Analyst

PAPER

View Article Online
View Journal | View Issue

Cite this: *Analyst*, 2014, **139**, 1383

Liquid biopsy of atherosclerosis using protoporphyrin IX as a biomarker

Monica Nascimento da Silva,[a] Letícia Bonfante Sicchieri,[b] Flávia Rodrigues de Oliveira Silva,[c] Maira Franco Andrade[b] and Lilia Coronato Courrol*[ab]

Protoporphyrin IX (PPIX), a derivative of hematoporphyrin, can accumulate in rapidly growing tissues, including tumors and atherosclerotic plaques. The objective of this study is to employ PPIX fluorescence to detect the changes in blood caused by the formation of atheromatous plaques in arteries; this measurement can function as a liquid biopsy. For this purpose twenty four rabbits were randomly divided into groups: control group (CG) – fed with a normal diet, and an experimental group (EG) – fed with a hypercholesterolemic diet (1% cholesterol). Blood samples were collected before (0 time) and after 22, 43, 64 days to measure biochemical factors. The aortas were removed after 22, 43 and 64 days to assess the atherosclerotic plaques. PPIX was extracted from the blood and fluorescence was measured in the 550–750 nm range from samples that were excited at 405 nm. Aminolevulinic acid (ALA) was administered intravenously to increase the PPIX fluorescence intensity in the arteries and consequently in the liquid biopsy of the atherosclerotic plaques. The results have shown that the PPIX fluorescence increased as the atheromatous plaques grew. The aorta fluorescence and the PPIX fluorescence increased in the animals in the experimental group that received ALA. PPIX that accumulates in atheromatous plaques transfers to the blood and can be analyzed by extracting porphyrin from total blood. Therefore, this method can aid in the early diagnosis of atherosclerosis with high sensitivity.

Received 16th October 2013
Accepted 4th December 2013

DOI: 10.1039/c3an01945d

www.rsc.org/analyst

A. Introduction

Most cases of chest pain and almost all heart attacks are caused by atherosclerosis, a narrowing and hardening of the arteries that results from the accumulation of fatty deposits known as atheromas or plaques.[1] The plaques typically comprise cholesterol-containing low-density lipoproteins (LDLs), smooth muscle cells, fibrous tissue, and occasionally calcium.[2] Plaques are not deposited homogeneously in the arteries. Coronary artery analysis revealed the presence of changes in the entire artery, but the obstructions that can limit blood flow tend to be localized. Thus, atherosclerosis does not have a specifically defined pattern. The variability of lesions complicates its diagnosis and treatment.[1]

The use of light to diagnose and treat atherosclerosis has been widely described in the literature. Edholm and coworkers suggested that quantitative optical techniques could be used to improve the detection of atherosclerosis *in vivo*.[3] The researchers measured the reflectance of the aorta between 500 and 550 nm in 15 patients. Kitrell and colleagues[4] demonstrated that fluorescence spectroscopy can be used to discriminate between a normal aorta and a fibrous plaque and suggested that for atherosclerosis, optical diagnosis (low-power illumination) and treatment (high-power illumination) could be combined in a single fiber optic device. Subsequently, several groups, many with the goal of developing a guidance system for catheters during angioscopic laser surgery,[5] investigated the usefulness of fluorescence spectroscopy for diagnosing atherosclerotic plaques.[6–8] The emission spectra of normal arterial tissue in atherosclerotic plaques were measured at various UV/VIS excitation wavelengths.[2,9–11] Agents, such as δ-aminolevulinic acid (ALA), that contribute to natural autofluorescence were used to enhance the contrast between health and diseased tissues.[12–14] The techniques developed for tumor studies[1,7] could be easily adapted to precisely demarcate atherosclerotic plaques and normal arterial walls.

It has been suggested that rapidly proliferating tissues in general may preferentially accumulate porphyrins.[15] Protoporphyrin IX (PPIX) fluorescence and changes in its amount in the

[a]*Universidade Federal de São Paulo, Laboratório de Lasers e Óptica Biomédica Aplicada (LOBA), Instituto de Ciências Ambientais Químicas e Farmacêuticas (ICAQF), Departamento de Ciências Exatas e da Terra (DCET), Rua Artur Riedel, 275 Eldorado, 09972-270, Diadema, SP, Brazil. E-mail: lccourrol@gmail.com; Fax: +55 1140436428*

[b]*Instituto de Pesquisas Energéticas e Nucleares, Centro de Lasers e Aplicações, São Paulo, SP, Brazil. E-mail: leticiabonfante@gmail.com; Tel: +55 1131339387*

[c]*Instituto de Pesquisas Energéticas e Nucleares, Centro de Ciência e Tecnologia dos Materiais, São Paulo, SP, Brazil. E-mail: flavia.rodrigues@ipen.br; Tel: +551131339228*

blood have been already considered as invasive, additional diagnostic parameters in oncological practice.[16] Spears *et al.* were the first to describe the accumulation of porphyrins in atheromatous lesions.[17] The researchers investigated animal models of atheroma, using both cholesterol-fed New Zealand white rabbits and a Patas monkey.

Peng *et al.*[18] recently showed that PPIX was detected in atheromatous plaques after an intravenous administration of δ-aminolevulinic acid (ALA) in rabbits subjected to a hypercholesterolemic diet. The PPIX fluorescence intensity in the plaque peaked 2 h after the ALA injection and was 12-fold higher than that of the adjacent normal vessel segment; the fluorescence was positively correlated with the macrophage content. Subsequently, photodynamic therapy has been performed (at 50 J cm^{-2}, 635 nm) on atherosclerotic plaques. The study demonstrated that PPIX can be measured to determine the macrophage content in plaques.

Recently, PPIX accumulation was identified in macrophages *in vitro*. This finding indicated that optimized PPIX-mediated sonodynamic therapy (SDT) exerts cytotoxic effects on macrophages through selectively induced apoptosis; this apoptosis occurs *via* intracellular singlet oxygen generation and disruption of the cytoskeleton with minimal necrosis.[12] PPIX-mediated SDT may become a treatment that can attenuate the progression of atherosclerotic plaques.

The objective of this study is to verify the correlation between the emission intensity of PPIX extracted from blood and the formation of atheromas in animals subjected to a high-cholesterol diet. The administration of ALA was also investigated to verify whether PPIX fluorescence can be used in clinical practice as a metric in the risk stratification for cardiovascular disease.

B. Materials and methods

Animal experimentation

A total of 24 adult white male rabbits (New Zealand species *Orytolagus cuniculus*, approximately 2.3 ± 0.1 kg, and ~3.5 months of age) were divided into the following two experiments shown in Tables 1 and 2.

The animals were individually housed in a controlled environment that was maintained at 19 °C; food and water were provided *ad libitum*. The Ethics Committee of UNIFESP approved the protocol of this study (protocol no. 0374/12).

All of the rabbits completed the experimental process. The animals were anesthetized by an injection of ketamine and xylazine and then euthanized by a heart injection of 1 mL of potassium chloride, according to the American Veterinary Medical Association guidelines for euthanasia.

Table 1 First experiment

First experiment (histological analysis)	Control group (CG) (commercial diet washed with chloroform)	Experimental group (EG) (diet containing 1% cholesterol (Sigma Aldrich) diluted in chloroform)
22 days	1	3
43 days	1	3
64 days	1	3

Artery excision and histological analysis (first experiment)

The arteries were excised and washed with PBS. Cryosections of the aortic arch specimens were cut in the vertical plane to a 10 μm thickness on a cryostat and then mounted on glass slides and stained with Oil Red O (O0625 SIGMA-ALDRICH). Lipids are soluble in the solvents used in standard histological tissue processing, therefore cryostat sections on fresh or fixed tissue are used. Oil-soluble dyes stain lipids by being more soluble in the lipids than in their solvents. The images were obtained using a Leica DMI6000 CS fluorescence microscope, Leica DFC360FX digital video camera and Leica AF6000 software (Laboratory of Applied Biomedical Optics at UNIFESP).

Biochemical analyses of serum (first experiment)

After 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 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 (Labtest Diagnostic S.A) with an automatic biochemical analyzer.

Porphyrin extraction (second experiment)

Total blood was collected for each animal after 0, 22, 43, 64, 82 and 89 days. Three parts by volume of analytical grade acetone were added to one part of the total blood collected and mixed well. The mixture was centrifuged at 4000 rpm for 15 min. The clear supernatant of the mixture was stored in a clean tube, and spectrofluorimetric analyses were performed on the same day.

Administration of ALA solution (second experiment, 89 days)

Solutions of ALA (Sigma) were freshly prepared at a concentration of 100 mg mL^{-1} in PBS (phosphate-buffered saline). The pH of the solutions was adjusted to 6.83 using NaOH (1 M). The dose per animal was 30 mg kg^{-1}, administered intravenously. The blood was collected before and 2 hours after the intravenous ALA administration.

Fluorescence spectral analyses (second experiment)

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 550–750 nm using a Horiba Jobin Yvon Fluorolog 3 Fluorimeter (Laboratory of Applied Biomedical Optics at UNIFESP).

Statistical analysis

The data are expressed as the mean ± standard error of the mean (S.E.M.). The statistical comparisons among the groups were performed using a one-way analysis of variance (ANOVA). Probability values of $p < 0.05$ were considered statistically

Table 2 Second experiment

Second experiment (PPIX analysis)	Control group (CG) (commercial diet washed with chloroform)	Experimental group (EG) (diet containing 1% cholesterol (Sigma Aldrich) diluted in chloroform)
89 days	4 (2 animals received commercial diet without chloroform) (blood collection after 0, 22, 43, 64, 82 and 89 days). 89 days ALA 2 animals PBS 1 animal	6 (blood collection after 0, 22, 43, 64, 82 and 89 days). 89 days ALA 2 animals PBS 1 animal

significant. All of the experiments were performed independently and repeated for a minimum of three times. GraphPad Prism 5 package was used.

C. Results and discussion

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 3.5 to 4.0 kg. Table 3 shows a comparison of serum lipids and glucose in the control and experimental groups (first experiment).

Fig. 1 shows images of aortas stained with oil red from the control and experimental groups. The images in A, B, and C were obtained after 22, 43, and 64 days, respectively. This figure shows that the aortas of the rabbits fed with a normal diet (control group) have normal thickness and that no lipid is present in the intima layer. For the rabbits fed with the high-cholesterol diet (experimental group), the intima layers are mainly thickened in the animals that were fed for 43 and 64 days.

The total blood PPIX accumulation response was measured in the rabbits fed with 1% cholesterol (second experiment). For this purpose, total blood porphyrin was extracted from the animals in which atheromatous plaques were observed. The results are shown in Fig. 2. In this figure, the signal emission area, which was obtained by integrating the endogenous PPIX emission spectra from 550–750 nm, was plotted as a function of days of plaque growth (22, 43, 64, 82 and 89 days after the diet began). Each point corresponds to an average of the signal from each studied group. For the control group, six animals were used, and two of which received food prepared without chloroform to exclude PPIX enhancement from possible inflammation, infection or toxic reaction (no evidence of toxicity was observed in the animals that were orally exposed to chloroform); the results from this group are indicated as CG. For the experimental groups (EG), six animals were used, and their blood was collected from 22 to 89 days after the hypercholesterolemic diet began. The mean intensity clearly increases as the arterial plaques grow, indicating that porphyrin accumulates in the blood.

Although the averaged sample spectra show different values for each group, which differed by ~20 days, some of the

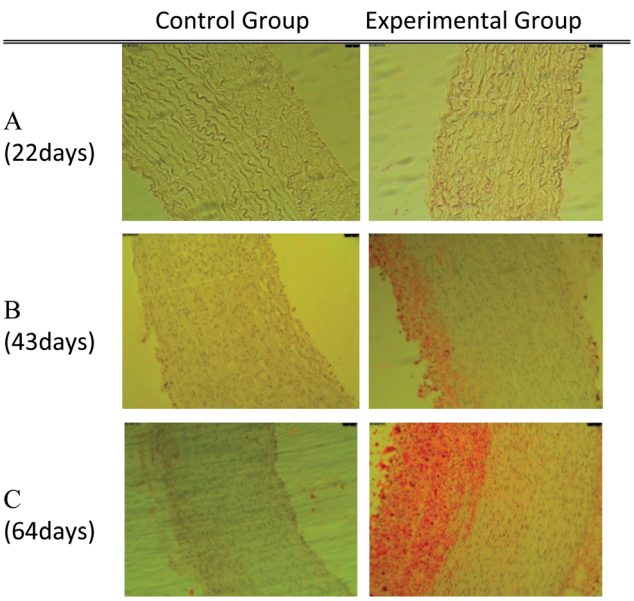

Fig. 1 Cross-section of thoracic aortas stained with oil red from control (left) and experimental (right) groups. (A) 22 days, (B) 43 days and (C) 64 days. The black box in the upper right corner of each image represents the scale of 25 μm ((A) left and (C) right) and 50 μm (magnification 40×).

Table 3 Serum lipids and glucose. Normal control group: 3 rabbits were fed a normal diet; experimental group: 9 rabbits were fed a high-cholesterol diet for 22 days (EG1), 43 days (EG2) and 64 days (EG3). The data are presented as the mean ± SEM; $n = 3$ for the control group, and $n = 3$ for each experimental groups. TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol and glucose

Group	Control	EG-22 days	EG-43 days	EG-64 days
TC (mg dL^{-1})	62.25 ± 4.26	1100.07 ± 87.43	1275.10 ± 109.00	1344.55 ± 200.13
TG (mg dL^{-1})	64.43 ± 3.56	545.61 ± 88.08	591.10 ± 34.96	445.36 ± 30.53
HDL-C (mg dL^{-1})	28.82 ± 1.99	28.17 ± 0.86	32.90 ± 4.52	34.41 ± 10.60
LDL-C (mg dL^{-1})	14.56 ± 1.44	405.43 ± 30.15	513.90 ± 50.30	558.30 ± 81.26
Glucose (mg dL^{-1})	105.12 ± 8.92	267.21 ± 29.54	188.67 ± 33.68	167.87 ± 24.04

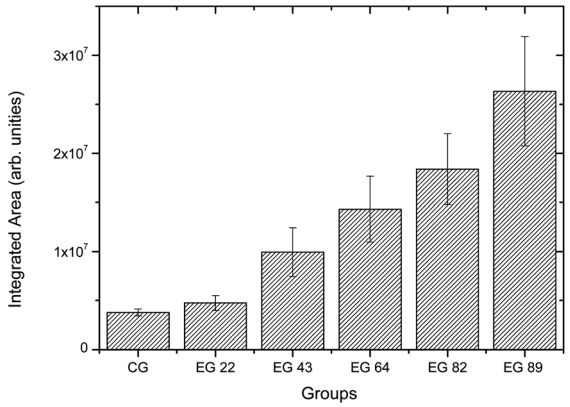

Fig. 2 The area of the emission spectra (550–750 nm) of PPIX extracted from the animal blood, plotted as a function of the days since the hypercholesterolemic diet began. CG indicates the control group, and EG 22, 43, 64, 82 and 89 indicate the experimental groups and the corresponding number of days.

standard error (SE) bars overlap in the analyzed intervals. The mean ± SE clearly differ between the control and experimental groups after ~40 days. To obtain better results it will be necessary to increase the number of animals in the experiment. Also the search for another solvent less volatile than acetone could improve the results.

To increase the PPIX fluorescence, an ALA solution was administered to two animals in the control group and two animals in the 89-day experimental group. One animal from each group received a PBS solution.

The autofluorescence spectra that were recorded from the aortas of control and experimental groups at 405 nm excitation, shown in Fig. 3, present a main emission band in the 430–600 nm range, which can be ascribed to flavins, lipopigments, NAD(P)H, fatty acids and vitamin A.[19] The emission intensity was enhanced in the animals that received ALA relative to that in the animal that received PBS solution. At longer wavelengths, three emission bands peaking at approximately 635 nm, 675 nm, and 710 nm were detected; the amplitude of these bands varied for different tissues. The PPIX emission band (Fig. 4) increased in the animals in the experimental group that received ALA.

Fig. 5 shows the evolution of the fluorescence intensity of the PPIX extracted from the blood before and after the ALA administration. The PPIX fluorescence increased in the animals that received ALA in both the control and experimental groups. The fluorescence intensity increased almost three-fold in the experimental group. Although experiments have been done only for 2 animals, the results obtained showed a close intensity for each animal of the same group and comparison of the results between PBS and ALA group showed a large difference (higher than the estimated experimental error, 5%).

Atherosclerosis is an inflammatory disorder initiated by the accumulation and subsequent oxidation of LDL in the arterial

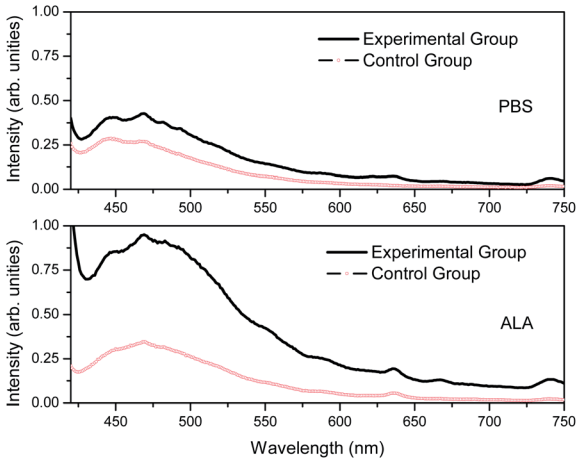

Fig. 3 Typical autofluorescence spectra recorded in the 420–750 nm region for the following groups: aorta without plaques (control group) and aorta containing atherosclerotic plaques (experimental group) from animals that received an intravenous PBS solution (upper figure) and animals that received an intravenous ALA solution (lower figure). The spectra were obtained with excitation at 405 nm; samples from one animal in the control group and an average from two animals in the experimental group that received ALA 2 hours before euthanasia were used for the spectral recording.

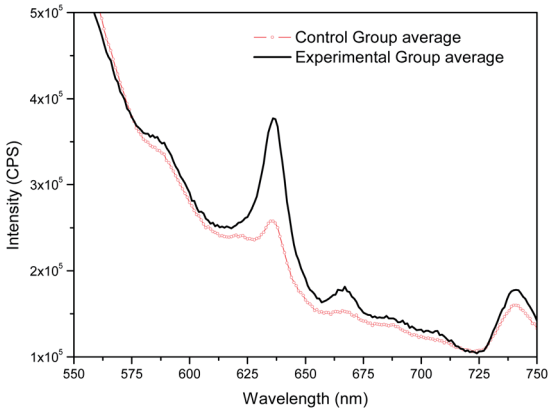

Fig. 4 Autofluorescence spectra recorded in the 550–750 nm region. Two animals in the control group and two animals in the experimental group received ALA two hours before euthanasia; the samples from these animals were used for the spectra, which were averaged.

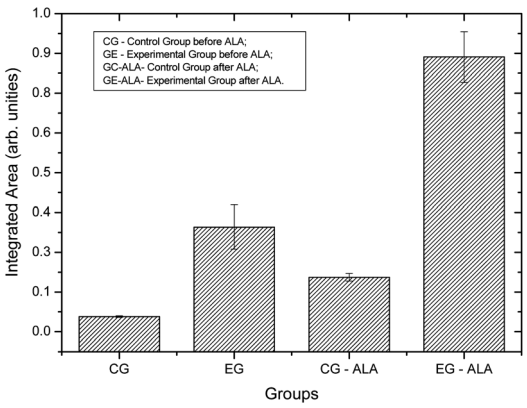

Fig. 5 Differences observed in the PPIX fluorescence in blood from the control and experimental groups before and after ALA administration.

intima.[16] Previous studies indicate that several types of porphyrins accumulate in significant amounts in rapidly growing tissues, including atheromatous plaques.[17]

The porphyrin accumulation in atheromatous plaques is due to the high levels of low-density lipoprotein (LDL) that are being deposited in the extracellular matrix, which is associated with cholesterol accumulation. LDLs are recognized by a specific receptor known as the apo B/E receptor; this recognition leads to the rapid internalization and delivery of the LDL particles to the lysosomal compartment. The PPIX accumulation leads to hepatic disease that is associated with changes in hepatic lipid metabolism, a feature of hyperlipidemia, and the development of atherosclerosis, creating a cycle.[20]

Atherosclerosis naturally occurs only in humans, a few non-human primates, and pigs. Rabbits, being herbivores, do not develop atherosclerosis naturally, but to develop an atherosclerotic condition that very closely resembles clinical observations by feeding intact rabbits a high-cholesterol diet. The rabbit models closely approximate a variety of aspects of human atherosclerosis and are commonly used to study atherogenesis, plaque instability and rupture, and myocardial infarction.[21] PPIX can be analyzed by porphyrin extraction from total blood.[22] The results (Fig. 2) show an increase in the PPIX fluorescence that accompanies the growth of atheromatous plaques. To the best of our knowledge, this is the first report of PPIX accumulation in the blood of animals with atherosclerosis.

In plasma, free hemin or PPIX is bound to apoB-lipoproteins, therefore the increase in extractable fluorescence observed in the hypercholesterolemic rabbits, can be caused by the large increase in apoB lipoproteins carrying hemin or protoporphyrins in the animals fed with cholesterol. In blood, any hemolysis may increase the levels of hemin and PPIX bound to lipoproteins causing a remarkable increase in the fluorescence around 635 nm.[23] In addition it should be noted that hypercholesterolemia increases the membrane fragility of erythrocytes increasing hemolysis.

Because ALA is a precursor in the heme biosynthesis pathway, ALA is converted to PPIX, which is fluorescent.[24] An ALA solution will reach macrophages, and atheromatous plaques will temporarily accumulate more PPIX than normal tissues.[13] The selective accumulation of PPIX in plaques provides a contrast between control animals and those with atherosclerosis.

After the animals received ALA, the arteries of the experimental groups significantly differed from the normal arteries (control group). For both the control and experimental groups, the PPIX fluorescence intensity increased almost three-fold after the ALA administration, relative to the PBS administration. The PPIX fluorescence intensity increased five-fold for the experimental group relative to the control group. These findings demonstrate a true exogenous accumulation of PPIX by macrophages that is caused by atherosclerosis-specific metabolic alterations and demonstrate that these PPIX molecules are transferred to blood.

At 22 days, the diet did not yet lead to atheromatous plaques in oil red-marked slices, but there was already an abnormality in the artery of the collected blood samples, most likely due to LDL accumulation. This finding indicates that this method can aid in the early diagnosis of atherosclerosis with high sensitivity.

Larger and better-designed studies are being carried out to further elucidate this matter and thus determine the most appropriate concentration of ALA solution and the best time for administering ALA to the animals.

D. Conclusions

PPIX can be analyzed by porphyrin extraction from total blood. The results showed an increase in the blood PPIX emission that accompanied the growth of atheromatous plaques. The aortic fluorescence in the 550–750 nm range increased in the animals from the experimental group that received ALA. This finding indicated that this method can aid in the early diagnosis of atherosclerosis with high sensitivity.

For human patients, larger individual variation is expected for non-atherosclerotic individuals due to their different physiology and pathology; this variation could reduce the diagnostic accuracy of PPIX fluorescence and affect its use as a screening tool. Nevertheless, the dependence of PPIX fluorescence on atherosclerosis-induced metabolic alterations should at least be a useful additional biomarker for diagnosing initial atheromatous plaque formation, thus allowing both a liquid biopsy and monitoring of therapeutic treatments.

Acknowledgements

This work was supported by the "Fundação de Amparo a Pesquisa do Estado de São Paulo" (FAPESP), Grant number 2010/016544-1. The authors also would like to thank Maira Franco de Andrade for help in veterinary procedures and Andrea Moreira Monteiro for help in biochemical analysis.

References

1 V. N. Borshch, E. R. Andreeva, S. G. Kuz'min and I. N. Vozovikov, *Russ. J. Gen. Chem.*, 2012, **82**, 554–563.
2 Y. Uchida, S. Kawai, R. Kanamaru, Y. Sugiyama, T. Tomaru, Y. Maezawa and N. Kameda, *J. Am. Coll. Cardiol. Img.*, 2010, **3**, 398–408.
3 P. Edholm, C. Grace and B. Jacobson, *Med. Biol. Eng.*, 1968, **6**, 409–413.
4 C. Kittrell, R. L. Willett, C. Delossantospacheo, N. B. Ratliff, J. R. Kramer, E. G. Malk and M. S. Feld, *Appl. Opt.*, 1985, **24**, 2280–2281.
5 M. Okada, M. Yoshida and Y. Tsuji, *Xxx World Congress of the International College of Surgeons*, 1996, vol. 1–2, pp. 893–902.
6 A. L. Alexander, C. M. C. Davenport and A. F. Gmitro, *Opt. Eng.*, 1994, **33**, 167–174.
7 K. Douma, R. T. A. Megens and M. van Zandvoort, *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.*, 2011, **3**, 376–388.
8 A. Manolopoulos, A. Vasileiou, V. Kokkinos, C. Athanasopoulos, E. Agapitos, N. Kavantzas and D. Yova, *Comparative Studies of Laser Induced Fluorescence and Intravascular Ultrasound for the Human Coronary Artery Diagnosis of Atherosclerosis*, 2000.

9 L. Marcu, M. C. Fishbein, J. M. I. Maarek and W. S. Grundfest, *Arterioscler., Thromb., Vasc. Biol.*, 2001, **21**, 1244–1250.
10 Y. Uchida, T. Sakurai, M. Kanai, S. Shirai and T. Morita, *Arterioscler., Thromb., Vasc. Biol.*, 2011, **31**, 1452–1460.
11 Y. Uchida, Y. Maezawa, Y. Uchida, N. Hiruta, E. Shimoyama and S. Kawai, *PLoS One*, 2013, **8**(2), e55188.
12 J. L. Cheng, X. Sun, S. Y. Guo, W. Cao, H. B. Chen, Y. H. Jin, B. Li, Q. N. Li, H. Wang, Z. Wang, Q. Zhou, P. Wang, Z. G. Zhang, W. W. Cao and Y. Tian, *Int. J. Nanomed.*, 2013, **8**, 669–676.
13 S. Y. Guo, X. Sun, J. L. Cheng, H. B. Xu, J. H. Dan, J. Shen, Q. Zhou, Y. Zhang, L. L. Meng, W. W. Cao and Y. Tian, *Int. J. Nanomed.*, 2013, **8**, 2239–2246.
14 F. P. Wang, Q. P. Gao, S. Y. Guo, J. L. Cheng, X. Sun, Q. N. Li, T. Y. Wang, Z. G. Zhang, W. W. Cao and Y. Tian, *BioMed Res. Int.*, 2013, **2013**, DOI: 10.1155/2013/737264.
15 R. L. Lipson, J. H. Pratt, E. J. Baldes and M. B. Dockerty, *J. Obstet. Gynecol.*, 1964, **24**, 78–84.
16 A. C. Croce, G. Santamaria, U. De Simone, F. Lucchini, I. Freitas and G. Bottiroli, *Photochem. Photobiol. Sci.*, 2011, **10**, 1189–1195.
17 J. R. Spears, J. Serur, D. Shropshire and S. Paulin, *J. Clin. Invest.*, 1983, **71**, 395–399.
18 C. H. Peng, Y. S. Li, H. J. Liang, J. L. Cheng, Q. S. Li, X. Sun, Z. T. Li, F. P. Wang, Y. Y. Guo, Z. Tian, L. M. Yang, Y. Tian, Z. G. Zhang and W. W. Cao, *J. Photochem. Photobiol., B*, 2011, **102**, 26–31.
19 A. C. Croce, A. Ferrigno, M. Vairetti, R. Bertone, I. Freitas and G. Bottiroli, *Photochem. Photobiol. Sci.*, 2004, **3**, 920–926.
20 V. Bloks, H. van Goor, H. Moshage, R. Havinga, P. L. M. Jansen and F. Kuipers, *Circulation*, 1998, **98**, 591.
21 Z. Bosze and L. M. Houdebine, *World Rabbit Science*, 2006, **14**, 1–14.
22 F. R. de Oliveira Silva, M. Helena Bellini, V. Regina Tristao, N. Schor, N. D. Vieira, Jr and L. C. Courrol, *J. Fluoresc.*, 2010, **20**, 1159–1165.
23 G. Camejo, C. Halberg, A. Manschik-Lundin, E. Hurt-Camejo, B. Rosengren, H. Olsson, G. I. Hansson, G. B. Forsberg and B. Ylhen, *J. Lipid Res.*, 1998, **39**, 755–766.
24 F. R. de Oliveira Silva, M. H. Bellini, C. T. Nabeshima, N. Schor, N. D. Vieira, Jr and L. C. Courrol, *Photodiagn. Photodyn. Ther.*, 2011, **8**, 7–13.