the PpIX concentration in tissues due to the plaque formation promotes an increase in the tissue lifetime. In this case the increased concentration of PPIX in the aortic tissue justify the enhancement in the observed lifetime in function of the time of high cholesterol diet administration.

5. CONCLUSION

The europium chlortetracycline complex was used as LDL fluorescent probe allowing the observation of formation of foam cells in aorta wall. In summary, our data demonstrate that optical techniques are useful adjuvants to detect pathological changes associated with atherosclerosis. Here we present a way to detect the metabolic changes associated with atherosclerosis at an early stage, which has enormous potential as a diagnostic tool using combined guided red fluorescence and FLIM technique exciting the tissue around 440 nm. Lifetimes longer than 1.75 ns suggest the presence of porphyrins in the tissue and consequently, inflammation and the presence of macrophages.

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