# Investigation of human plasma low density lipoprotein by three-dimensional flourescence spectroscopy

Ernst Koller, Oswald Quehenberger, Günther Jürgens[+], Otto S. Wolfbeis[°] and Hermann Esterbauer*

*Institute of Biochemistry, University of Graz, Schubertstraße 1, [+]Institute of Medical Biochemistry, University of Graz, Harrachgasse 21/III* and *[°]Institute of Organic Chemistry, University of Graz, Heinrichstraße 28, A-8010 Graz, Austria*

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Human plasma LDL exhibits a diffuse fluorescence (excitation 360 nm) in the 400–600 nm range. Application of three-dimensional fluorescence spectroscopy shows the presence of 7 fluorophores in the lipid and 6 fluorophores in the protein domain. The 430 nm fluorescence in freshly prepared LDL and its apo-B is most likely indicative for remnants of in vivo lipid peroxidation.

*Three-dimensional fluorescence spectroscopy LDL Lipid peroxidation*

## 1. INTRODUCTION

Human plasma and aortic low density lipoproteins (LDL) possess a native fluorescence in the ultraviolet (UV) region due to tryptophan residues contained in apolipoprotein-B (apo-B) [1–3]. This fluorescence and its quenching were used to study some aspects of the LDL structure [2] and the change induced by lipid peroxidation [3]. Dobretsov et al. [1] reported on the fluorescence of aortic LDL in the near-UV and visible regions which was attributed to the presence of lipid peroxidation products and Schuh et al. [4] found that plasma LDL develops a fluorescent chromophore at around 450 nm, when subjected in vitro to autooxidation conditions. We have shown [5] that native plasma LDL also exhibits fluorescence in the region 400–600 nm. However, the broad and diffuse shape of the spectrum did not allow conclusions to be made regarding the number of fluorescent LDL constituents and their possible origin.

Here, we report on the application of 3-dimensional fluorescence spectroscopy to resolve the multi-fluorescent component system of the LDL complex in the 390–590 nm emission range. The results indicate that at least one fluorophore in native plasma LDL may be due to lipid peroxidation. Since modification of plasma LDL by in vivo lipid peroxidation is frequently discussed as a significant event in atherogenesis [6,7], our results might be important for a better understanding of the complex phenomena occurring during lipid peroxidation-linked alterations of LDL.

## 2. MATERIALS AND METHODS

Human plasma LDL was prepared by step-wise ultracentrifugation within a density cut-off of $d = 1.020–1.050$ g/cm$^3$ as described [8]. Four preparations were used, of which two were from pooled plasma of 5 healthy, male and female donors under 25 years of age (LDL nos 2,3) and two from single donors, both female under 25 years of age (LDL nos 1,4). EDTA (1 mg/ml) was present throughout all steps of the preparation. Immediately after drawing blood, plasma was made 200 $\mu$M in butylated hydroxytoluene. LDL prepared under these conditions revealed the same electrophoretic mobility on agarose gel electrophoresis (Lipidophor, kindly supplied by Im-

* To whom correspondence should be addressed

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muno AG Vienna, Austria) as the LDL in the parent plasma stored at 4°C for the time needed for LDL preparation (5 days). In 3.75% SDS-PAGE (staining with Coomassie blue R250) only the apo-B 100 band was detectable in freshly prepared LDL [8]. For fluorescence measurements LDL was immediately dialyzed for 48 h at 4°C against degassed 0.01 M phosphate buffer (pH 7.4) containing 0.16 M NaCl, 1 mg/ml EDTA and 0.1 mg/ml chloramphenicol. The sample was diluted with the dialysis buffer to a final concentration of 1.5 mg total LDL/ml and the spectra were then recorded. The LDL lipid fraction was prepared by extracting 3 ml LDL solution (1.5 mg/ml), prepared as described above, 4 times with 2 ml of a mixture of $CHCl_3/CH_3OH$ (2:1, v/v). Phase separation was facilitated by centrifugation. The pooled extract was concentrated to 3 ml by gassing with nitrogen and this lipid solution was then used for fluorescence measurements. The aqueous phase of the chloroform/methanol extraction was removed and the precipitated apo-B washed in centrifuge tubes twice with 3 ml water, dried under nitrogen and redissolved in 3 ml of 3% aqueous SDS. This solution of apo-B was then used for the fluorescence spectroscopy.

Auto-oxidation of LDL was performed essentially as described by Schuh et al. [4]. Freshly prepared LDL was freed from EDTA by dialysis against 0.01 M phosphate buffer (pH 7.4) containing 0.16 M NaCl and 0.1 mg/ml chloramphenicol. The LDL was then adjusted to 1.5 mg/ml, transferred to a dialysis bag and placed in a 3-fold amount of the above-described dialysis buffer which was continuously gassed with oxygen. The auto-oxidation in the dialysis bag was performed at room temperature and lasted 24 h. Thereafter, fluorescence spectra of the total LDL complex, lipid and apo-B fraction were recorded. The concentrations in the final measuring solutions were always equivalent to 1.5 mg parent LDL/ml.

Fluorescence spectra were scanned on a Jobin Yvon JY3 D spectrofluorimeter. A slit width of 10 nm was used. The instrument was standardized with quinine sulfate (0.1 $\mu$g/ml in 0.1 N $H_2SO_4$) to give a fluorescence intensity of 100 at 450 nm when excitation was done at 350 nm. For 3-dimensional fluorescence studies the samples were diluted 1:1 with the respective solvent. The absorbance of these solutions did not exceed 0.2 in the range 300–500 nm. The contour plots (to which we refer as fluorescence intensity topograms, FITs) in the 300–500 nm excitation and 390–590 nm emission ranges were obtained on an Aminco SPF-500 spectrofluorimeter as in [9]. Band passes of the instrument were 10 nm in excitation and emission. The

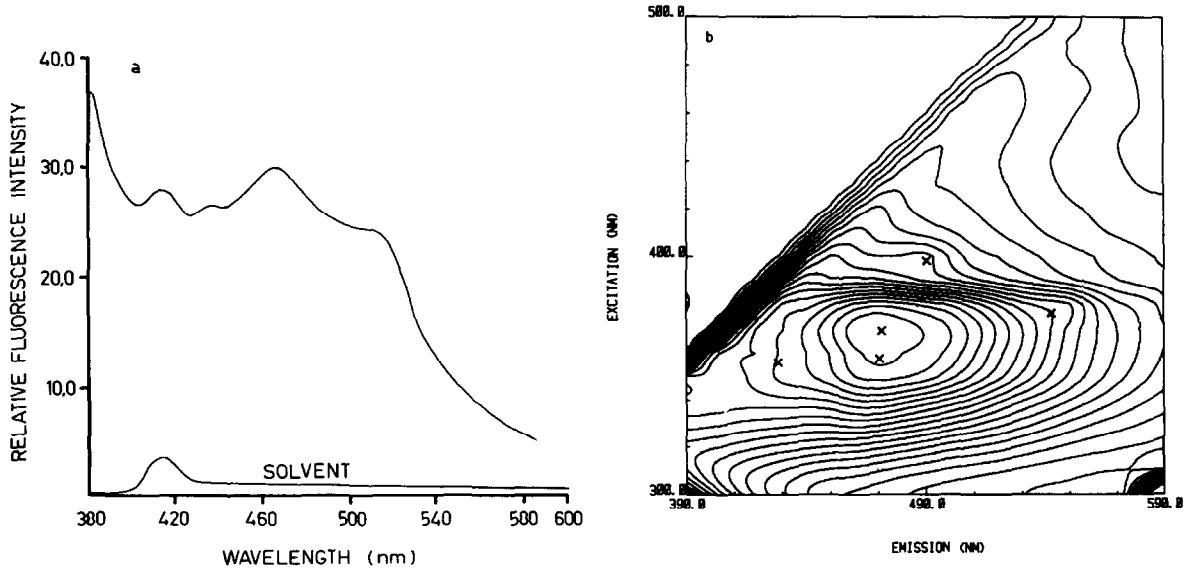


Fig.1. Uncorrected emission spectrum (a) at 360 nm excitation and solvent background corrected FIT (b) of total LDL isolated from pooled plasma (LDL no.3). (×) Probable positions of the intensity maxima.

elements of the excitation/emission matrix were acquired with 10-nm increments of both emission and excitation wavelengths. Solvent background correction was achieved by subtracting the excitation/emission matrix of the solvent from that of the sample. The contour lines were from 5 to 95% in 5% increments of the highest peak.

## 3. RESULTS

Freshly prepared human plasma LDL exhibits a weak fluorescence in the visible range (400–600 nm) when excitation is performed at 360 nm (fig.1a). The spectrum shows an apparent maximum at around 470 nm and shoulders at 430 and 520 nm. The sharp peak at 410 nm is not due to a fluorescent chromophore but to Raman scattering. The LDL also gives a strong UV fluorescence, typical for tryptophan-containing proteins with an emission maximum at 342 nm and an excitation maximum at 290 nm (not shown). Spectra similar to that shown in fig.1a were found for the other 3 plasma LDL samples analyzed. At a fixed concentration of 1.5 mg/ml the relative fluorescence intensity at 470 nm varied from 27.0 to 40.0 intensity units, and at 430 nm from 22.0 to 30.0.

A much deeper insight into the fluorescence characteristics of LDL was obtained by 3-dimensional fluorescence spectroscopy. This technique gives a plot of fluorescence intensity as a function of both excitation and emission wavelengths. The superiority of this method is clearly evident from the FIT shown in fig.1b, which was measured with the same LDL sample used for the spectrum shown in fig.1a. From the FIT it is obvious that the predominant fluorescent component of LDL possesses an excitation maximum at 370 nm and an emission maximum at 470 nm.

As against a single fluorophore the LDL pattern is not characterized by symmetrical contour lines in the FIT it is understood that LDL contains several fluorophores. A more detailed inspection of the shape of the contour lines reveals at least 4 additional fluorophores with excitation and emission maxima at 355/470, 355/430, 380/540 and 400/490 nm. The diagonal lines from 350/390 to 500/520 nm are contributions from stray light which could not be removed quantitatively by background subtraction.

To determine whether the fluorescence is associated with the lipid and/or protein portion of LDL the two components were separated and

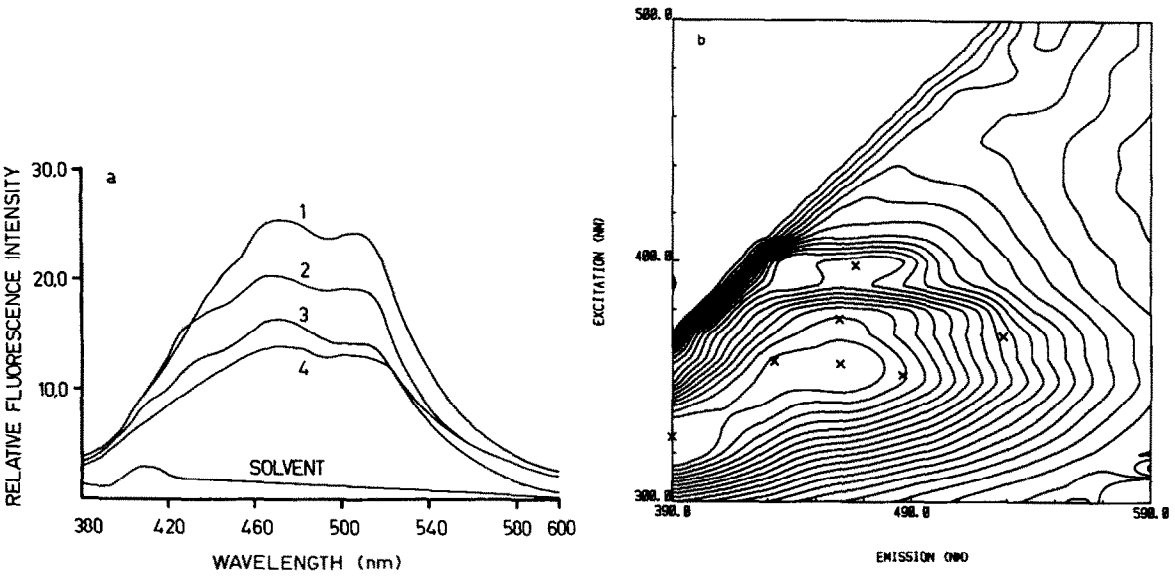


Fig.2. Uncorrected emission spectra (a) at 360 nm excitation and solvent background corrected FIT (b) of the LDL lipid material. The emission spectra were obtained from the lipids of LDLs from plasma of two single donors (nos 1,4) and two from pooled plasma (nos 2,3). The FIT was recorded from the lipid material of LDL no.3. (×) As in fig.1.

analyzed again. The spectra shown in figs 2a and 3a indicate that fluorophores are contained in both fractions. The apparent emission maxima identified in the spectra are at 470 and 520 nm in the lipid fraction and at 415 (Raman band), 440 and 520 nm in the apo-B fraction. The lipid and apo-B fractions from all 4 LDL samples resulted in more or less the same spectral form with some variation in the relative fluorescence intensity.

Representative FITs for the lipid and protein fraction of LDL are shown in figs 2b and 3b. According to these topograms the lipid fraction contains a major fluorescent chromophore with spectral properties (excitation maximum/emission maximum) at 356/462 nm. Several minor fluorophores are at 350/490, 375/460, 370/530, 355/430 and 400/470 nm. The shape of the FIT is also indicative of a further fluorescent component possessing a maximum at about 325/390 nm which, however, is just at the short-wavelength limit or even outside the measured range. The protein fraction (fig.3b) possesses main fluorophores with coordinates at 330/390, 355/430 and 365/460 nm and additional fluorophores with low fluorescence intensity at 380/540, 400/530 and 420/490 nm.

The fluorescence properties of native LDL did not change upon storage at 4°C. Thus, the emission spectrum (excitation 360 nm) and the FIT of a freshly prepared LDL were identical with the spectra recorded from the same preparation but stored at 4°C under nitrogen for 3 weeks as a 4% solution in 0.1 M Tris-HCl buffer (pH 7.5) containing 1 mg/ml EDTA. It was ascertained that fluorescence did not result from contaminants in the buffers and chemicals used for isolation of LDL.

When the LDL was subjected to conditions known to induce auto-oxidative degradation of polyunsaturated lipids, the fluorescence in the 430 nm range (excitation 360 nm) dramatically increased, whereas in the other parts of the spectrum it remained more or less unchanged. This effect of auto-oxidation on the 430 nm fluorescence could be seen on both total LDL and the apo-B, isolated from an auto-oxidized LDL sample. With the lipid fraction the fluorescence decreased in the 470 nm range and increased in other parts of the spectrum, particularly at 410 nm. From FIT analyses, auto-oxidized LDL and its apo-B possess an emission maximum at 430 nm and an excitation maximum at 355 nm. All LDL preparations investigated so far responded to auto-oxidation conditions with this strong increase of the 430 nm fluorescence. On average, the 430 nm fluorescence intensity of auto-oxidized LDL and the apo-B isolated therefrom

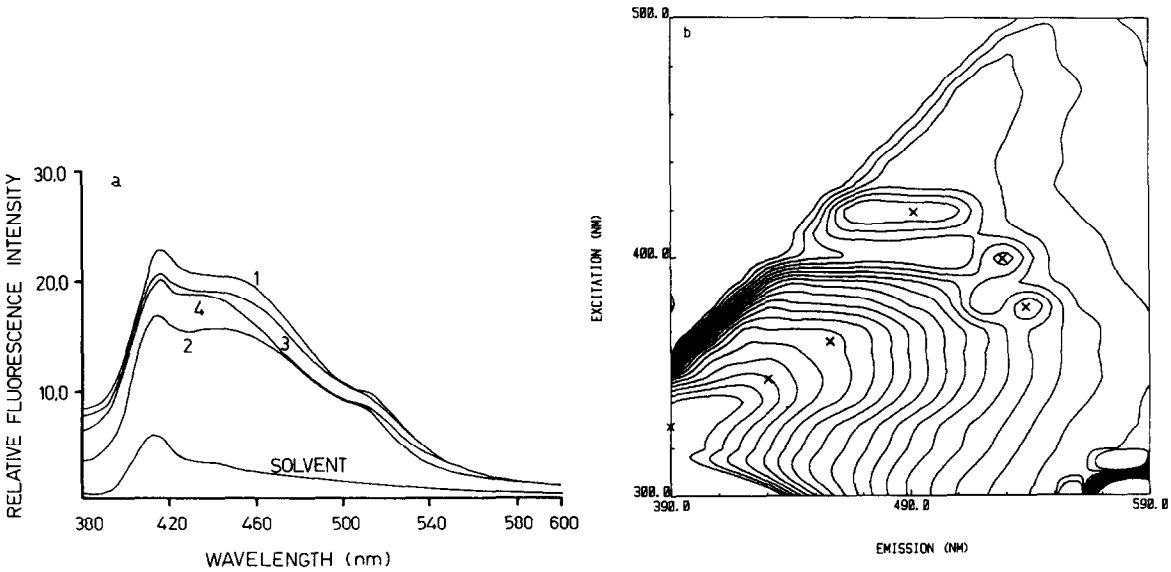


Fig.3. Uncorrected emission spectra (a) at 360 nm excitation and solvent background corrected FIT (b) of apo-B. 1–4 refer to the 4 LDL preparations shown in fig.2. The FIT was recorded from the apo-B of LDL no.3. (×) As in fig.1.

was 3-times higher than in the corresponding control sample.

## 4. DISCUSSION

We have focused our interest on the near-UV and visible part of the LDL fluorescence spectrum (300–500 nm excitation, 390–590 nm emission), which is not overshadowed by the strong tryptophan fluorescence and should therefore be more indicative of subtle alterations of the protein and lipid domain under physiological or pathophysiological conditions. Human plasma LDL represents a multicomponent system with several fluorescent constituents. The application of 3-dimensional fluorescence spectroscopy enabled us to resolve the diffuse fluorescence of total LDL and its lipid and protein fraction into distinct peaks or shoulders and to determine the spectral properties of the fluorophores contained in LDL. The FIT of freshly prepared LDL clearly showed the presence of at least 5 fluorescent components. Separate analyses of the lipid fraction and the apo-B revealed firstly that the number of potential fluorophores in normal plasma LDL was much higher than detectable by the FIT analysis of the total LDL and, secondly, that both the apo-B and the lipid fraction contained fluorescent constituents. The approximate number deduced from the FITs was 6 in apo-B and 7 in the lipid fraction.

The precise assignment of the various excitation and emission maxima to specific substances or chemical structures was not within the scope of this initial work. Vitamin E, a known constituent of LDL, fluoresces maximally at 335 nm (excitation 290 nm) and is therefore outside the FIT range measured here. Retinol and vitamin A derivatives are most likely responsible for the emission around 470 nm in the lipid fraction. The 430 nm fluorescence (excitation maximum 355 nm) found in freshly prepared LDL and its apo-B is most likely indicative of the presence of remnants from lipid peroxidation which had occurred in vivo prior to isolation of the LDL from plasma of the donors. This assumption is supported by several findings. (i) Peroxidation of LDL in vitro reveals a strong fluorescence with maximum excitation at 355 nm and maximum emission at 430 nm. (ii) LDL isolated from aortic tissue was also shown to have an intense fluorescence at around 420 nm which was tentatively attributed to in vivo lipid peroxidation [1]. (iii) It has been reported that LDL is the main carrier for plasma lipid peroxides [6]. (iv) LDL of the two single donors isolated simultaneously and LDL from the pooled plasma LDL, all prepared under identical conditions, showed different relative fluorescence intensities at 430 nm indicating variations between different individuals.

The fluorescence at around 430 nm of in vivo or in vitro peroxidized biological samples has been ascribed to the aminoiminopropene structure -N=CH-CH=CH-NH- formed by the interaction of the lipid peroxidation product malondialdehyde with amino functions in proteins or phospholipids [10]. We have shown that malondialdehyde reacts with LDL to give fluorescent products [5]. The excitation and emission maxima (400/470 nm) were, however, not identical with those of the fluorophore produced during LDL auto-oxidation (355/430 nm). The fluorescence of native or auto-oxidized plasma LDL and aortic LDL at 430 nm [1] must therefore be ascribed to products which are not derived from malondialdehyde. It is known [11,12] that a great diversity of reactive aldehydes other than malondialdehyde are produced during the peroxidative degradation of polyunsaturated lipids and it seems reasonable to assume that one (or more) of them can interact with proteins or lipids [8] to give the 430 nm fluorophore.

The biological significance of this study lies in the possibility of detecting by 3-dimensional fluorescence spectroscopy alteration of LDL related to peroxidation processes. It has been shown that LDL exposed to endothelial cells [7] or smooth muscle cells [13] is altered by lipid peroxidation, assumes an increased negative charge, becomes toxic to fibroblasts and bypasses the classical LDL receptor [7]. Many other in vitro and in vivo observations (review [8]) suggest but of course do not prove that lipid peroxidation of LDL is in some way involved in the multi-step process of atherogenesis.

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