Study of Tryptophan Lifetime Fluorescence Following Low-Density Lipoprotein Modification

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In this paper we report the effects of the irradiation of low-density lipoprotein (LDL) by ultra-short laser pulses to obtain in vitro alterations mimicking proatherogenic modifications occurring in vivo in LDL. The modifications by metallic ions (copper and iron) and ultra-short laser pulses were studied by fluorescence steady state and time-resolved lifetime measurements. The results demonstrate that the modifications caused by ultra-short laser pulses and by iron affect the tryptophan residues of apolipoprotein B-100 (Apo-B), slightly decreasing fluorescent lifetimes, with almost no modifications in pre-exponential factors, indicating preservation of structural properties around the fluorophore. On the other hand, oxidation by copper strongly affects the Apo-B protein associated with LDL. We describe a fast, inexpensive, and nondestructive fluorescence-based method that is readily accessible to provide the LDL particle characterization.

Index Headings: **Low-density lipoprotein (LDL); Time-resolved fluorescence; Tryptophan; Ultra-short pulsed laser.**

INTRODUCTION

Cardiovascular diseases are closely related to increased serum cholesterol levels, or more precisely, increased low-density lipoprotein (LDL) serum levels. The LDL structure includes a surface layer composed of phospholipids, unesterified cholesterol, the apolipoprotein B-100 (Apo-B), and an inner core composed mainly of cholesterol esters and triglycerides.[1] LDL in its native state is not atherogenic;[2,3] however, oxidized LDL plays an intrinsic role within atherosclerotic plaque formation and atherosclerosis progression.[4,5] Various studies suggested that native LDL is oxidized within the sub-endothelial space by various reactive species including superoxide, myeloperoxidase, 15-lipoxygenase, and peroxynitrite.[6]

Several methods[7–9] are used to examine LDL in vitro by simulating what occurs in the human body. The most used method (established by Esterbauer et al.[10]) for continuous monitoring of in vitro oxidation of human LDL consists of adding metal ions such as iron (Fe) and copper (Cu) to LDL solutions.

LDL oxidation can be monitored by the increase of conjugated dienes,[11] thiobarbituric reaction assays,[10] fluorescence development,[12] and changes of Apo-B electrophoretic mobility.[13] Most of these characterization methods require either special equipment or lengthy analytical time.[14]

In this work, we introduce an alternative method to modify LDL particles, the irradiation by ultra-short laser pulses, and compare its results with the ones obtained with traditional LDL modification by metallic ions. The characterization of the oxidation obtained by both methods was done by traditional techniques (thiobarbituric acid reactive substances [TBARS] and fast protein liquid chromatography [FPLC]) and by a time-resolved spectroscopic technique that follows the LDL oxidation state rapidly and inexpensively.

MATERIALS AND METHODS

LDL was extracted from blood plasma obtained from healthy blood-donor volunteers from COLSAN, a Brazilian Benevolent Association blood bank. Added to the plasma were benzamidine (2 mmol/L), gentamicin (0.5%), chloramphenicol (0.25%), phenyl-methyl-sulfonyl-fluoride (0.5 mmol/L), and aprotinin (0.1 unit/mL). The LDL was isolated from the resulting solution by sequential ultracentrifugation at 100 000 $\times g$, at 4 °C, by using a 75 Ti rotor (Beckman Instruments), and dialyzed at 4 °C against pH 7.4 phosphate-buffered saline (PBS). Last, the LDL was sterilized via filtration through a 0.22 μm-pore filter. The protein concentration was determined by means of a BCA protein assay kit (Pierce) by using bovine serum albumin as the standard.

The LDL was oxidized by two different methods, the traditional one that involves metallic ions, and the alternative technique here introduced, which uses ultra-short laser pulses. Both are described below.

LDL Oxidation by Metallic Ions. To obtain oxidized LDL samples by copper ions (CuLDL), LDL was dialyzed at 2.0 mg of protein/mL overnight against PBS without EDTA, followed by overnight incubation with 20 μM $CuSO_4$ for 18 h at 37 °C.[10] LDL oxidized by iron ions (FeLDL) was produced by dialysis at 2.0 mg protein/mL in 4 μM $FeSO_4 \cdot 7H_2O$ in pH 7.2 PBS, for 48 h at room temperature in the dark. Both oxidation protocols were terminated by addition of EDTA at 1 mmol/L.

LDL Oxidation by Laser Irradiation. Samples of native LDL were irradiated for 15 min by ultra-short pulses generated

Received 13 July 2012; accepted 2 November 2012
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DOI: 10.1366/12-06780

0003-7028/13/6704-0379/0

by an amplified Ti:Sapphire CPA laser system (FEMTOPOWER Compact Pro CE-Phase HP/HR, from FEMTOLASERS). The 25 fs (full width at half-maximum) pulses were centered at 785 nm, in a 4 kHz pulse train, with energies in the range of 200–750 μJ, resulting in peak powers from 8 to 30 GW. For irradiations, the pulses were confined to a 4 mm-diameter collimated beam, producing fluences in the range of 1.6–6.0 mJ/cm^2. The beam went through a 10 mm optical path glass cuvette, in which 1 mL of LDL solution (sample) was poured. The irradiations were done at 22 °C, followed by sample storage at 0 °C, and protected from ambient light until absorption and fluorescence measurements were performed.

The LDL samples modified by both methods were then characterized by the well known TBARS and FPLC techniques, and also by time-resolved fluorescence, and the results compared. The characterization methods are detailed below.

Determination of Lipid Peroxidation. Lipid peroxide formation was determined with TBARS assays.[14] To quantify the degree of oxidation by this method, 50 μL samples were incubated with 200 μL of TBARS reagent (1% thiobarbituric acid, 562.5 μM HCl, and 15% trichloroacetic acid, all from Merck, Germany). The mixture was boiled for 15 min and centrifuged at 1000 × *g* for 10 min. The absorbance of the supernatant at 540 nm was measured with a spectrophotometer (GENIOS TECAN, Austria) and compared with a malondialdehyde (MDA) standard.

Conjugated dienes, which exhibit absorbance at 234 nm, were measured according Esterbauer et al.[14] Data were analyzed by a one-way analysis of variance, followed by multiple comparisons tests.

Determination of Protein Modification. Molecular-exclusion FPLC was performed with a column of Superdex 200 HR in an FPLC system (Pharmacia Biotech, Sweden). The column was stabilized with 0.05 M PBS. The 500 μL LDL samples were diluted in Milli-Q water (Millipore) at a concentration of 1 mg/ml water and injected into the column. The reading of 0.05 M PBS was performed at a continuous flow rate of 0.5 mL/min. The sample absorbance at 280 nm was measured with a Diode Array detector (Shimadzu).

Spectroscopic Characterization. The absorption spectra of all samples were measured in the range of 200–600 nm by a Varian Cary 17D, and their emission spectra were obtained with a Jobin Yvon Fluorolog 3 by exciting the samples inside a 1 mm optical path cuvette.

Time-resolved fluorescence intensity and anisotropy decay profiles were measured with an apparatus based on a time-correlated, single-photon counting technique. The excitation source was a Spectra Physics Tsunami 3950 Ti:Sapphire laser, and the pulse repetition rate was set to 4.0 MHz by using a Spectra Physics 3980 pulse picker. The laser was tuned so that a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) originated the 295 nm excitation pulses, directed to an Edinburgh FL900 spectrometer. The spectrometer was set in L-format configuration, the emission wavelength was selected by a monochromator, and the emitted photons were detected by a refrigerated Hamamatsu R3809U micro-channel plate photomultiplier. A Soleil–Babinet compensator in the excitation beam and a Glann–Thomson polarizer in the emission beam were used in the anisotropy experiments. The instrument response function was typically 100 ps (full width at half-maximum), and measurements were made with 12 ps of time resolution per channel. The software provided by Edinburgh Instruments was used to analyze the individual decays, which were fitted to multi-exponential curves. The quality of the fit was judged by the reduced χ^2 value and inspection of the residuals distribution. The average lifetimes, τ, were calculated according to:

$$\tau = \frac{\sum b_i \tau_i^2}{\sum b_i \tau_i} \quad (1)$$

where τ_i and b_i are the lifetime and the pre-exponential factor of the *i*th decay component.

RESULTS

The absorption spectrum of the native LDL solution (2mg/ml), shown in Fig. 1a, exhibits an ultraviolet absorption band around 285 nm, originated mainly by the tryptophan (Trp) residues contained in Apo-B,[15] and absorption bands between 350 and 600 nm, mostly due to the β-carotene–binding protein.[16] The inset graph shows that the β-carotene absorption band is bleached after LDL is irradiated by 712 μJ laser pulses for 15 min. Figure 1b depicts the fluorescence spectra of Trp from the native LDL, CuLDL, FeLDL, and LDL irradiated by 350 and 712 μJ laser pulses (fsLDL), under excitation at 280 nm. Trp fluorescence exhibits a band centered at 340 nm for native LDL, both laser-irradiated samples (350 and 712 μJ), and FeLDL. The oxidation by Cu is the one that promotes most modifications in the LDL particle, and a red shift to 360 nm is observed for the CuLDL sample, demonstrating that when large changes occur in the LDL particle, the emission band is displaced to longer wavelengths.

The time-resolved fluorescence measurements were performed by exciting the LDL at wavelengths corresponding to Trp absorptions, and setting the emission monochromator at several wavelengths, spanning the Trp emission band in the range of 330–370 nm. Figure 2 shows the 350 nm luminescence temporal behavior for the native LDL, FeLDL, CuLDL, and both fsLDL samples, and it is seen that the CuLDL decay has distinctive features when compared with the other samples emission, which are very similar.

Table I shows the fitted parameters and the average lifetimes calculated by Eq. 1, with three exponential decays, as judged by the reduced χ^2 values close to 1, for emission lifetimes at wavelengths from 335 to 365 nm for the native LDL sample. As native LDL in FeLDL and laser-irradiated LDL samples, there is a lengthy lifetime, around 5.7 ns, an intermediate close to 1.9 ns, and a short lifetime around 0.5 ns. In CuLDL, these lifetimes were significantly lower, around 3.5, 1.1, and 0.15 ns.

Table II presents the average lifetimes (τ) calculated from the data for many emission lines for all samples, evidencing that this technique detects a lifetime decrease, consistent for all wavelengths emission, when the sample is modified (oxidized). Statistical *t*-testing of the data presented in Table II reveals that there is a significant difference ($P < 0.05$) in lifetimes for different LDL oxidation states.

Figure 3 presents anisotropy decay curves measured at 350 nm for all samples. Isolated Trp in aqueous medium anisotropy presents fast decay, which could be fitted to a mono-exponential decay, with rotational correlation times around 80 ps, and the residual anisotropy for extended times (>2 ns) is zero. Two rotational correlation times were needed to fit the data for the LDL samples, as opposed to only one for the Trp,

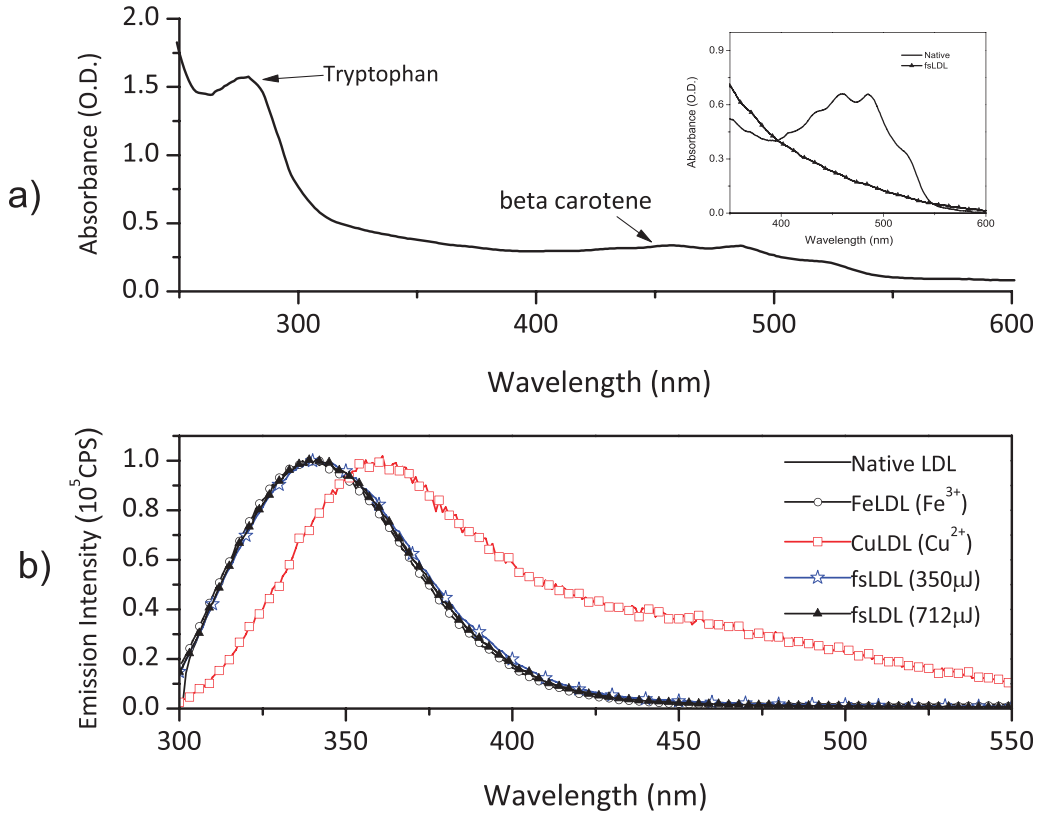

FIG. 1. (**a**) Absorption spectrum of native LDL and (**b**) fluorescence spectra, under 280 nm excitation, from native LDL, CuLDL, FeLDL, and 15 min laser-irradiated LDL samples (350 and 712 μJ).

as can be seen in Table III. A common interpretation usually attributes[17] the short component (<100 ps) to local Trp motion, and the long correlation time (ranging from 2.3 ns in native LDL to 1.6 ns in the irradiated and Cu-oxidized LDL) to overall tumbling of the region of the macromolecule containing the fluorescent residue.

Validation Method. To validate the ultra-short laser pulse irradiation method to modify LDL, all LDL samples had their oxidation product measured by FPLC, and the results were compared. Once the oxidation levels were determined by these established methods, the results were compared with those obtained from the Trp fluorescence lifetime in order to validate this measurement technique.

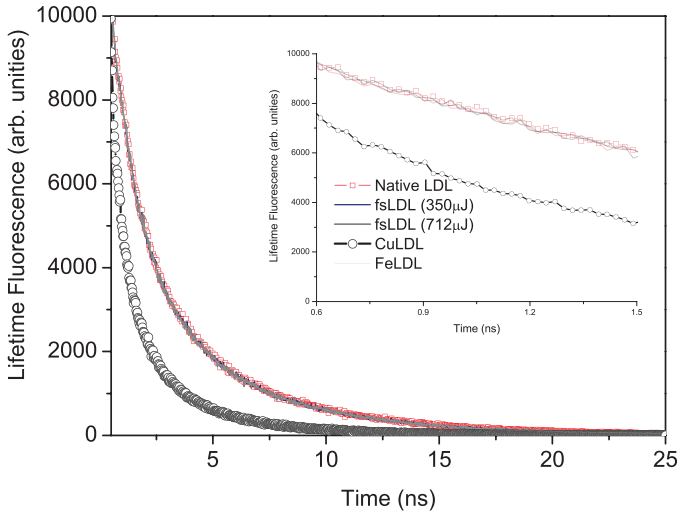

FIG. 2. Lifetime fluorescence decays obtained for native LDL, CuLDL, FeLDL, and 15 min laser-irradiated LDL samples (350 and 712 μJ), exciting the samples in the Trp absorption band.

Table IV shows the values of conjugated dienes and TBARS, which prove the lipid peroxidation of all LDL samples. These results demonstrate that all modification methods were effective in oxidizing the LDL, validating laser irradiation for this purpose. It is worth mentioning that the Cu promotes a drastic oxidation when compared with the other methods, as evidenced by the higher values measured for this technique. The results obtained from molecular-exclusion FPLC and from the detection of protein absorption at 280 nm are shown in Fig. 4. This figure shows that the laser irradiation results in the appearance of low-molecular materials that are similar, but not identical, to those generated by transition metals oxidation. These results corroborate that the irradiation by ultra-short laser pulses oxidizes the LDL in a milder way when compared with the metallic ions process and confirm that the lifetime measurements results (shown in Table II) constitute a technique to measure the promoted modifications.

DISCUSSION

Despite the similarities in the fluorescence decay profiles for different forms of LDL (shown in Fig. 2), a consistent trend is observed in the average lifetimes values, independent of emission wavelength (Table II): The longest values, around 4 ns, correspond to the native LDL, decreasing in the oxidized

TABLE I. Fitting parameters for the LDL luminescence decay to a three-exponential function: lifetime (τ_1 in ns), normalized pre-exponential factor (b_i), and reduced χ (χ^2_{red}), and average lifetimes (τ in ns).

	Parameter							
λ_{em} (nm)	τ_1	τ_2	τ_3	b_1	b_2	b_3	χ^2_{red}	τ
335	5.52	1.83	0.45	0.20	0.44	0.36	1.16	3.69
340	5.58	1.81	0.44	0.22	0.46	0.32	1.27	3.84
345	5.70	1.97	0.54	0.23	0.43	0.34	1.12	3.96
350	5.78	1.91	0.46	0.25	0.45	0.30	1.18	4.11
355	5.78	1.94	0.52	0.27	0.45	0.28	1.16	4.18
360	5.69	1.82	0.42	0.30	0.46	0.24	1.10	4.25
365	5.84	2.07	0.57	0.30	0.43	0.27	1.10	4.33

TABLE II. Average lifetime (ns) calculated from parameters of decay profile fitting to three exponential decay curves, measured at different emission wavelengths. A typical error of 5% is considered in the presented values.

Wavelength (nm)	Emission lifetime (ns)				
	LDL	FeLDL	fsLDL (350 μJ)	fsLDL (712 μJ)	CuLDL
340	3.84	3.78	3.70	3.77	2.19
350	4.11	4.01	3.94	3.89	2.36
360	4.25	4.19	4.20	4.06	2.39
370	4.45	4.25	4.28	4.25	2.38

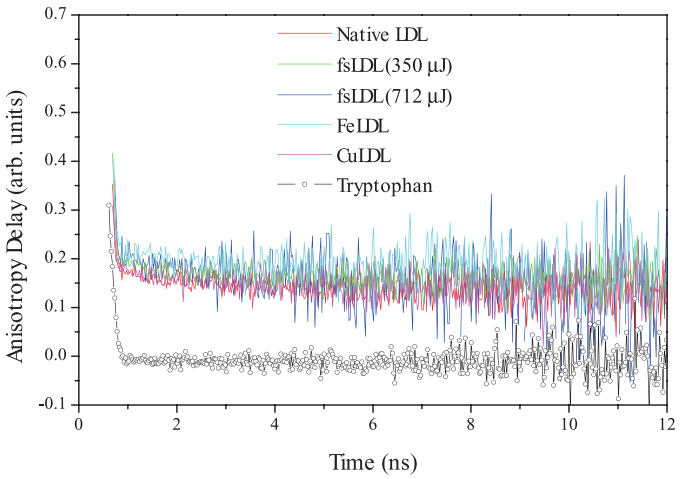

FIG. 3. Anisotropy decay of the 350 nm emission under excitation at 295 nm for native LDL, CuLDL, FeLDL, and 15 min laser-irradiated LDL samples (350 and 712 μJ). The anisotropy decay of tryptophan in aqueous solutions is also shown.

TABLE III. Parameters (rotational correlation times [θ_1], initial anisotropies [r_i] and residual anisotropy [r_∞]) obtained from fitting of anisotropy decays.

	Parameter				
Sample	θ_1 (ns)	θ_2 (ns)	r_1	r_2	r_∞
Native LDL	0.047	2.3	0.23	0.045	0.134
LDL (Fe)	0.037	1.8	0.26	0.042	0.177
LDL (Cu)	0.028	1.6	0.27	0.058	0.153
fsLDL (350 μJ)	0.040	1.9	0.20	0.042	0.134
fsLDL (712 μJ)	0.040	1.6	0.29	0.039	0.157
Trp	0.076	—[a]	0.29	—	0.0

[a] — = no data.

and the laser-irradiated samples; a drastic decrease to just above 2 ns was observed for the CuLDL.

Although the oxidation of LDL by Fe and laser irradiation did not affect the processes leading to Trp fluorescence intensity decay, the rotational kinetics of the residue are sensitive to oxidation, as is observed by the decrease of the long correlation time (θ_2 in Table III) from 2.3 ns in the native LDL to less than 1.9 ns in all modified lipoproteins, showing that the rotation kinetics of the protein region containing the Trp are faster in the oxidized samples. It is also worth noting that the residual anisotropy values at lengthy times (r_∞ in Table 3) are not zero, instead ranging from 0.134 in native LDL to 0.177 in FeLDL. Those large values were originated from the fit of the experimental anisotropy decays, and result from the fact that the residue is part of a protein with very long rotational diffusion time, so that the Trp residue (lifetimes around 4 ns) de-excited before the tumbling of the macromolecule as a whole. From these results, it can be concluded that structural changes in proteins promoted by oxidation do affect its rotational dynamics.

Several processes such as proton and electron transfers, quenching, indole conformations within the protein, or time-dependent relaxations of the local microenvironment surrounding the indole residue can be involved in the complex fluorescence response of Trp in proteins. The presence of three lifetime components for Trp is commonly observed in peptides, and it has been attributed to the occurrence of different rotational conformers of the indole ring around the C_α–C_β bond of the alanyl side chain.[18] In amphipathic peptides

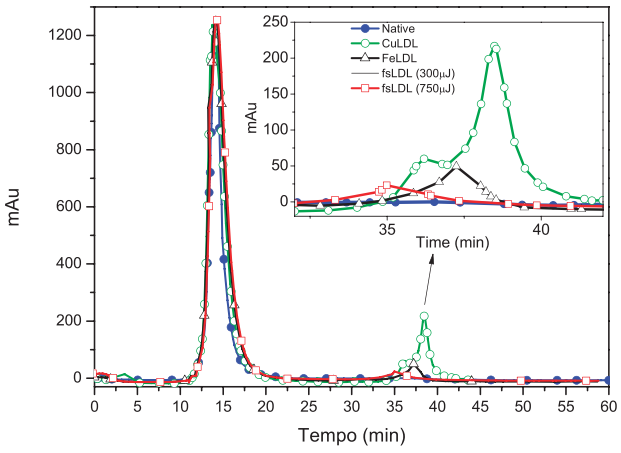

FIG. 4. Fast performance liquid chromatography (FPLC) fractions of Native LDL, CuLDL, FeLDL, and fsLDL.

TABLE IV. Generation of conjugated dienes and thiobarbituric acid reactive substances (TBARS) during oxidation of LDL. Conjugated dienes and TBARS are expressed in nanomoles per milligram of protein. Values are means ± standard deviations of triplicate determinations.

	LDL				
Sample	Native LDL	fsLDL (350 μJ)	fsLDL (750 μJ)	FeLDL	CuLDL
Conjugated dienes	0.68 ± 0.03	0.72 ± 0.01	0.890 ± 0.003	1.69 ± 0.01	2.45 ± 0.01
TBARS	1.00 ± 0.01	7.45 ± 0.05	25.5 ± 0.5	29.5 ± 0.5	81.2 ± 1.2

containing a single Trp molecule, crystallographic data supported a correlation of the lifetimes with rotamer distributions,[19] and in cyclic hexapeptpides, the triple-exponential fluorescence emission can be analyzed in view of Trp side-chain rotamer populations, determined by proton nuclear magnetic resonance spectroscopy.[20] In the peptide α-melanocyte–stimulating hormone (and analogs), the pH-dependent modifications in the pre-exponential factors were associated with different Trp rotamers,[21] whose distributions changed due to alterations in the protonation state of side-chain residues.[22]

It is known that Cu^{+2} oxidation can completely remove the Trp molecule from the LDL;[15] hence, it could also be this removal that ultimately causes the fluorescence changes in this oxidation. The Trp removal could eventually explain the large difference between the Cu^{+2} oxidation and the remaining methods.

The ultra-fast laser was chosen to irradiate LDL, due to the fact that the very short duration of the pulses easily induces nonlinear processes in materials transparent to the pulses wavelength. This condition, allied to pulse durations under the thermal vibration periods of the molecules, minimizes heat transfer to the target differently from what occurs with continuous-wave or nanosecond lasers. The nonlinear induced phenomena can also promote local electronic density modifications (and also ionizations), leading to modifications in the target. In the present work, it was observed that irradiating LDL for 15 min with 750 μJ pulses efficiently bleaches the antioxidant molecules, allowing the oxidation of the lipoprotein. It is known that these antioxidants—β-carotene and α-tocopherol—are capable of capturing free radicals and breaking lipid peroxidation chain reactions, thereby preventing lipid destruction. LDL oxidation involves the peroxidation of polyunsaturated fatty acids, cholesteryl esters, and un-esterified cholesterols, and is accompanied by fragmentation of Apo-B into smaller peptides and the derivatization of some of its lysine amino groups by aldehydes such as MDA, which are end products in the peroxidation of fatty acids.

Finally, the advantage of using ultra-short pulses over the traditional methods to modify lipoproteins comes from the possibility of precisely reaching spots away from the light source or behind a transparent barrier such as the cellular membrane. Also, the possibility of using a laser to remotely induce and measure a luminous emission confers the same advantages to a detection–characterization method based on the Trp fluorescence lifetime characterization. Additionally, Trp fluorescence can be a more efficient and faster method of mapping protein modifications when compared with traditional methods such as molecular-exclusion FPLC or gel electrophoresis. These attributes make the methods introduced in this work suitable to modify and characterize lipoproteins in living tissues and even inside cells, where ion methods would not be applicable. Nevertheless, more studies are necessary to verify that a laser would not disrupt metabolic processes in such environments.

CONCLUSION

Novel methods for oxidizing LDL and monitoring the evolution of the oxidation products were presented here.

LDL modification by ultra-short laser-pulse irradiation, described for the first time to our knowledge, is an alternative method to modify LDL in vitro, with potential in vivo application in tissues and cells. It was shown that irradiating LDL for 15 min with 25 fs, >712 μJ pulses efficiently bleaches the antioxidant molecules, β-carotenes, and α-tocopherols, resulting in the oxidation.

The time-resolved fluorescence monitoring of oxidation products demonstrated that oxidation by Cu strongly affects the Apo-B protein associated with LDL, probably due to the removal of Trp, causing the fluorescence changes in the Cu^{+2} oxidation; it also showed that laser-irradiation iron oxidations have milder effects on the residues of Apo-B in TRP, slightly decreasing its fluorescence lifetimes without significantly changing the pre-exponential factors. This indicates that the structural properties around the fluorophore are preserved. Regarding the constraints to Trp residues rotational dynamics, as revealed by time-resolved anisotropy decay, the oxidation process facilitates the rotational diffusion of the protein region containing the residue (decrease in rotational correlation time), and at the same time, imposes restrictions to the overall tumbling of Trp (increase in the lengthy residual anisotropy times). Considering the obtained results, we conclude that time-resolved spectroscopy is a readily accessible, fast, inexpensive, and nondestructive method to follow the LDL particle characterization.

ACKNOWLEDGMENTS

The authors thank the Fundação de Amparo à Pesquisa do Estado de São Paulo, the Instituto Nacional de Ciência e Tecnologia de Fluidos Complexos, and the Conselho Nacional de Desenvolvimento Científico e Tecnológico for the financial support.

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