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Oxidation-induced Spin Probes in Low-density Lipoproteins*

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PBN
spin trap
EPR spectroscopy

PBN trapping indicated the presence of at least two types of radicals induced in LDL upon slow oxidation, as observed with EPR spectroscopy. The EPR spectra are very similar to those of the spin probes or spin labels, which are routinely used in the studies of macromolecular systems and supramolecular assemblies. The parameters of the spectral line shapes, treated in two different theoretical models, did not provide conclusive evidence for the phase transition in the LDL particles, known to be present at about 25 °C. It is concluded that both types of trapped radicals, or at least one of them, are associated with the apolipoproteins, with the spin probe sticking into the lipid domain of phospholipids.

INTRODUCTION

The mechanism of oxidation of low-density lipoprotein (LDL) in the artery wall and possibly in plasma is not yet completely understood. However, there is general agreement that oxidized LDL has an important role in atherogenesis.[1–5] A certain amount of circulating LDL could be mildly or »minimally« oxidized. There is already solid evidence for the existence of the circulating LDL particles containing selected oxidation specific epitopes, or chemically identified oxidation products.[6–10] It is possible that in plasma mildly oxidized particles have a longer life-time than natural LDL, and consequently higher probability to react with the endothelium and penetrate the intima.[11]

Oxidation of LDL by copper ions has been extensively studied *in vitro*. In those experiments the number of added cupric ions greatly exceeded the number of LDL particles, leading to heavily oxidized lipoprotein.[12–14] The LDL oxidation under such conditions could not be an adequate model for the *in vivo* LDL oxidation in the artery wall. This stimulated us to investigate slow and mild oxidation. For that purpose, we studied oxidation in closed systems, with a defined and limited amount of oxygen.[15–17] After making a large number of experiments under various conditions, we were able to characterize a general pattern for the time dependent behavior of various oxidation parameters. We could interpret the observed consumption of molecular oxygen, very small increase of lipid hydroperoxides, monotonic growth of trapped free radicals and the unchanged level of α-tocopherol.[17]

More than a decade ago[18] we showed that the radicals formed during the LDL oxidation could be detected

* Dedicated to Professor Nenad Trinajstić on the occasion of his 65th birthday.
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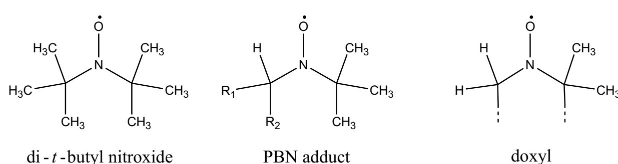
and analyzed with the electron paramagnetic resonance (EPR) spectroscopy. Subsequently, the LDL oxidation has been the subject of numerous EPR studies performed by us[14–17] and by other researchers.[19–23] These studies have revealed that the radicals are formed in the lipid and protein domains.

The present study is aimed at demonstrating that trapped radicals (spin-adducts), induced by oxidation and subsequent trapping, could be used as probes for monitoring the radical environment in the LDL particles. Spin traps are nitrone or nitroso compounds, and consequently the trapped radicals are nitroxides, just as spin probes and spin labels, which are commonly used in investigating the local environments in macromolecular or supramolecular structures, as shown in the Scheme below.



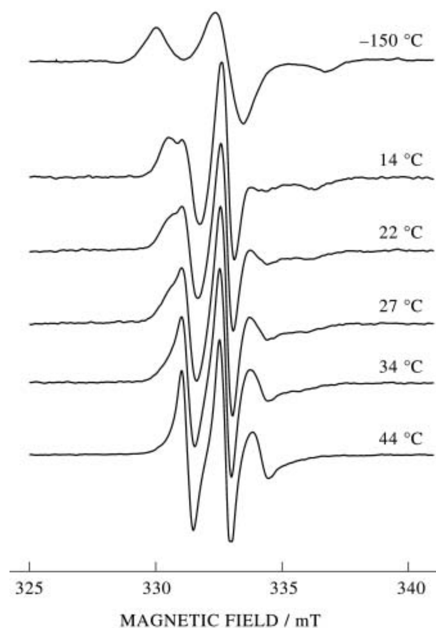


Figure 1. EPR spectra of the PBN-trapped free radicals, generated in LDL during the incubation at 37 °C for 2 days. The spectra were recorded at the microwave frequency of 9.375 MHz and 10 mW power, at temperatures as indicated.

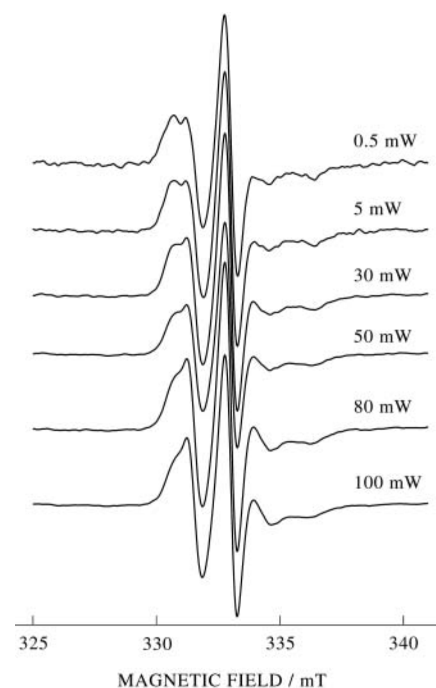


Figure 2. The EPR line shapes of the PBN-trapped free radicals, for various levels of the microwave power applied upon recording. The spectra were recorded at 15 °C. Other conditions are the same as for Figure 1.

EXPERIMENTAL

The process of LDL isolation from plasma of healthy individual donors was described earlier.[17] After isolation, LDL was dialyzed at 4 °C against phosphate-buffered saline (PBS), pH 7.4, containing EDTA (1 mg/mL). For the experiments LDL preparations were first concentrated to 80–100 mg LDL/mL using Minicon B–15 concentrators, and then diluted with a 100 mM *N*-*t*-butyl-α-phenyl nitrone (PBN) solution. The samples with final 1:1 ratio of the two solutions (purposely without any oxidation initiator added) were incubated for two or three days in tightly closed capillary tubes at 37 °C. The trapped radicals generated during that period could be found in all the phases of the sample: water, protein and lipid. The water-soluble spin-adducts were removed by additional dialysis. Hence, the recorded EPR spectra represented only the radicals associated with the LDL particles.

The EPR measurements were performed with a Varian E-109 EPR spectrometer equipped with a variable temperature control unit. The spectra were routinely recorded in the temperature range 0–45 °C, and in some cases at temperatures below the sample freezing point. The EPR resonance of diphenyl-picryl-hydrazyl (DPPH) was used as a marker for the line position determination.

RESULTS AND DISCUSSION

The EPR spectra of the LDL spin-adducts at several different temperatures are presented in Figure 1. The spectrum at –150 °C is typical of a frozen sample. The spectra change gradually as the temperature is raised to about 50 °C. In the temperature range from 0 °C to about 40 °C the observed EPR spectra consist of two separate resonance patterns, attributed to two distinct spin-adducts, or to chemically the same species located in two different domains in the LDL particles. The existence of the two distinct components in the composite EPR spectrum

could easily be proven by their different microwave power saturation behavior (Figure 2). The two radicals differ in their spin-lattice relaxation time, and consequently in their response to the change of the microwave power.[24]

The EPR spectra of the spin-adducts are usually characterized by the dominating triplet structure due to the coupling of the unpaired electron with the ^{14}N nucleus, and the additional doubling of the lines due to the β-proton coupling. The latter coupling is of the order of 0.3 mT,[25–27] depending essentially only on the nature of the trapped species and the polarity of the medium. In both of the two constituent EPR patterns in the present system, the β-proton coupling is obviously too small, compared to the line widths, to be resolved or even noticed. Thus, the shape of both components in the EPR spectra should look very similar to the spectra of purposely attached or added spin labels or probes under appropriate conditions.[28,29]

As can be seen from Figure 2, the recorded EPR spectra consist of a narrow-line component and a broad-line component. The exact shapes of the two components and their partition in the total pattern could not be equivocally determined. The analysis of the line shapes of the constituent components depends on the specific molecular arrangement of the spin probe environment and on the modes of the restricted motion of the probe. Since at this stage the details of the probe environment and the probe modes of motion are largely unknown, we have applied two models for the line shape analysis. One of them is the formalism of Schindler and Seelig,[30] originally developed for the nitroxides in the liquid crystalline environment. In this model three order parameters (S_i) and three correlation times (τ_{2m}) for each component are used for the fitting procedure, with a very strong influence of S_3 and τ_{20}. Figure 3 illustrates the use of that procedure for the decomposition of the experimental EPR spectrum recorded at 30 °C. The fit is almost perfectly attained with the use of 64 % of the broad component and 36 % of the narrow component. A good fit could also be obtained for the spectra recorded at other temperatures. Of course, for different temperatures different S_i and τ_{2m} values, but always the same ratio of the two components, have to be used. The temperature dependence of the most sensitive parameter S_3 for the two components is presented in Figure 4. An almost monotonous decrease of S_3 is observed in the entire temperature range studied (12–40 °C). The temperature profile of the two τ_{20} parameters are similar (not shown).

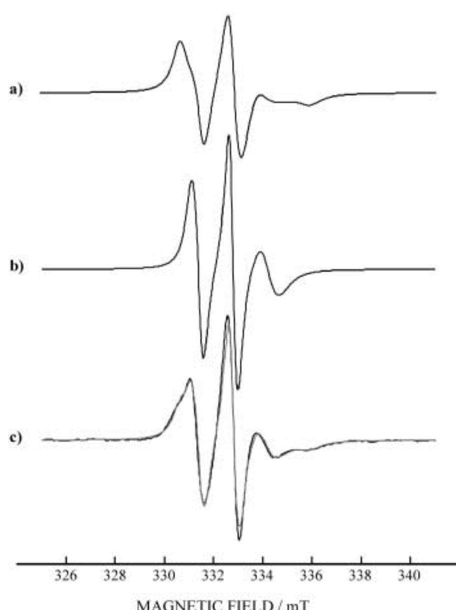


Figure 3. The EPR spectrum recorded at 30 °C (a), composed of two distinct patterns, the broad-line pattern (b) and the narrow-line pattern (c). Other parameters are the same as for Figure 1. The line shape of the composite spectrum (broader dotted line in (c)) was computed in the framework of the liquid crystalline model.

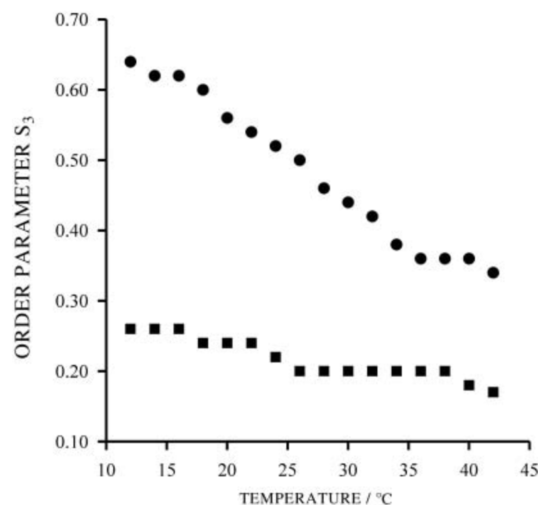


Figure 4. The temperature dependence of the S_3 order parameter for the slow (●) and fast (■) tumbling components, computed for the liquid crystalline model.

An alternative approach is to assume the broad component to be representative of a spin-adduct (spin probe) undergoing slow molecular motion. We assume that the radical is covalently bound to the protein (apo B) and that its motion is predominantly a Brownian isotropic rotational diffusion. For such a model the empirical parameter S, defined as:

$$S = \frac{2A_{zz}(\tau_R)}{2A_{zz}(\tau_{R\to\infty})}$$

and the related rotational correlation time, τ_R,

$$\tau_R = a(1 - S)^b$$

define the motional freedom of the spin probe in its environment.[31] $2A_{zz}(\tau_R)$ and $2A_{zz}(\tau_R \to \infty)$ refer to the separation between the outer extrema in the EPR spectrum at the measured temperature (and related correlation time)

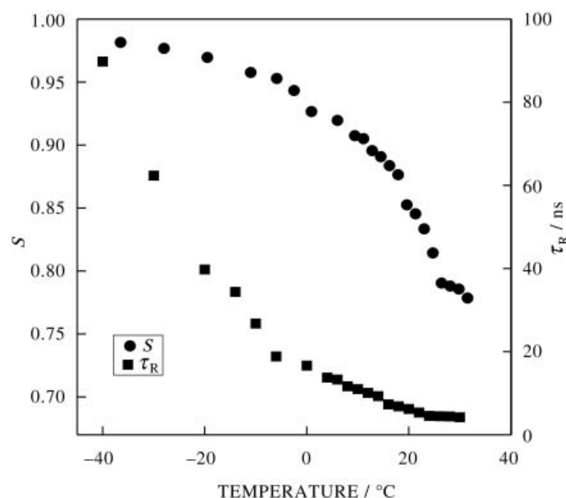


Figure 5. Temperature dependence of the order parameter (*S*) and rotational correlation time (τ_R) computed in the model of slow tumbling.

and for the spectrum of the immobilized radical, respectively. For constants *a* and *b*, the following empirical values are used: $a = 5.4 \times 10^{-10}$ s, $b = -1.36$. The values of the *S* and τ_R parameters as a function of temperature are shown in Figure 5. It is seen that the molecular motion gradually decreases upon lowering the temperature. Quantitatively slightly different and qualitatively similar behavior is predicted if the model of random jumps is applied.[32] A significant motional freedom at temperatures much below the freezing point is worth noting. Also, there is no abrupt change of the order parameter *vs.* temperature curve at the freezing temperature of water. These observations indicate that the trapped radicals stick into the lipid domain rather than into the aqueous domain.

The present experimental results, interpreted either in the liquid crystal model[30] or in the model of slow tumbling,[31,32] do not offer a clear sign for the phase transition, known to occur in both natural[33] and oxidized LDL[34] at about 25 °C. This fact suggests that the spin probes are embedded in the phospholipid domain rather than in the cholesteryl ester domain, which is responsible for the phase transition.[35,36]

CONCLUSIONS

The above results demonstrate that the trapped oxidation-induced radicals in LDL could be used as spin probes for studying the properties of the LDL particles. The similarity of the EPR spectra of the spin-adducts, formed in LDL by mild oxidation and subsequent trapping by PBN, and the spectra of the purposely added spin probes or attached spin labels[28,29] means that all the methods developed for the use of spin probes and spin labels could as well be applied to the use of spin-adducts for the same purpose. Our procedure induces some, although very mild, disturbance in the system studied.[17] It is probably not larger than the disturbances induced by insertion of standard spin probes or attachment of spin labels.

Both models for the molecular motion of the oxidation-induced spin probes used in the present study are probably inadequate. The parameters deduced should be considered only as some empirical markers, and not as reliable physical quantities. For an efficient use of the spin adducts as spin probes in the lipoprotein studies, a more adequate model for the molecular environment and modes of motion is required.

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SAŽETAK

Spinske sonde u lipoproteinima male gustoće, uvedene sporom oksidacijom

Dubravka Krilov, Marta Žuvić-Butorac, Nataša Stojanović i Janko N. Herak

Pri sporoj oksidaciji lipoproteina male gustoće (LDL), EPR spektroskopijom s primjenom spinske stupice (PBN) opažena su dva tipa slobodnih radikala. EPR spektri radikala stabiliziranih stupicom vrlo su slični spektrima spinskih sondi ili spinskih obilježivača, stabilnih radikala u standardnoj uporabi u istraživanju makromolekula ili supramolekularnih struktura. Parametri oblika EPR linija, određeni primjenom dvaju različitih teorijskih modela, ne pokazuju znakove znanoga faznoga prijelaza u LDL česticama pri temperaturi oko 25 °C. Zaključeno je da su obje vrste radikala (ili barem jedna) povezane s apolipoproteinom na način da im se spinske glave nalaze u lipidnoj domeni fosfolipida.