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INTERACTION BETWEEN METHEMOGLOBIN AND SOME BIOGLASS SYSTEMS STUDIED BY EPR SPECTROSCOPY

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In this work were analyzed continuous wave X-band EPR spectra of methemoglobin spin labeled in position β-93, before and after interaction with bioactive glasses. The aim of this study is to use EPR SDSL for study the binding of methemoglobin on bioactive glass surfaces. EPR spectra recorded in solution can be compared directly to those obtained after adsorption to extract structural information. A consistent analysis of EPR spectra revealed that the adsorption of methemoglobin is influenced by the chemical composition of the bioactive glass. A fitting of the different spectral components was carried out, addressing the possible origin of those components.

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1. Introduction

The adsorption of proteins on solid surfaces is one of the most studied subjects in the field of development of biocompatible materials for implants and tissue engineering [1,2]. Protein adsorption is known to be the first biological event at the surface of an implant after implantation. All the other reactions to or from the biomaterial will be influenced by these interactions and the first step in evaluating the blood and tissue compatibility of any medical device is to study its behavior in terms of interactions with proteins. The structure of the protein in the adsorbed state is an important piece of information, knowing that the inflammatory responses are caused by the uptake and concomitant denaturing of proteins at the surface of the biomaterials [3-5].

Bioglass and other bioactive ceramics have an excellent bone-bonding capability due to their ability to deposit hydroxyapatite, which has a high capacity to bind proteins [6, 7]. The use of protein coupling agents allows the control of protein release kinetics and maintains almost completely the native protein structure [8, 9]. However, the bioactive function of bioactive glasses can be hampered by crystallization [10]. Therefore, it is necessary the chemical treatment applied to the surface for maintaining the protein-binding ability of the bioglass [11]. Specifically, the bioglass systems were silanized in 3-aminopropyl-triethoxysilane (APTS) [12] and after that the protein coupling agent, glutaraldehyde (GA) was attached. Each APTS molecule introduces one Si atom to the surface. After surface modification the reference and modified samples exhibit a different Si content, the ratio being 1:2, which verifies the successful surface silanization [13]. The coupling of GA to the surface was confirmed by a typical reddish color of the samples obtained after GA treatment.

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The APTS presence on a surface is known to be the prerequisite for the successful GA coupling. It has been shown in the literature [12, 14] that GA has a very high affinity to APTS. On average it is assumed that one GA molecule docks to one APTS molecule. Glutaraldehyde is known to be cytotoxic if not bound and it can be replaced by other coupling agents such as genipin for *in vitro* and *in vivo* studies [15].

The interaction of proteins and surfaces involves both protein binding and unfolding. There were used many methods to study this subject, like crystallography and NMR spectroscopy (limited to special cases [16]), surface-sensitive vibrational spectroscopy [17-19], fluorescence spectroscopy [20, 21], circular dichroism [22], surface force measurements [23] and microscopy [24] and neutron reflectivity [25].

The method of site-directed spin labeling (SDSL) in combination with electron paramagnetic resonance (EPR) spectroscopy was chosen because it has the advantage that it can provide information about the structure and conformational dynamics of the proteins under conditions close to the physiological state of the system under investigation. In this technique, a spin label side chain is introduced at a selected site via cysteine substitution mutagenesis followed by modification of the unique sulfhydryl group with a specific paramagnetic nitroxide reagent [26]. The continuous wave (cw) EPR spectrum yields information about the nitroxide side chain mobility, the solvent accessibility, the polarity for its immediate environment, and the distance between the nitroxide and another paramagnetic center in the protein [26, 27]. The mobility analysis is based on the fact that the room temperature EPR spectral shape is sensitive to the reorientational motion of the nitroxide side chain due to partial motional averaging of the anisotropic components of the g- and hyperfine tensors. For the spin-labeled sites exposed to the solution, the nitroxide mobility refers to rotational correlation times of the nitroxide in the ns range, resulting thus an EPR spectra characterized by small line widths of the center lines and small apparent hyperfine splitting. If the mobility of the spin label side chain is restricted by interaction with neighboring side chains or surfaces, the line widths and the apparent hyperfine splitting are increased [28].

Until now, EPR was poorly used for studying the interaction between proteins and solid surfaces. There are few studies where the method was used to investigate the proteins adsorbed to planar lipid bilayers [29, 30], and one study which investigates the partial unfolding of lysozyme on quartz [31]. In these studies, the authors suggested proved that this technique can monitor successfully the structural changes of a protein adsorbed to a solid surface.

2. Experimental

Bioglass preparation

Three bioglass systems were used in this study, all of them were prepared by sol-gel method. The first two systems have the same composition (in weight %): 55%SiO_2, 41%CaO, 4%P_2O_5, but they suffered different drying processes. The starting reagents for both systems were (TEOS), $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2{\cdot}HPO_4$. The first system (will be named from further on as “system 1”) was aged for 70 days at room temperature and after that it was heat treated at 310°C for 1 hour in air. The second system (will be named from further on as “system 2”) was dried at 80°C for 50 minutes, then aged for 70 days at room temperature and after that it was heat treated at 310°C for 1 hour in air. The third system (will be named from further on as “system3”) used in this study was 45S5 Bioglass®, with the following composition (in molar %): 45% SiO_2, 24.5% Na_2O, 24.5% CaO, 6% P_2O_5. As starting reagents were used (TEOS), $Ca(NO_3)_2{\cdot}4H_2O$, $(NH_4)_2{\cdot}HPO_4$ and Na_2CO_3. The sample was aged for 30 days at room temperature, and then treated at 310°C in air for 1 h. In the end, the bioglasses were milled to obtain fine powders.

After preparation, the samples were chemically treated by immersion into an aqueous APTS solution (0.45mol/L, pH adjusted to 8) at 80ºC. After 4h the samples were taken out, cleaned in deionised water, then immersed for 1h in GA solution (1mol/L) at ambient conditions and finally cleaned again in deionised water[11, 13].

Protein adsorption

Powder samples were incubated for 4 hours at 37°C in 3.22 mg/ml (or 0.05mM) solution of methemoglobin in phosphate buffer (0.01M, pH 8.0) with a salt concentration of 0.5M of NaCl, for 4 hours. After the immersion they were washed three times with the buffer solution.

EPR SDSL spectroscopy

Horse methemoglobin provided from Sigma-Aldrich was spin-labeled with (4-(2-Iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy) free-radical in the position β-93 (in this position horse methemoglobin has a native cysteine).

For the X-band continuous wave EPR measurements, sample volumes of 15 µl were filled into EPR glass capillaries with 0.9 mm inner diameter. Room temperature (296–299 K) continuous wave (cw) EPR spectra were recorded using a Magnettech Miniscope MS200 X-band spectrometer equipped with a rectangular TE102 resonator, with the microwave power set to 10 milliwatts, and B-field modulation amplitude adjusted to 0.15 mT.

The simulation of the X-band cw EPR spectra presented here was performed with Multicomponent299 by Christian Altenbach [32].

The g-values and the A-values were obtained from the W-band continuous wave (cw) EPR spectrum of the hemoglobin, recorded at 166 K. The high-field continuous wave (cw) EPR measurement was performed on a home-built W-band EPR spectrometer (3.4 T, 95 GHz, cw) equipped with a TE_{011} cavity whose setup was described elsewhere [33]. The sample suspension was filled into quartz capillaries with an inner diameter of 0.6 mm (VitroCom Inc., N.J., USA). The presented spectrum is the average of eleven measurements and each was detected with a 0.02 mT resolution. The simulated powder spectrum was fitted to experimental spectra using a modified version of the program DIPFIT [34].

3. Results and discussion

Hemoglobin is a globular protein with a molecular mass of 66.5 kDa, composed of four polypeptide chains (Alpha 1, Beta 1, Alpha 2, Beta 2), which are structurally similar, each binding one heme group (Fig. 1). Most of the amino acids in hemoglobin form alpha helices, connected by short non-helical segments. The hemoglobin protein consists of four polypeptide chains: two alpha types and two beta types. Each protein chain subunit contains a heme group with the iron attached. Each iron ion binds reversibly with one oxygen molecule, so each hemoglobin molecule can bind to a total of four oxygen molecules. Maintenance of this heme iron in the reduced state (Fe^{2+}) is imperative for oxygenation to occur; oxidation of the iron into Fe^{3+} can result in the formation of methemoglobin and, subsequently, inhibit the oxygen-binding capabilities of the protein.

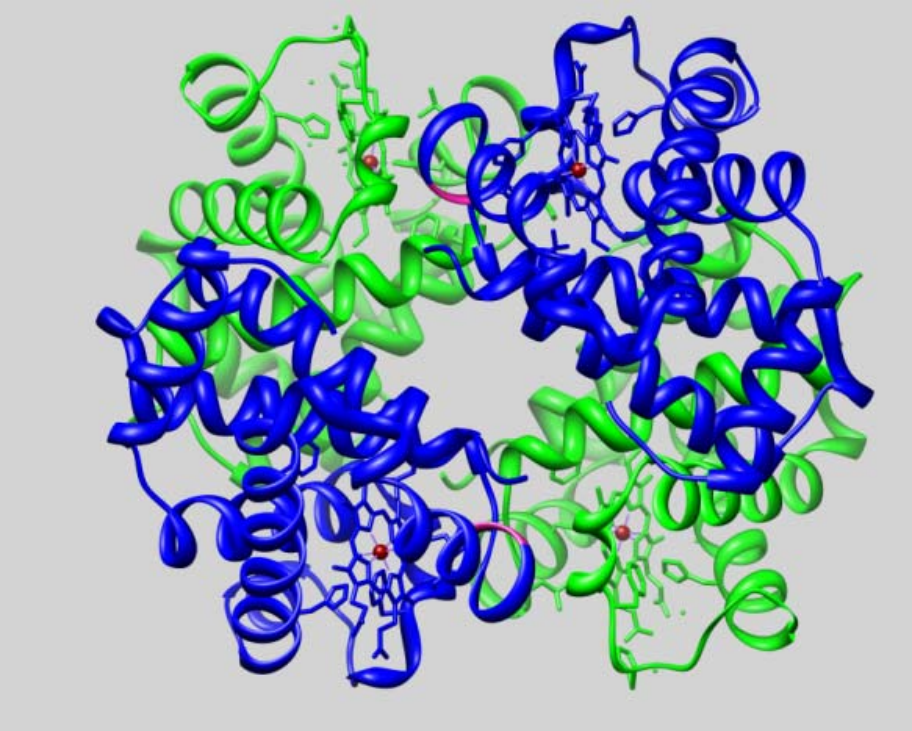

Fig. 1. Structure of methemoglobin obtained by X-ray crystallography. The α-chains are colored in green and the β-chains are colored in blue. The iron from the heme group is colored in dark red. The native cysteins from positions β-93 are colored in magenta.

Horse methemoglobin has two native cysteins in position 93 from the two β-chains, which were labeled with (4-(2-Iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy) free-radical. Horse methemoglobin has other two native cysteins, in position α-104 from the two α chains, but this position is buried in the interior of the protein, thus being less accessible to the spin labels.

The sulphydryl group of cysteine β-93 is in equilibrium between two alternative positions: one external and the other half-buried in the "tyrosine pocket" between helices F and H. The sulphydryl group of Cys swings around the Cα-Cβ bond from a mainly external position in methemoglobin to a less accessible one in a pocket between helices F and H, from which it displaces the side chain of Tyr [35,36]. This position is thus located inside the protein but close to the surface, therefore is suitable for studying the interaction between the protein and the surface of the bioglass.

Fig. 2 shows the X-band cw-EPR spectra of spin-labeled 93*methemoglobin in solution (black line) and in the adsorbed state on the three bioglass system investigated (red line). All spectra exhibit a typical signal-to-noise ratio.

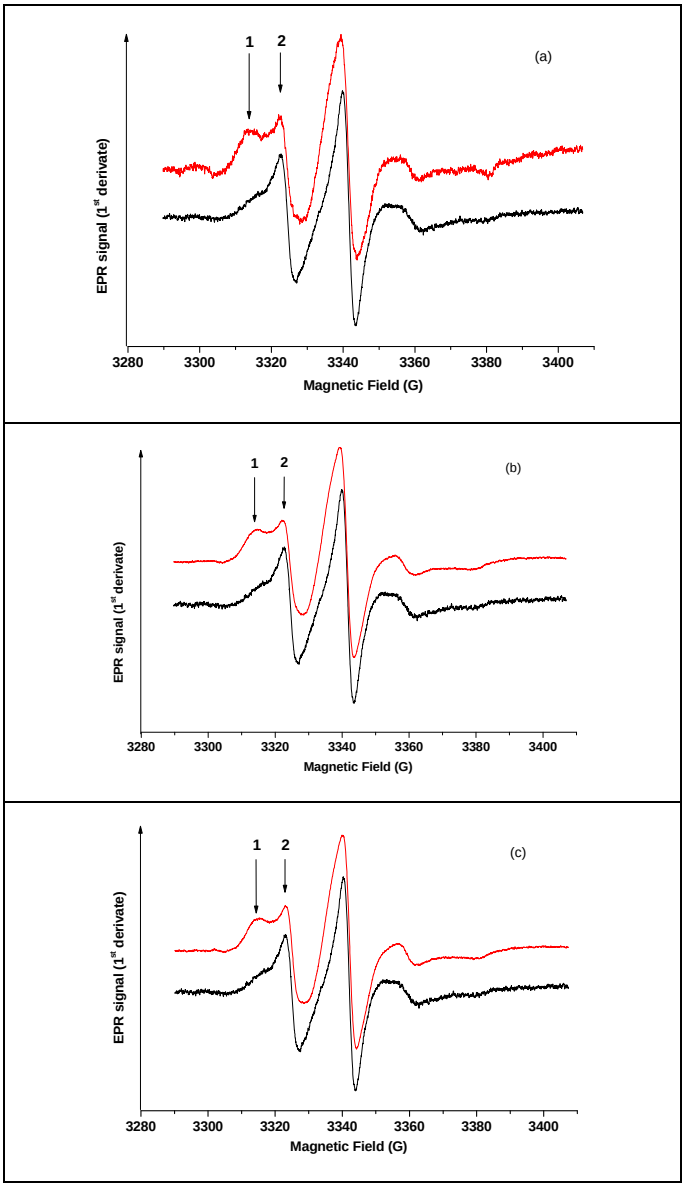

Fig. 2. X-band EPR spectra of horse methemoglobin spin-labeled in position β-93 in aqueous solution (a, b, c the black lines), and in the adsorbed state (red lines) on system 1 (a), 2 (b), and 3 (c). The immobile and mobile components are indicated by arrows 1 and 2 respectively.*

In all the EPR spectra we can identify two components, representing two populations of spin labels, with different orientation: one which is strongly immobilized (arrow 1 in the spectra, fraction f_1) and one which is free on the surface of the molecule and is weakly immobilized (arrow 2 in the spectra, fraction f_2). The origin of these two components is the swinging of the spin label between the two alternative positions: the external one, from the surface of the protein, and the half-buried one, in the "tyrosine pocket" between helixes F and H. One can see that there are significant differences between the spectra recorded in solution and the spectra recorded in the adsorbed state. In the adsorbed state the immobile component increases while the mobile component decreases, compared with the spectra recorded in solution (Fig. 2). This is a clear indication that the fraction of populations of the immobilized spin labels is higher in the adsorbed state, proving that the protein binds to the bioglasses surfaces, in all three cases. The prominent change of the line shape between the spectra

recorded in solution and the spectra recorded after adsorption, for all three systems, indicates also that the environment of this residue 93* is strongly perturbed by the adsorption process.

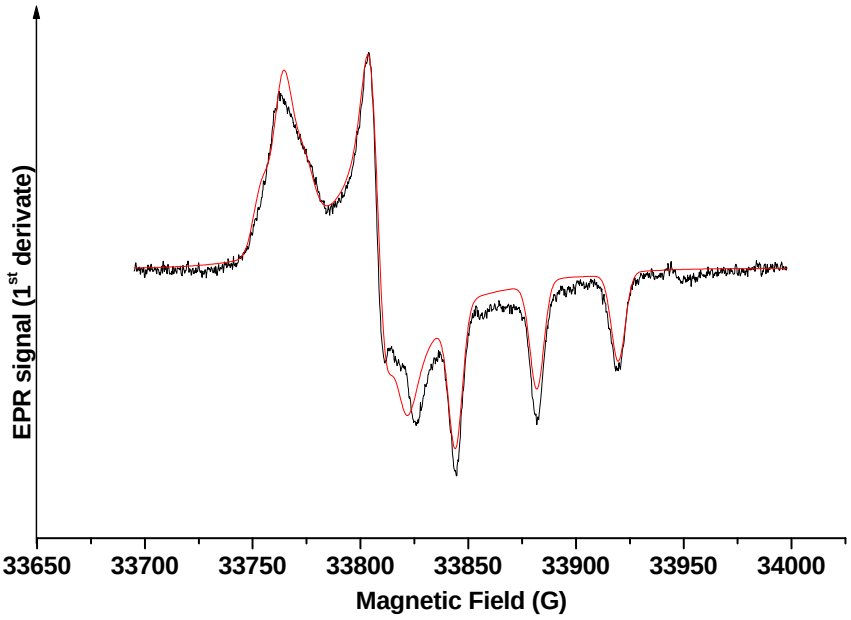

Fig. 3. Rigid-limit experimental high-field EPR spectrum (continuous lines, first-derivative representation, T = 166 K, ν = 95 GHz) for methemoglobin. Red line represents the calculated line shape obtained by a modified version of the program DIPFIT (fittings were performed with two sets of g_{xx} parameters and only one set for g_{yy}, g_{zz} and A-tensor parameters).

For fitting the spectra recorded in X-band it was needed to find out the proper values of the g- and A- tensor. These values were determined from the W-band cw-EPR spectrum of methemoglobin (the sample was prepared with the same concentrations as for protein solution used for the immersion of the bioglasses), recorded at 166K (Fig. 3). In this temperature regime (between 160-175K) the dynamics of proteins exhibit glasslike behavior, and the reorientational correlation time of an otherwise unrestricted spin label side chain exceeds 100 ns [37], i.e., the nitroxide may be considered as immobilized on the EPR time scale. Hence, g- and A-tensor components may be determined by fitting simulated powder spectra to the experimental spectra [38].

W-band EPR simulated powder spectrum was fitted to experimental spectra using a modified version of the program DIPFIT [39]. Parameters varied during fitting were the g- and A-tensor components, and the orientation-dependent line width parameters of a Voigtian line shape. The following values were obtained by W-band EPR spectroscopy for g- and A-tensor components of spin-labeled methemoglobin: g_{xx1} = 2.00941, g_{xx2} = 2.00880, g_{yy} = 2.00614, g_{zz} = 2.00217, A_{xx} = 0.52 mT, A_{yy} = 0.45 mT, A_{zz} = 3.774 mT.

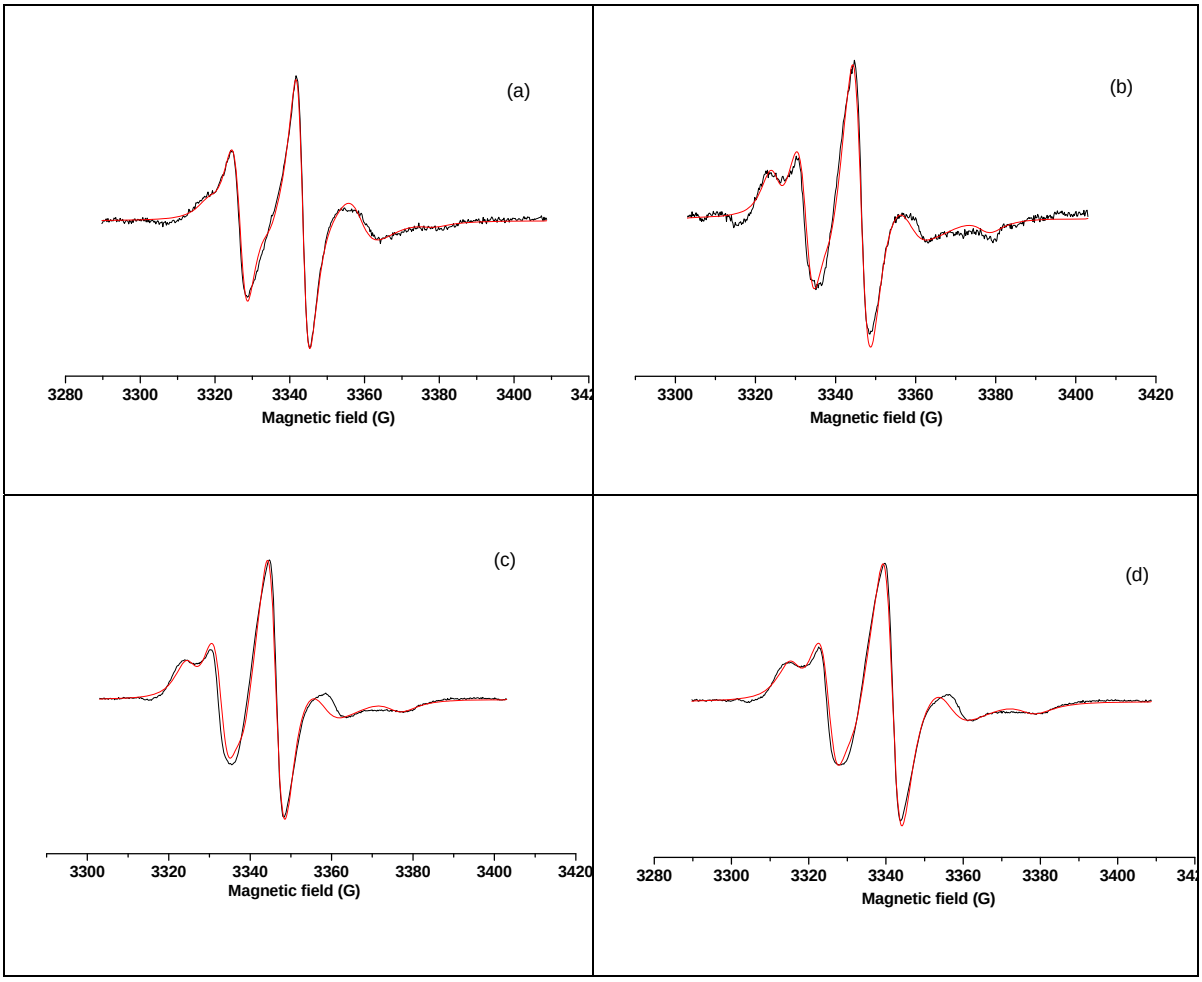

Fig. 4. X-band EPR spectra of methemoglobin spin-labeled in position β-93 in solution (a) and in the adsorbed state on bioglass system 1(b), system 2 (c), and system 3 (d). The red lines represent the best fits of simulated spectra to the experimental ones, using the g and A-tensor values determined by W-band EPR spectroscopy.*

These values were used further to fit the X-band cw-EPR spectra obtained for methemoglobin in solution and in the adsorbed state, for all the three bioglass systems. The features observed in the X-band EPR spectra are well reproduced by the fits shown in Fig. 4. The fractions of the two spin label populations (Table.1) were calculated from the simulated spectra and one can see that they are clearly different in the solution compared with the adsorbed state.

For all three systems the number of the weakly immobilized spin labels is decreasing, while the number of the strongly immobilized spin labels is increasing, but there are also differences induced by the chemical composition of the bioglass system.

In order to check the stability of the protein bound to the bioglass, after 30 days of storing at 4°C the samples were taken out and ultrasonicated for 45 minutes. After ultrasonication, the samples were washed three times with buffer solution, to remove the protein which was eventually detached from the surface during the ultrasonication. There are no (or slightly) differences between the spectra recorded right after immersion and the spectra recorded after ultrasonication (Fig. 5.), some of them may be due to the poor signal-to-noise ratio in the spectra recorded after ultrasonication, proving the high binding stability of methemoglobin on all three bioglass systems.

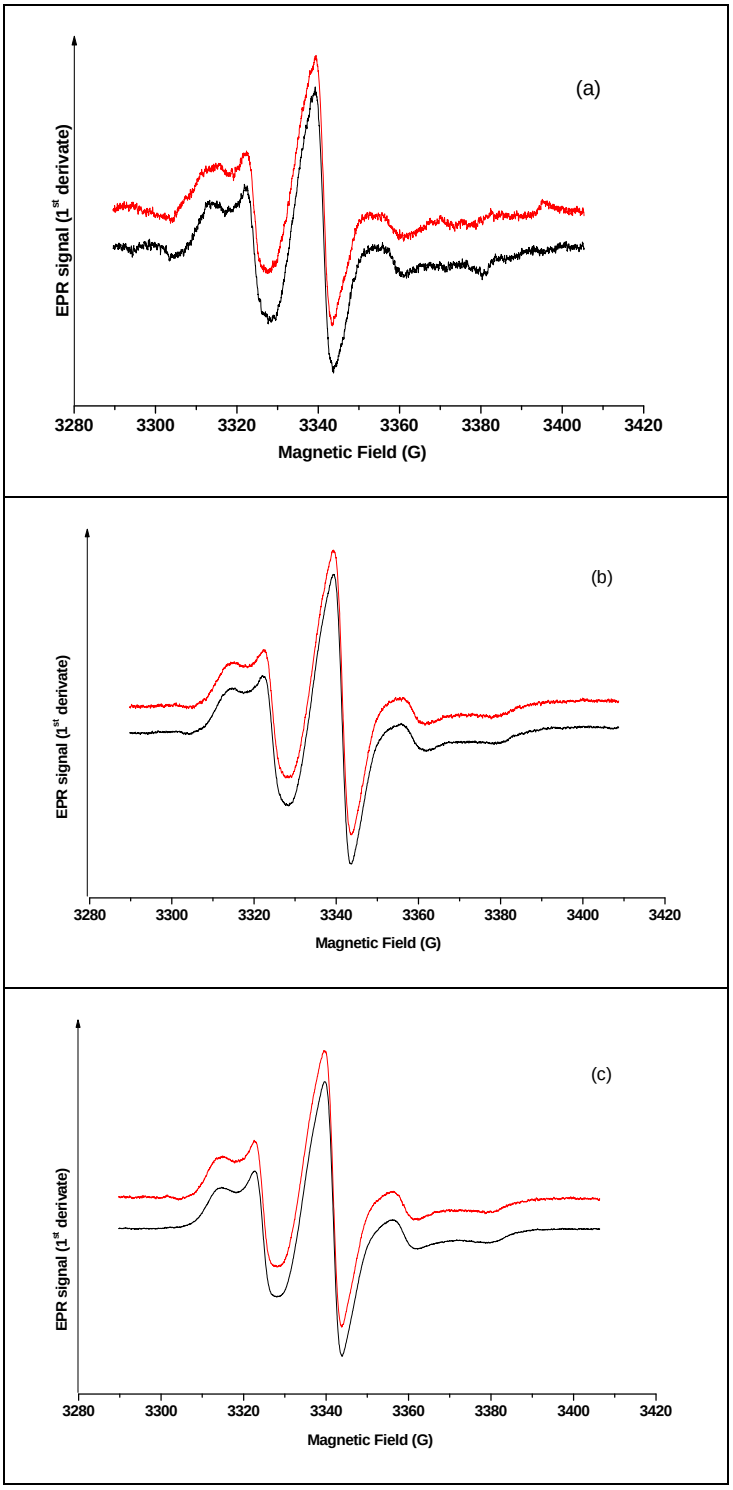

Fig. 5. X-band EPR spectra of horse methemoglobin spin-labeled in position β-93 in the adsorbed state on system 1(a), 2(b), and 3(c) right after immersion (the black lines), and after ultrasonication (the red lines).*

Table. 1
Values of the mobile and immobile fractions of populations for the spin-labels in methemoglobin β-93 in solution and in adsorbed state, determined by X-band EPR spectroscopy.*

Methemoglobin	$\mathbf{f_{mobile}}$ **(%)**	$\mathbf{f_{immobile}}$ **(%)**
in solution	72.0	28.0
adsorbed on system1	63.3	36.7
adsorbed on system 2	52.9	47.1
adsorbed on system 3	51.3	48.7

In a study on the adsorption of proteins to organic siloxane surfaces it was found that methemoglobin shows an enhanced adsorption [40]. They explained this based on the fact that hemoglobin displays relatively high surface activity. The methemoglobin form of the protein generated due to replacement of ferrous heme with its ferric state may be the reason for its enhanced adsorption because it was found that the hemoglobin mutants show increased surface activity compared to the normal protein. They also found that, in spite of its pronounced surface activity, the protein is less tightly held on organosiloxane surfaces. On the investigated bioglasses, on contrary, we found that the protein remains bound even after 30 days, and even after the samples were washed again with buffer and kept for 45 minutes in ultrasonic bath, denoting that the protein is irreversible bound to the bioglass.

4. Conclusions

In this work X-band EPR measurements were carried out to study the adsorption of methemoglobin on three different bioglasses. The EPR spectra revealed structural changes of the proteins and its surroundings in the adsorbed state for all three bioglasses investigated, but it seems that the well-known 45S5 Bioglass® has the highest affinity for the binding of methemoglobin.

X-band EPR spectra recorded for spin-labeled methemoglobin adsorbed on bioglasses revealed that in the adsorbed state the immobile component increases, proving that the protein binds to the bioglass in all three cases. The broad immobile component of the spectra can be explained by an interaction of the spin label with the surface or adjacent unfolded parts of protein and it also suggests that the environment of the β-93* residue is strongly perturbed by the adsorption process. We can conclude that this region of the protein has an important role upon interaction with the bioglass surface.

The EPR results also proved that the protein is irreversible bound to these bioglasses.

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