New blood markers for staging and prognostics of atherosclerosis

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ABSTRACT

Analysis from the plasmas of rabbits subjected to high-cholesterol diets were performed by fluorescence spectroscopy using three biossensors: Europium-Chlortetracycline (EuCTc), Evans Blue (EB), and Thioflavin T (ThT). For this purpose an animal experimentation was done with New Zealand rabbits divided into two groups: control group of 6 rabbits that received a regular diet for 60 days; and experimental group of 9 rabbits, that were fed with 1% cholesterol for 60 days. The results from spectroscopic analysis have shown that the EuCTc marker emission intensity increases in the presence of plaque formation. The EB emission intensity remained constant for control and experimental groups. The ThT presented an increase in the emission intensity and a modification in the spectra shape with 60 days of diet. The studied biomarkers may not yet be specific in the identification of unstable plaques, but can provide additional information on the patients risk for plaque formation.

Keywords: Europium-Chlortetracycline, Evans Blue, Thioflavin T, fluorescence, biomarker, rabbit, plasma

1. INTRODUCTION

Atherosclerosis is a progressive and inflammatory disease which is characterized by accumulation of lipids and fibrous elements in the artery wall [1]. It's considerate one of the main causes of morbidity and mortality worldwide. To help predictions in cardiovascular events, numerous biomarkers were studied like inflammatory markers, coagulation markers and others [2].

Europium-Chlortetracycline has been used as low density lipoproteins biosensor [3], and in a recent study it was used to marker the formation of the plaque in arteries of rabbits with a hypercholesterolemic diet [4]. This complex has absorption around 400 nm and a strong emission at 617 nm in the presence of cholesterol, is easily synthesized and operates in pH neutral [5,6] .

Tioflavin T (ThT) is composed by three fragments: benzthiazole ring, benzene ring, and dimethylamino group, and it is considered a molecular rotor [7,8]. The dye has absorption around 400 nm and in the presence of fibrils has a shift in the absorption band to 450 nm and an emission around 480 nm[8]. ThT is used to marker amyloid-like structure [7,8]. The restriction on rotation of the fragments of ThT causes an increase in the emission of the dye in the presence of these fibrils [7,8]. Griffin et.al studied the risk of cardiovascular event with ThT [9].

Evans Blue (EB) is a dye nontoxic used commonly to marker extravasation of protein [10], cellular permeability and *in vivo* vascular leakage [11]. EB binds strongly with albumin [12]. This dye has absorption around 600 nm with an emission band in the red region. Uchida et al. [13] used EB to localize the low density lipoprotein oxidize in plaques of atherosclerosis.

In this study, these three biosensors were used and their potentiality to mark atherosclerotic plaque formation were investigated by measuring their fluorescence proprieties in the presence of a small quantity of plasmas of rabbits with a hypercholesterolemic diet.

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2. MATERIALS AND METHODS

2.1 Materials Preparation

The Europium-Chlortetracycline (EuCTc) complex was prepared in 10 mmol/L 3-(N-Morpholino) propanesulfonic acid (Mops, from Carl Roth) buffer with pH 6.9, in the ratio 1.5 Eu: 1.0 CTc, (31.5µmol/L, 21 µmol/L). The Thioflavin T (ThT) and Evans Blue (EB) were prepared in water bi-deionizate with 8 µmol/L and 10^{-5} mol/L, respectively. All the solutes used were prepared starting from inorganic salts with analytical purity, obtained from Sigma Aldrich and Molecular Probe.

For the fluorescence measurements in presence of plasma, 1 mL of each biosensor was mixed with 20 µL of plasma of each animal. All the experimental were made in duplicate.

2.2 Animal experimentation

New Zealand rabbits were divided into two groups: CG (control group) of 6 rabbits received a regular diet for 60 days; EG (experimental group) of 9 rabbits fed with 1% cholesterol diet (Sigma-Aldrich) for 60 days. 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 oil red.

2.3 Biochemical analyses of plasma

After 12 h of fasting, blood was drawn from the marginal ear vein at the baseline time point and at the end of 60 days after begging high cholesterol diet protocol. Blood samples were centrifuged (1200 rpm, 15 min, 4 °C) to obtain plasma and serum. The total cholesterol (TC), TG, HDL-C and low-density cholesterol (LDL-C) levels in the serum samples were assessed using an enzymatic colorimetric assay with an automatic biochemical analyzer.

2.4 Instrumentation

The analyses of fluorescence were obtained with a fluorimeter of Jovin Yvon and the excitation font was a xenon lamp. The excitation and emission regions analyzed for EuCTc, ThT and EB were 400 nm and 617 nm, 413 nm and 481 nm, and 606 nm and 665 nm, respectively. All experiments were perfomed in a cuvette with 1 mm optical-path.

2.5 Statistical Analysis

The data were expressed as mean ± standard error of the mean (S.E.M.). All of the experiments were performed independently and repeated a minimum of two times.

3. RESULTS

3.1 Biochemical Results

The rabbits lipid profile is shown in the Table 1. The results indicate an increase of total cholesterol (TC) and low density lipoprotein (LDL-C) and Glucose for the animals from the experimental group when compared with the control group.

Table 1- Serum lipids and glucose. Normal control group: rabbits were fed a normal diet; experimental group: rabbits were fed a high-cholesterol 60 days (EG3). Data are presented as the mean ± SEM. TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol and glucose.

Group	Control	EG-60 days
TC (mg/dL)	52.61 ± 5.11	1425.40 ± 163.13
TG (mg/dL)	48.10 ± 2.49	52.88 ± 10.28
HDL-C (mg/dL)	28.42 ± 2.31	10.29 ± 2.78
LDL-C (mg/dL)	13.36 ± 2.22	343.84 ± 53.85
Glucose (mg/dL)	148.23 ± 10.62	171.50 ± 10.00

3.2 Microscopic analysis

In Figure 1 it was shown the images of aortic arch stained with Oil Red for control and experimental groups. It can be observed the formation of the plaque in the experimental group with 60 days of diet.

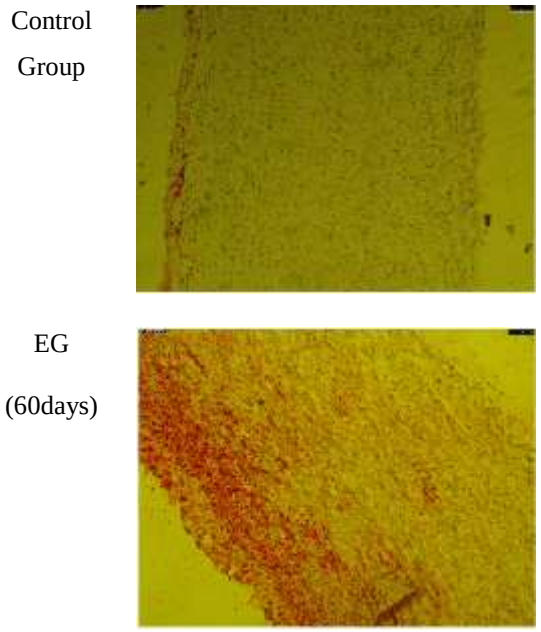

Figure 1- Cross section of thoracic aortas from control group and experimental group - 60 days stained with oil red.

3.3 Europium Chlortetracycline

It was obtained the absorption spectra of chlortetracycline, europium-chlortetracycline and europium-chlortetracycline in the presence of rabbit`s plasma with 60 days of diet (Figure 2). The chlortetracycline presents a principal absorption band at 374 nm, but when complexed with Europium the absorption band presents a shift to 400 nm. In the presence of plasma, it could be observed light scattering but it can't be observed a shift in the absorption band.

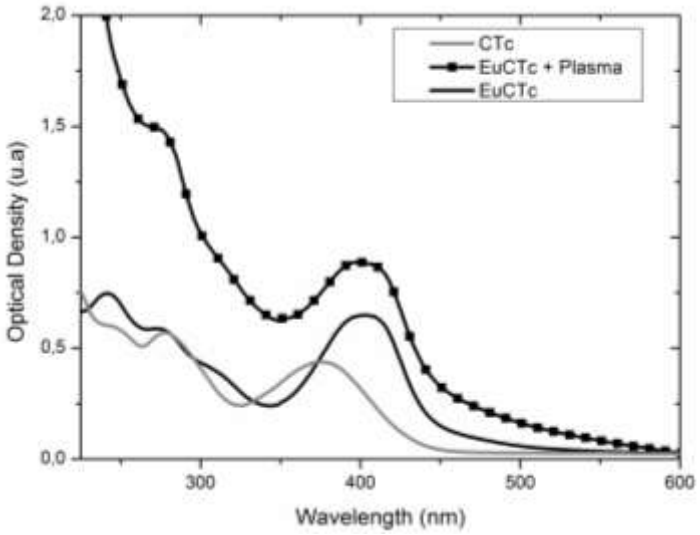

Figure 2 - Absorption Spectra of EuCTc in the presence and absence of plasma.

For the analysis of EuCTc fluorescence in the presence of plasma, samples for control and experimental groups were excited at 400 nm. In Figure 3 it can be seen that the average of emissions of EG group are more intense than the CG. The emission spectra of EuCTc is in accordance with the images of aortic arch stained with oil red.

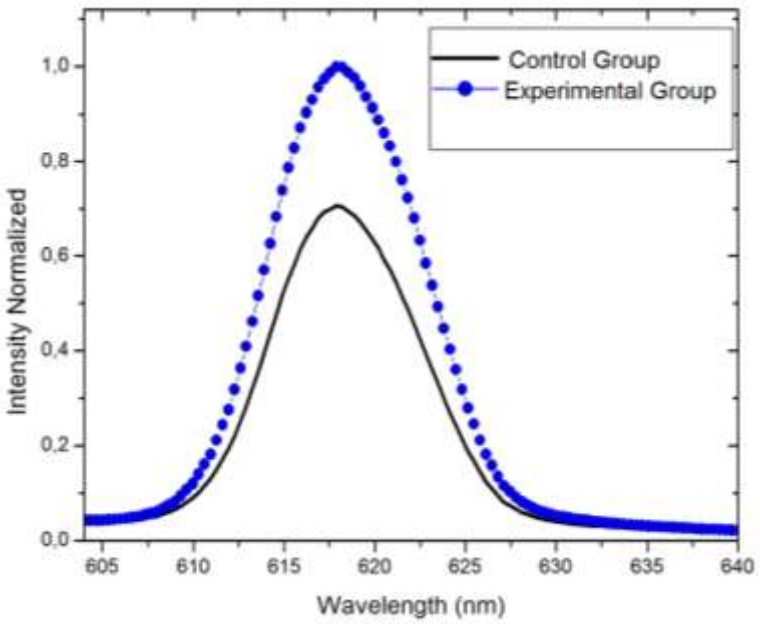

Figure 3- Emission spectra of EuCTc in presence of plasma for animal of control and experimental groups.

3.4 Evans Blue

The dye EB presents three absorption bands around 317 nm, 400 nm and 606 nm with and without animals' plasmas (Figure 4).

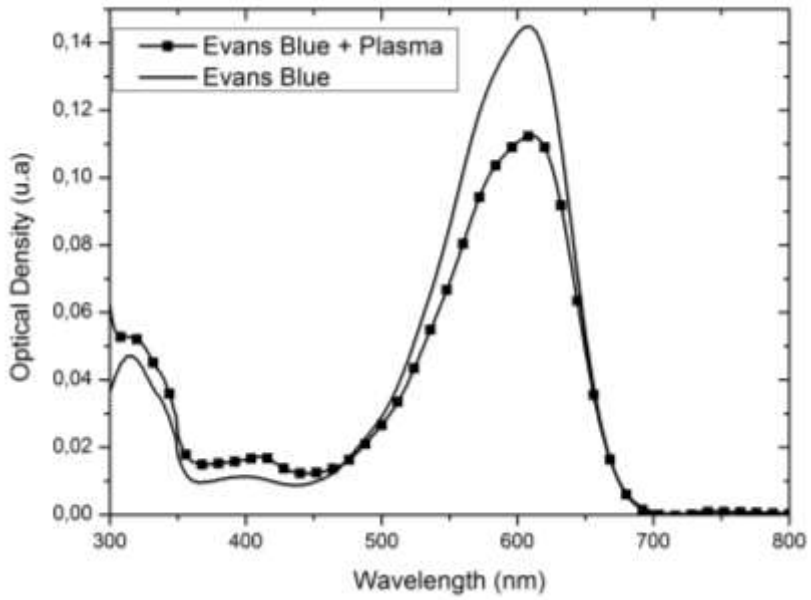

Figure 4 - Absorption spectra of Evans Blue dye.

It was obtained the emission spectra of EB in the presence of plasma. For this purpose, the samples were excited with 606 nm and the emission bands were performed between 625 – 750 nm. In the Figure 5, it can be observed that the average of EB emissions remain practically constant in the presence of plasma of CG and EG.

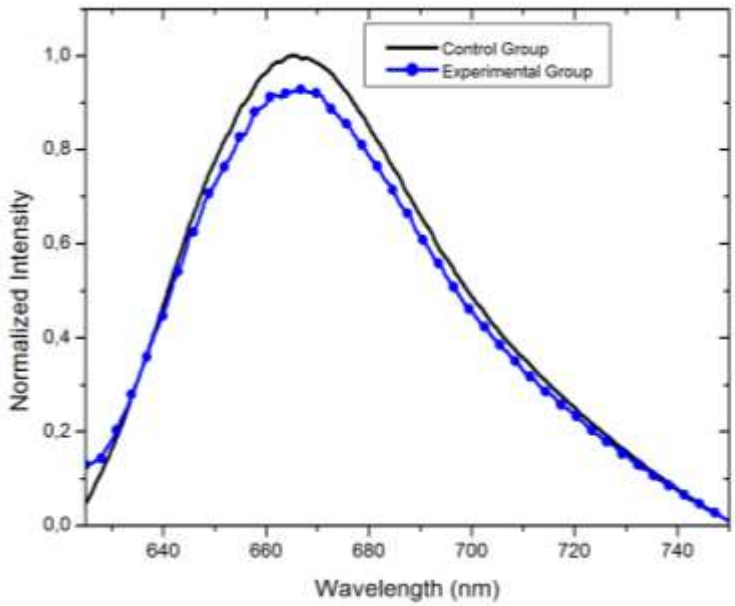

Figure 5 - Emission spectra of EB in the presence of plasma for animal of control and experimental groups.

3.5 Tioflavin T

It was obtained the absorption spectra of ThT and ThT in the presence of plasma Figure 6. The ThT presents an absorption band in 413 nm and in the presence of plasma it can't be observed a shift in this band.

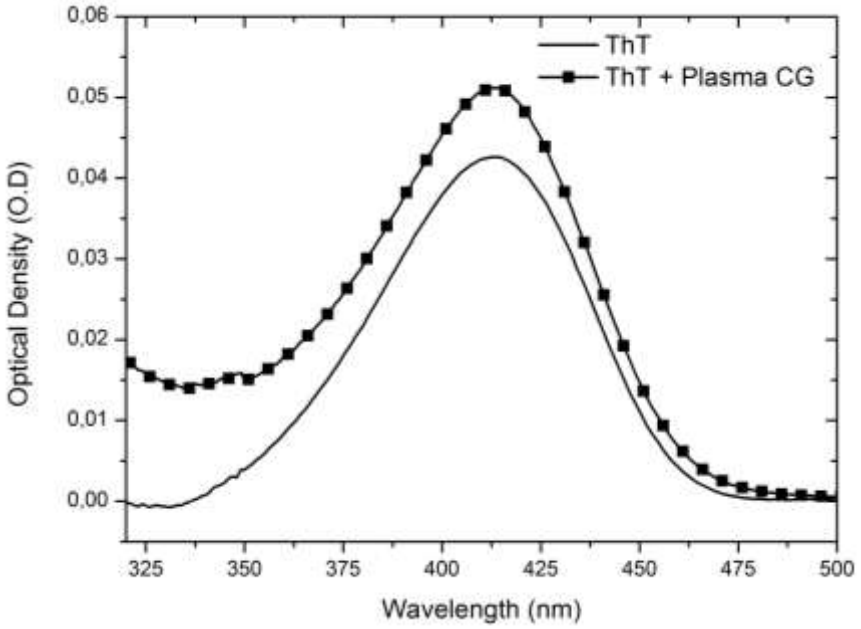

Figure 6 - Absorption spectra of ThT and ThT in the presence of plasma.

In Figure 7, it was shown the average of ThT emission spectra in the presence of plasmas of control and experimental groups. For that, the samples were excited at 413 nm and the emission bands were measured between 430 – 600 nm. It can be seen an increase in the ThT emission intensity in the presence of plasma rich in cholesterol when compared to control group, and also it is observed different spectrum shape indication the presence of a new emission band for the emissions of experimental group. The increase in the ThT emission intensity is in accordance with the images of microscopy stained with Oil Red.

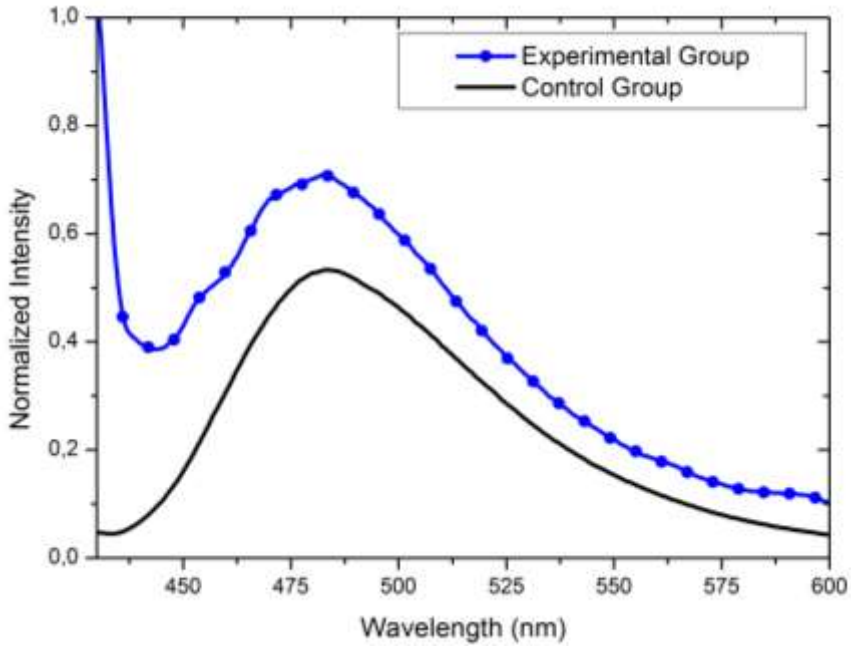

Figure 7 - Emission spectra of ThT in the presence of plasma for animal of control and experimental groups.

The Figure 8 shows the ration of emission intensities of experimental and control groups for EuCTc, EB and ThT. For EB the EG/CG emission ration remains constant (almost 1.0) but for both EuCTc and ThT it was an increase of ~40% of emission intensities of experimental groups in comparison with control groups.

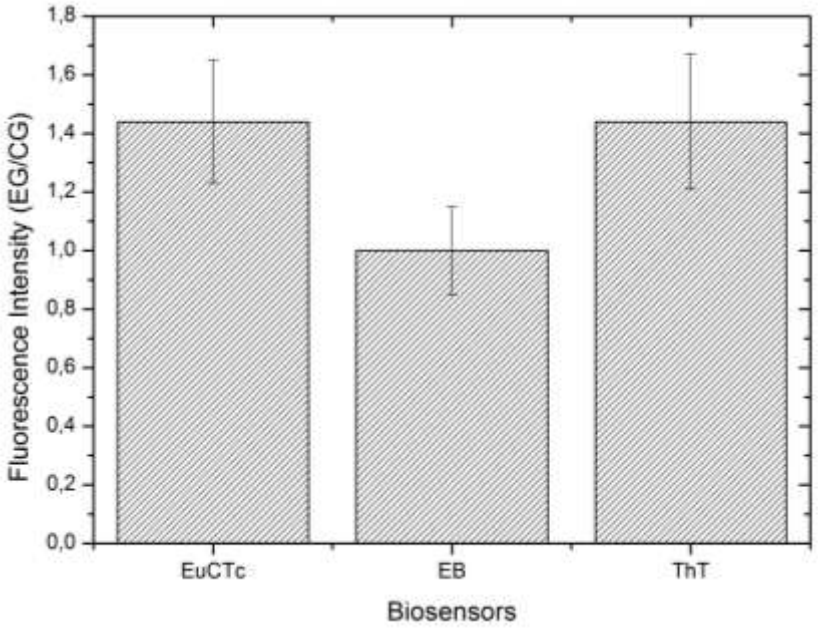

Figure 8 – Rate of emissions intensities of experimental and control groups for EuCTc, EB and ThT biosensors.

4. DISCUSSION

The biochemical analysis indicated that the protocol to induce an increase in the lipid profile of the animals was appropriated, and there was an increase in the CT, and LDL-C for experimental group when compared to the control group. The microscopy images of aortic slices stained with Oil Red showed that the animals with 60 days of diet presented atherosclerotic plaque.

For Europium-Chlortetracycline it can be seen an increase in the europium emission spectra for experimental group in comparison to control group. The increase in the lipoproteins particles present in the plasma can displace water molecules in the Europium neighborhood inducing an increase in the europium emission intensity [3]. So, the complex EuCTc can be used to predict the formation of plaque in arteries.

Evans Blue is a dye with high affinity to albumin [12], and in some works had to be used marker the localization of ox-LDL in atherosclerosis plaque [13, 14]. In this study the emission of EB in the presence of plasma remains practically constant. The results indicated that the affinity of EB with albumin is greater than the affinity of EB with the lipoproteins which masks changes in the emission intensity due to the presence of atherosclerosis.

ThT has been used to predict cardiovascular disease [9]. In the literature is described a shift in the absorption band of ThT in the presence of amyloid structure [8], but in this study this shift did not occur. The measured emission spectra a presented a different shape for ThT in the presence of the plasma of EG probably due to oxidation of low density lipoproteins, and this can be considerate a form to monitoring the accumulation of lipids.

More experiment will be necessary and performed to understand better this association of the biosensors and plasma with high concentration of cholesterol.

5. CONCLUSION

This study showed the spectroscopy analysis of three biosensors in the presence of plasma of rabbits submitted to a rich cholesterol diet and with atherosclerosis formation. It could be seen that EuCTc and ThT can be used to predict plaque formation. The EuCTc is very easy to prepare, presents good stability and a large Stokes shift that facilitate the

fluorescence analysis. The ThT shows a modification of the emission spectra shape for animals with increased LDL values indicating specificity.

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REFERENCES

[1] Lusis, A.J., "Atherosclerosis," Nature 407, 233–241 (2000).
[2] Brown, T.M., and Bittner, V., "Biomarkers of atherosclerosis: clinical applications.," Current cardiology reports 10(6), 497–504 (2008).
[3] Teixeira, L.D.S., Grasso, A.N., Monteiro, A.M., Neto, a M.F., Vieira, N.D., Gidlund, M., and Courrol, L.C., "Enhancement on the Europium emission band of Europium chlortetracycline complex in the presence of LDL.," Analytical biochemistry 400(1), 19–24 (2010).
[4] Courrol, L.C., Sicchieri, L.B., and Cristine, D., "Cholesterol accumulation in the cornea and in the aorta : imaging using europium chlortetracycline complex fluorescent probe," in Proc. SPIE 8591 8591, 85910C (2013).
[5] Courrol, L.C., and Samad, R.E., "Applications of Europium Tetracycline Complex: A Review," Current Pharmaceutical Analysis 4(4), 238–248 (2008).
[6] Silva, F.R.D.O., Samad, R.E., Gomes, L., and Courrol, L.C., "Enhancement of europium emission band of europium tetracycline complex in the presence of cholesterol.," Journal of fluorescence 18(1), 169–74 (2008).
[7] Stsiapura, V.I., Maskevich, A. a, Kuzmitsky, V. a, Uversky, V.N., Kuznetsova, I.M., and Turoverov, K.K., "Thioflavin T as a molecular rotor: fluorescent properties of thioflavin T in solvents with different viscosity.," The journal of physical chemistry. B 112(49), 15893–902 (2008).
[8] Sulatskaya, A.I., Kuznetsova, I.M., and Turoverov, K.K., "Interaction of thioflavin T with amyloid fibrils: stoichiometry and affinity of dye binding, absorption spectra of bound dye.," The journal of physical chemistry. B 115(39), 11519–24 (2011).
[9] Griffin, M.D.W., Wilson, L.M., Mok, Y.-F., Januszewski, A.S., Wilson, A.M., Karschimkus, C.S., Romas, E., Lee, A.B., Godfrey, T., et al., "Thioflavin T fluorescence in human serum: correlations with vascular health and cardiovascular risk factors.," Clinical biochemistry 43(3), 278–86 (2010).
[10] Saria, A., and Lundberg, J.M., "Evans blue fluorescence: quantitative and morphological evaluation of vascular permeability in animal tissues.," Journal of neuroscience methods 8(1), 41–9 (1983).
[11] Moitra, J., Sammani, S., and Garcia, J.G.N., "Re-evaluation of Evans Blue dye as a marker of albumin clearance in murine models of acute lung injury.," Translational research : the journal of laboratory and clinical medicine 150(4), 253–65 (2007).
[12] Yao, J., Maslov, K., Hu, S., and Wang, L. V, "Evans blue dye-enhanced capillary-resolution photoacoustic microscopy in vivo.," Journal of biomedical optics 14(5), 054049 (2009).
[13] Uchida, Y., Maezawa, Y., Uchida, Y., Hiruta, N., Shimoyama, E., and Kawai, S., "Localization of Oxidized Low-Density Lipoprotein and Its Relation to Plaque Morphology in Human Coronary Artery," PloS one 8(2), e55188 (2013).
[14] Uchida, Y., Uchida, Y., Kawai, S., Kanamaru, R., Sugiyama, Y., Tomaru, T., Maezawa, Y., and Kameda, N., "Detection of vulnerable coronary plaques by color fluorescent angioscopy.," JACC. Cardiovascular imaging 3(4), 398–408 (2010).