

Investigation of atherosclerotic plaque by high-frequency EPR

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Abstract. We present a comparative study of samples of aorta walls from male patients with atherosclerosis and hydroxyapatite powders with the average size of crystallites of 30 nm synthesized by the wet precipitation technique by using 94 GHz pulsed EPR. Origin of the observed paramagnetic centers is discussed. Supported by the electron microscopy and microanalysis, it is shown that EPR spectra from the calcified biological tissues correlates with those obtained in inorganic hydroxyapatites. The hypothesis about the important role of (nano)hydroxyapatite in formation of the mineral deposits and atherosclerotic plaque instability is further sustained.

1. Introduction

Interest to this study is governed by the search for reliable prognostic markers for atherosclerotic plaque development. *Plaque* (also known as *atheroma*) stands for fatty deposits within the coronary arteries and other large arteries in arteriosclerotic cardiovascular diseases (ASVD). ASVD is a condition in which an artery within the heart wall thickens and hardens. According to the current understanding, a large number of diseases with totally different clinical signs are basically atherosclerosis related: myocardial infarction, stroke, abdominal aneurysms, lower limb ischemia, etc. The mortality rate in Russian Federation from the diseases of the blood circulatory system is reported to be about 800 on 100 000 (2009) [1-3].

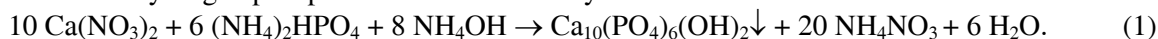
Most of the acute manifestations of atherosclerosis share a common pathogenetic feature: rupture of an atherosclerotic plaque. Atherosclerotic plaque contains multiple components including lipidic, fibrotic, thrombotic, and calcific materials. The calcium deposits in cardiovascular tissues are comprised of various calcium salts, such as calcium carbonate, calcium oxalate, calcium phosphate, calcium pyrophosphate, and hydroxyapatite (HAp). It was proposed that the microcalcification is tightly connected with the instability of the plaque, leading to the plaque rupture [4]. Namely, as it was assumed in [5], it goes hand-to-hand with the presence and size of the HAp component in the organomineral matrix of aorta deposits and, therefore, the nanosized HAp components of the aorta tissues could serve at least as markers for the early diagnosis of the plaque rupture risks.

To support or kick out the last hypothesis, in present work we use the advantages of the increased resolution and sensitivity of high-frequency (94 GHz) electron paramagnetic resonance (EPR) together with energy dispersive spectrometer (EDS) analysis combined with the scanning electron microscopy (SEM) to compare the results from the samples of aorta walls from male patients with atherosclerosis at different stages with those from the modified synthetic HAp.



2. Materials and Methods

Plaque tissues of aorta walls from male patients with atherosclerosis at different stages were gathered in Interregional Clinic and Diagnostic Centre, Kazan, Russia following the rules and under control of the responsible Ethics Committee. Sample preparation was the same as described in paper [5]. The HAp-based powders with the average size of crystallites of 30 nm were synthesized in the group of V.I. Putlyaev (Department of Material Sciences, Moscow State University, Moscow, Russia) by the wet (precipitation) chemical procedure involving the aqueous solutions of calcium nitrate, diammonium hydrogen phosphate and ammonium hydroxide:



X-ray diffraction (XRD) spectra show that all the synthesized samples contain only one phase corresponding to the space group $P6_3/m$ with the parameters of the unit cell close to $a \approx b \approx 0.942$ nm and $c = 0.688$ nm typical for the bulk crystals of the hydroxyapatite [6]. Corresponding EDS investigations ensure the ratio $\text{Ca/P} = 1.66(2)$ within the accuracy of our measurements in the powder grains serving as an evidence of the stoichiometry of the investigated HAp species. Details of the synthesis of the HAp samples and their modifications containing manganese and carbonate ions, post-synthesis treatment and results of the analytical characterization are described in [7-9].

The micromorphology of the biological samples, their Ca- and P- contents was examined and defined by scanning electron microscopy (SEM, Merlin, Carl Zeiss) combined with the electron dispersive spectrometer (EDS, AZtec, Oxford Instruments) in the group of Yu.N. Osin (Interdisciplinary Center of Analytical Microscopy, Kazan Federal University, Kazan, Russia). The samples were coated by Au/Pd = 80:20 thin film of about 20 nm, (registration conditions: 20 kV, 300 pA, imaging mode: detectors SE, Inlens, AsB, EDS).

To create stable paramagnetic centers in EPR silent samples, X-ray irradiation of the species was performed using URS-55 tube ($U = 55$ kV, $I = 16$ mA, W-anticathode) at room temperature with the estimated dose of 10 kGy to have an ability to apply different EPR techniques. XRD patterns before and after X-ray irradiation were checked in Bruker D2 Phaser diffractometer (Bruker, Germany) and were the same.

Pulsed EPR done in high-frequency (94 GHz, W-band) Bruker Eleksys 680 spectrometer (Bruker, Germany). EPR spectra were recorded by means of field-swept two-pulse echo sequence $\pi/2$ - τ - π with the minimal pulse length of π pulse of 4 ns and time delay $\tau = 220$ ns.

3. Results and Discussion

Strong correlation between the calcification of the aorta samples and possibility to obtain EPR was revealed in our experiments. EPR spectra in solid samples at room temperature were obtained only in the calcified tissues. Figure 1 presents the micromorphology of the sample that gives the detectable EPR signals before as well as after X-ray irradiation. Some segments were chosen for the EDS microanalysis (marked by squares in figure 1). C, O, Ca and P were detected there. While the content of Ca reached up to 25 wt %, the ratio Ca/P (in at %) for them was found to be in the range (1.80-2.20). The last values are slightly higher than those for the stoichiometric hydroxyapatite ($\text{Ca/P} = 1.66$) and are within the range commonly reported for the biogenic carbonated hydroxyapatite (1.70-2.30; see [10] and references therein). Thus one could expect the obtained values to suggest that (10-20) % of PO_4 groups in HAp are substituted by the carbonate, even though the presence of the other forms of calcium phosphates in the studied samples also should not be excluded. In our opinion, this finding could serve as a piece of evidence that the investigated aorta tissues contain (hydroxy)apatite like material. Therefore, we have supposed that the EPR signals are due to some paramagnetic centers incorporated into the HAp. (An example of SEM and EDS for the sample from the aorta wall that gives a low-intensive EPR signals is presented in accompanying paper [11]).

Different paramagnetic centers could be detected in the synthetic and natural hydroxyapatite based materials but only few of them in the HAp of biogenic origin, especially without X-ray irradiation. Figure 2 shows the electron spin echo (ESE) signal detected during the magnetic field sweep (field-sweep ESE) in W-band at room temperature of atherosclerotic plaque and synthesized

$\text{Mn}_{0.0005}\text{Ca}_{9.9995}(\text{PO}_4)_6(\text{OH})_2$ (MnHAp) powder with the average grain size of 30 nm. The obtained six-line pattern signal is undoubtedly stemmed from the hyperfine interaction between electron ($S = 5/2$) and nuclear ($I = 5/2$) spins of the incorporated manganese (Mn^{2+}) in non-oriented powders. Taking into account the accuracy of our experiments originating from the different sources of the magnetic field inhomogeneity at the sites of the studied paramagnetic centers, the closeness of the observed spectroscopic parameters (g-factor of 2.001(1) and hyperfine splitting constant of 9.3(3) mT) points out that manganese in aorta samples is most probably located in HAp component of the plaque organomineralic matrix.

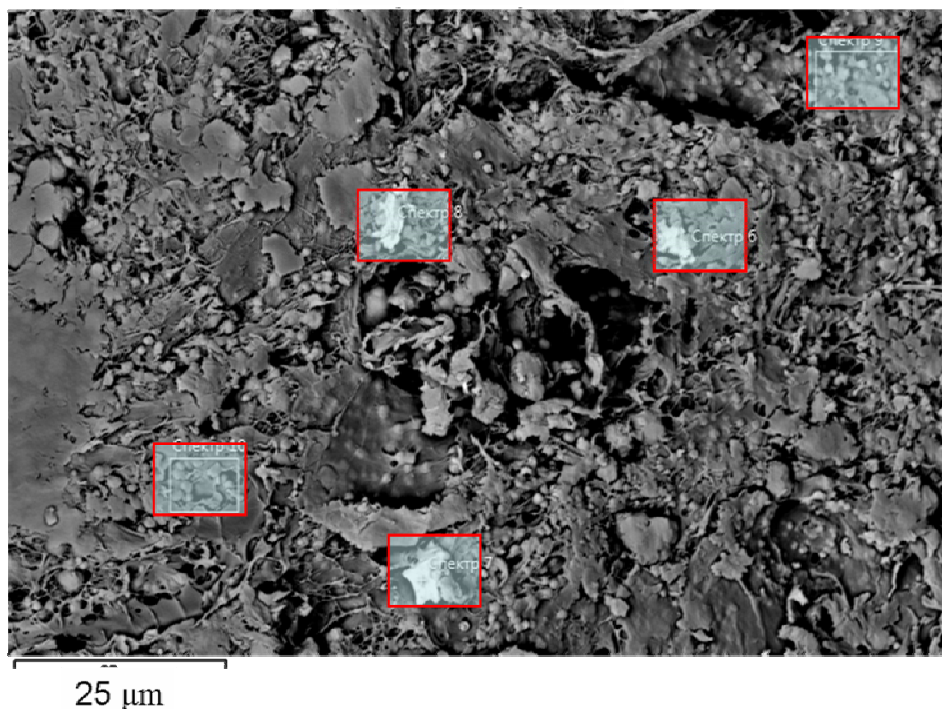


Figure 1. Electron microscopy of the part of the calcified deposit from the aorta wall. The squares mark the segments chosen for the microanalysis.

The small concentration of manganese neither in biological tissues nor in synthetic HAp was observed by the microanalysis or by the conventional X-band (9 GHz frequency) EPR. To detect the trace amounts of elements other methods of atomic spectroscopy (like AAS – atomic absorption spectroscopy) often linked with the mass-spectrometry are usually applied [12-14] which are initially the destructive techniques. In this sense, the presented results emphasize the advantages of high-frequency EPR as a sensitive, requiring very low (of less than 1 μg) amount of tissue, non-destructive tool to study different paramagnetic species in different materials, their (electronic) structure as well as a variety of types of interactions between them [15, 16].

After X-ray irradiation, in contrary, a variety of radiation-induced defects could be created which EPR spectra might overlap each other complicating, therefore, their interpretation [6, 17]. In this context, by choosing the appropriate parameters of the detection in the pulsed mode (such as a temperature, pulse lengths, etc.), one can distinguish between the paramagnetic species with different relaxation times. In such a manner we could manage to separate the carbonate spectra which are presented in figure 3. The spectra are due to the most stable and long-relaxing CO_2^- radical [5, 17]. As a reference, the carbonated HAp with the carbonation level of about 13(1) wt % was taken.

The relationship between a few percent carbonate and its location in the lattice structure of apatite-like materials is still a “bone of contention” both from the theoretical and experimental points of view [7, 17], while the location of manganese ions is believed to be determined perfectly [7]. We hope that

our further experiments could give a key to experimental determination of carbonate location by advanced EPR methods [5, 7, 16, 18].

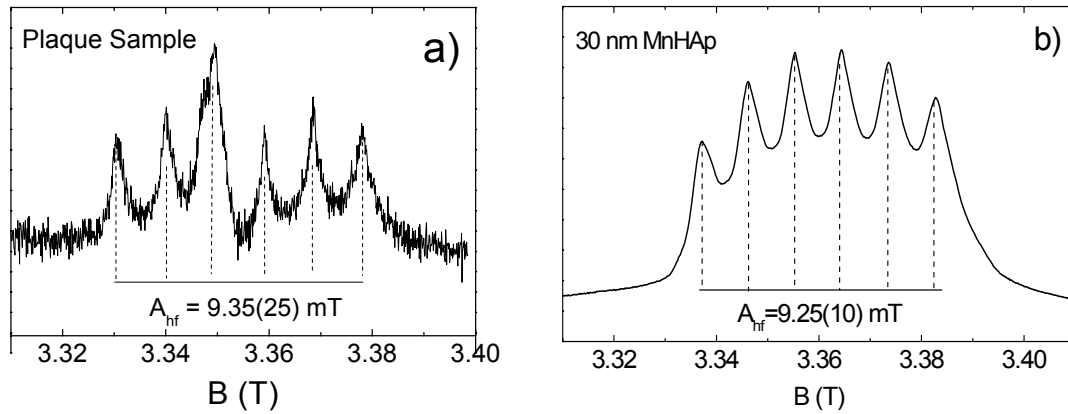


Figure 2. ESE-detected EPR spectra of the atherosclerotic plaque (a) and of the nano-MnHAp (b) at $T = 293$ K.

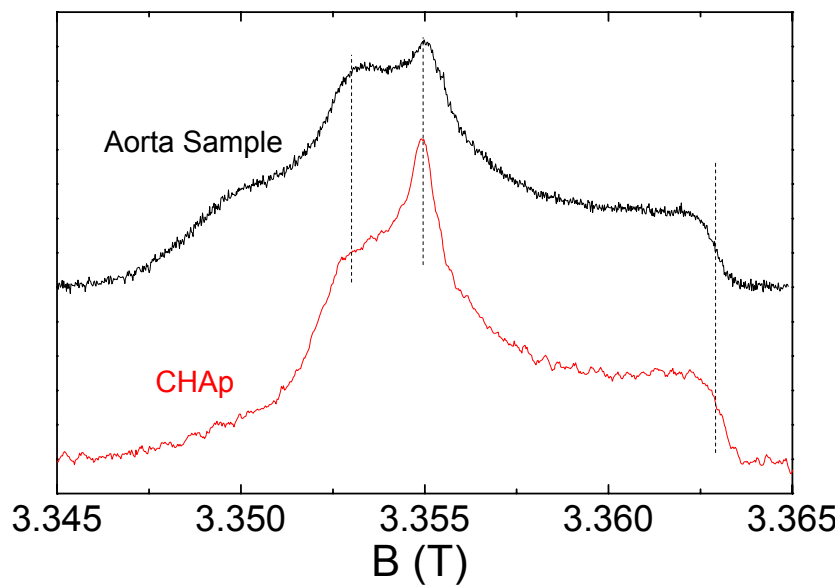


Figure 3. ESE-detected EPR spectra of the long-relaxing CO_2^- radiation-induced radical in aorta sample (upper curve) and in the carbonated HAp (CHAp, lower curve) at $T = 293$ K.

4. Summary

1. A high sensitivity of modern commercially available high-frequency spectrometers along with the pulsed methods open new horizons for using EPR methods in biomedical research. In particular, for early ASVD diagnosis.
2. EPR of manganese ions (Mn^{2+}) in the atherosclerotic plaques was detected. It is shown that the

spectroscopic parameters of Mn^{2+} ions are practically the same for the investigated atherosclerotic plaques and synthesized HAp nanoparticles.

3. EPR of the radiation induced CO_2^- radical in the atherosclerotic plaque was separated. The spectroscopic parameters of the radical are revealed to be the same as in the carbonated HAp.
4. A correlation between the calcification of the aorta wall deposits and intensity (possibility to detect) of the mentioned above EPR signals is observed.

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