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EPR study of self-organized magnetic nanoparticles in biomaterials

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Abstract. An innovative approach based on the effect of bio-mineralization as a response reaction of cells to decrease their damage under stress was applied to *Juniperus communis* (JC) and JC-based biomaterials (Nefrovil and Immunostan drugs with properties increasing the antioxidant activity and improving the immune system of human organism, respectively). Electron paramagnetic resonance spectroscopy (EPR), also called electron spin resonance (ESR), was used as the main experimental tool for detecting paramagnetic species resulted from the existence of antioxidant activity system, represented by superoxide dismutase with manganese, catalase *etc*., as well as formation of superparamagnetic iron oxide nanoparticles (SPIONs). The influence of temperature and microwave power on the intensity of EPR signals detected in JC, Nefrovil and Immunostan was examined. Obtained *g*-factor values of EPR signals from JC shell and seeds as well as from Nefrovil and Immunostan were attributed to the paramagnetic species of Mn (g = 2.0), Fe_3O_4 SPIONs (g = 2.17…2.60), Fe aggregates (g = 3.22…3.94) and Fe^{3+} ions (g = 4.3). The EPR signals from SPIONs and Fe^{3+} ions in Immunostan were found to be fully correlated, showing an additional experimental evidence of the bio-mineralization effect (*i.e*., transformation of Fe^{3+} ions to Fe_3O_4 SPIONs). The results of the EPR study of Fe_3O_4 SPIONs incorporated into polymer matrix were taken into account in comparative analysis. The results reported in the present work support well self-organization of magnetic nanoparticles in the investigated biomaterials.

Keywords: EPR, self-organization, superparamagnetic iron oxide nanoparticles, biomaterials.

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1. Introduction

Recently, an innovative approach based on the effect of bio-mineralization [1–4] as a response reaction of cells to decrease their damage under stress was applied to *Juniperus communis* (JC) of the Carpathian region of Ukraine and related biomaterials, namely, Nefrovil and Immunostan drugs, prepared by Roslyna Karpat Co., Ltd (Ukraine) [5–9]. Existence of such antioxidant enzymes as catalase and superoxide dismutase (SOD) [10], containing manganese (MnSOD), copper (CuSOD), zinc (ZnSOD), iron (FeSOD), and nickel (NiSOD) in *Juniperus communis*, makes the former model biomaterial objects with natural externally-attained functionality that may be useful for biomedical applications. Moreover, Nefrovil and Immunostan drugs were prepared to increase the antioxidant activity and to improve the immune system of human organism, respectively. Therefore, they may also serve as the model objects. Electron paramagnetic resonance spectroscopy (EPR) or electron spin resonance (ESR) method was used as the main experimental tool for detecting paramagnetic species resulted from the existence of antioxidant activity system represented by MnSOD as well as formation of superparamagnetic iron oxide nanoparticles (SPIONs) [8, 9].

In this work, the influence of temperature and microwave power on the intensity of EPR signals detected in JC, Nefrovil and Immunostan has been studied. A good correlation of EPR signals from SPIONs and Fe^{3+} ions in Immunostan is found, which provides an additional evidence of the bio-mineralization effect (*i.e.*, transformation of Fe^{3+} ions to magnetite (Fe_3O_4) SPIONs) in the investigated biomaterials.

2. Experimental

JC samples were taken from the Carpathian region of Ukraine. The JC based Nefrovil and Immunostan drugs were used as provided by Roslyna Karpat Ltd. (Ukraine). ESR spectra of the biomaterials studied were registered using the ESR spectrometer ECS-106 (Bruker, Germany) in the X-range under the following conditions: HF-modulation amplitude of magnetic field of 0.5 mT, microwave power of 20, 40, 80 and 126 mW, field center of 260 mT, field scanning of 290 mT, temperature of 293 and 77 K, and gain of 10^4 and 5×10^5, respectively. Micro-/nanoscopic structure of samples was observed using the scanning electron microscope (SEM) ZEISS EVO 50.

3. Results and discussion

EPR spectra of JC shell and seeds as well as their mixture along with a differential spectrum between shell and seeds are shown in Fig. 1a. It should be emphasized that two EPR signals are registered in biomaterials. Namely, the EPR signal at g = 2.01 is attributed to the paramagnetic species of Mn [3, 11], characterized by six-component hyperfine structure, in the Mn-containing enzymes. This signal is found only in JC seeds. At the same time, the broad ESR signal at g = 2.28 is attributed to the paramagnetic species of SPIONs [3, 12] and is found only in JC shell.

A narrow signal is observed in the center of the EPR spectrum corresponding to Mn (Fig. 1a). Due to the fact that the g-factor values of MnSOD and paramagnetic centers of photosystem are close, it is not possible to separate them in the EPR spectrum. In other words, change of the registration parameters such as power and modulation amplitude leads to a fail in registration of the typical EPR spectrum of MnSOD. Superoxide anion radical (SAR, $O_2^{\bullet-}$) is a dangerous molecule for living organisms, as it takes part in the process of one-electron reduction of oxygen [13]. SAR can attach one or two protons to form peroxide. The decay of the latter to a hydroxyl anion and OH radicals is catalyzed by copper and iron. Superoxide dismutase (SOD) is an enzyme that converts superoxide anion radical to oxygen and hydrogen peroxide. A MnSOD active site contains 5 manganese atoms, 3 of which are bound to histidine, one to aspartate residue and the rest one to a water molecule or an OH group. Numerous experiments proved the presence of a 4-nuclear Mn cluster in the active center of the photosynthetic apparatus of plants. This cluster is directly responsible for the release of oxygen. Complexes of metals with terminal oxido-ligands play an important role in various transformations, including those in high-valent manganese-oxide units at the stage of formation of O-O bonds during photosynthetic oxidation of water [14, 15]. Theoretically, presence of MnIV-oxyl radicals is also assumed, but such species were not observed experimentally. Using a combination of experimental measurements and theoretical calculations, it was demonstrated [14, 15] that the bond within a Mn oxido-group is best described as highly covalent with 0.45 spins on the oxido-ligand. These results explain the presence of putative high-valent manganese species in photosynthesis in the form of an energetically available high-spin MnV-oxide unit instead of MnIV-oxyl radicals. It may be suggested therefore that the narrow signal with g-factor of 2.01 is responsible for various paramagnetic centers, quinones, as well as dependent on reactive oxygen species (superoxide anion radicals, singlet oxygen, hydroxyl radicals) and carbon-centered ones (alkyls or hydroxyalkyls). The rate of formation of these radicals and, consequently, the rate of degradation of proteins and carbohydrates depend on the possibility of electron transfer from manganese cluster ions to Photosystem II [14, 15].

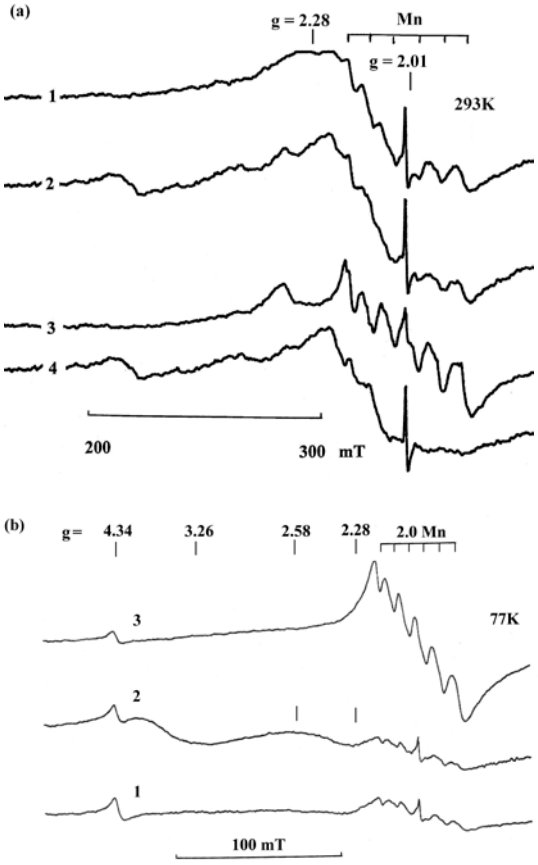

Fig. 1. EPR spectra of JC shell, seeds and their mixture measured at the temperature of (a) 293 K (*1* – mixture of shell and seeds, *2* – shell, *3* – seeds and *4* – differential spectrum (*2* and *3*)) and (b) 77 K (*1* – mixture of shell and seeds, *2* – shell and *3* – seeds). The estimated EPR signal widths are: g = 2.28, $\Delta H_{1/2}$ = 15.9…16.9 mT; g = 2.58, $\Delta H_{1/2}$ = 26.6 mT.

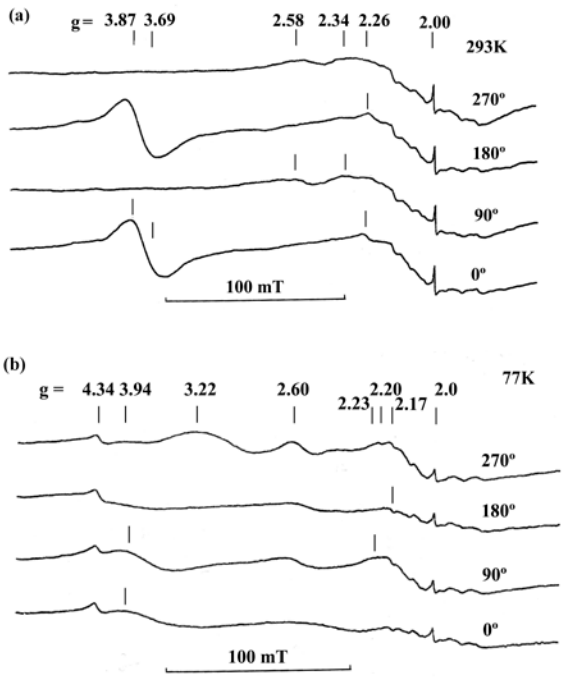

Fig. 2. EPR spectra of JC shell measured at different orientations of samples relative to the magnetic field (0°, 90°, 180°, and 270°) and the temperatures of (a) 293 K and (b) 77 K. The estimated EPR signal widths are: g = 2.58, $\Delta H_{1/2}$ = 26…32 mT; g = 2.60, $\Delta H_{1/2}$ = 12.1 mT; g = 3.22, $\Delta H_{1/2}$ = 34.5 mT; g = 3.87, $\Delta H_{1/2}$ = 17.2 mT; g = 3.94, $\Delta H_{1/2}$ = 29.3 mT.

SPIONs detected in the investigated materials have an important role in the development of pathological conditions. They are frequently used for biomedical applications [16–25] and in food science [26–28]. SPIONs also lead to the appearance of magnetic properties in plant systems and the emergence of EPR signals [3, 29–32]. The stress factors affecting JC shell are also directly confirmed by EPR detected SPIONs, which stems from the bio-mineralization effect under stress. In its turn, MnSOD, which indicates the antioxidant activity, is normally identified in JC seeds (Fig. 1a).

EPR spectra of JC shell, seeds and their mixture measured at low temperature are shown in Fig. 1b. As expected, the measurements at 77 K show the enhancement of the EPR signal from enzymes (g = 2.0) for the seeds. At the same time, the intensity of the EPR signal from SPIONs (g = 2.28) for the shell is reduced at 77 K. The intensity of the broad EPR signal at g = 3.26 for the shell is found to decrease at low temperature. In all dried soil samples, a broad EPR signal at g = 3.3 is observed at room temperature. The intensity of this signal decreases upon reducing the temperature down to 80 K [3]. Registration of the broad EPR signal at g = 3.3 in soil samples is attributed to high content of iron aggregates, including SPIONs [3]. As can be seen in the case of shell, behavior of the EPR signals at g = 2.28 and g = 3.26 at 77 K is quite similar, supporting the finding [3] that these both signals are related to SPIONs and iron aggregates (containing SPIONs). Besides, the weak signal of Fe^{3+} at g = 4.3 (recorded at 80 K in [3]) is also detected in JC samples at 77 K (Fig. 1b). The broad EPR signal at g = 2.58 is also assigned to SPIONs, since this signal is the closest to the one at g = 2.69 detected in the samples with magnetic nanoparticles [3].

Fig. 2 shows EPR spectra of JC shell measured at different sample orientations relative to the magnetic field (0°, 90°, 180° and 270°) at 293 and 77 K. It is clearly seen that at both temperatures the intensity of EPR signals from SPIONs and iron aggregates (g = 2.26, 2.38, 2.54, 3.69 and 3.87 at 293 K and g = 2.17, 2.20, 2.23, 2.60, 3.22, and 3.94 at 77 K) depends on whether the sample is oriented parallel (0° and 180°) or perpendicular (90° and 270°) to the magnetic field. It is found that the intensity of the weak EPR signal of Fe^{3+} at g = 4.34 (77 K) is independent on the sample orientation.

Room-temperature EPR spectra of JC shell measured at the microwave power of 20 and 126 mW are shown in Fig. 3. As expected, the intensity of EPR signals from SPIONs (g = 2.26) and iron aggregates (g = 3.87) is strongly dependent on the microwave power.

In contrast to SPIONs and iron aggregates, the intensities of EPR signals from Mn-containing compounds (g = 2.0, characterized by six-component hyperfine structure) in the JC seeds measured at 293 and 77 K are independent of the sample orientation as shown in Fig. 4.

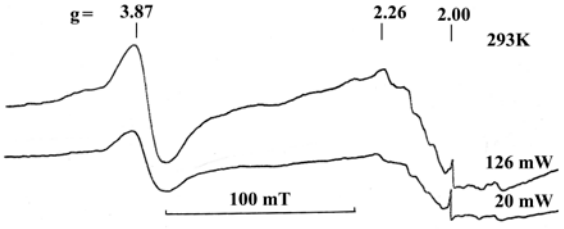

Fig. 3. EPR spectra of JC shell measured at different values of the microwave power (20 and 126 mW) at the temperature of 293 K. The estimated EPR signal width is: g = 3.87, $\Delta H_{1/2}$ = 17.2 mT.

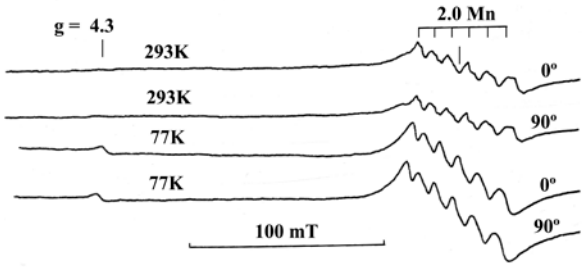

Fig. 4. EPR spectra of JC seeds for different sample orientations relative to the magnetic field (0° and 90°) and temperatures (293 and 77 K).

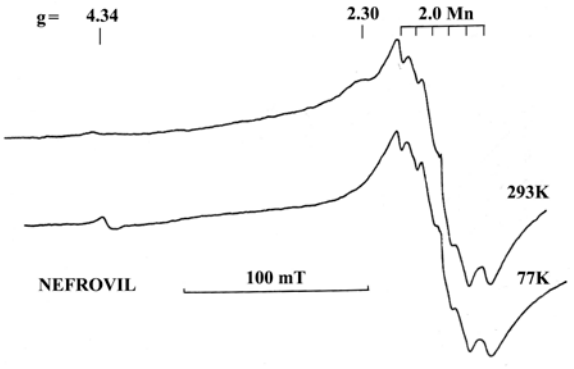

Fig. 5. EPR spectra of Nefrovil measured at the temperatures of 293 and 77 K.

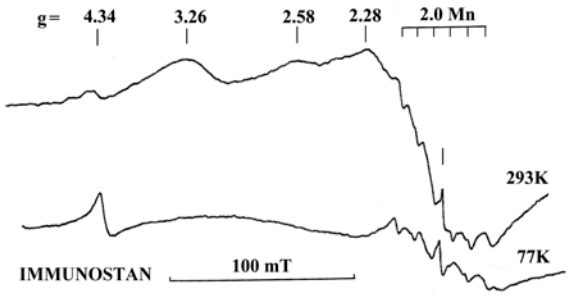

Fig. 8. EPR spectra of Immunostan measured at the temperatures 293 and 77 K. The estimated EPR signal widths are: g = 2.58, $\Delta H_{1/2}$ = 26…34 mT; g = 3.26, $\Delta H_{1/2}$ = 22.4 mT.

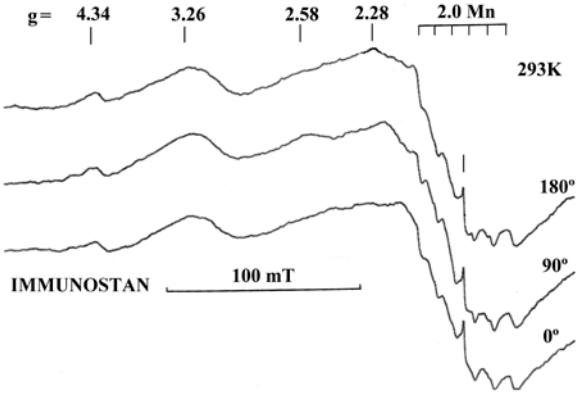

Fig. 9. EPR spectra of Immunostan measured at different sample orientation relative to the magnetic field (0°, 90°, and 180°) and the temperature of 293 K. The estimated EPR signal width is: g = 3.26, $\Delta H_{1/2}$ = 22.4 mT.

Fig. 6. EPR spectra of Nefrovil for different sample orientations relative to the magnetic field (0° and 90°) and the temperature close to 293 K.

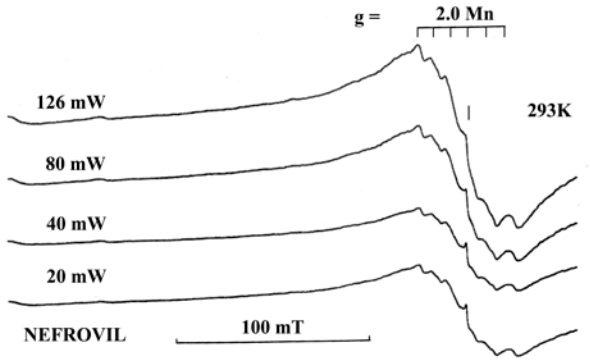

Fig. 7. EPR spectra of Nefrovil for different values of the microwave power (20, 40, 80, and 126 mW) measured at the temperature close to 293 K.

Fig. 5 shows EPR spectra of Nefrovil as the functions of magnetic field measured at the temperatures of 293 and 77 K. It is clearly seen that the dominant contribution to these spectra are made by Mn-containing compounds (g = 2.0, characterized by six-component hyperfine structure) as compared to the contribution of SPIONs (g = 2.30). Presence of both Mn-related and SPIONs signals in the Nefrovil drug is conditioned by that a mixture of JC shell and seeds was used for drug preparation. Hence, the obtained results testify that the antioxidant activity of Nefrovil is increased when it is prepared according to the procedure developed in this work.

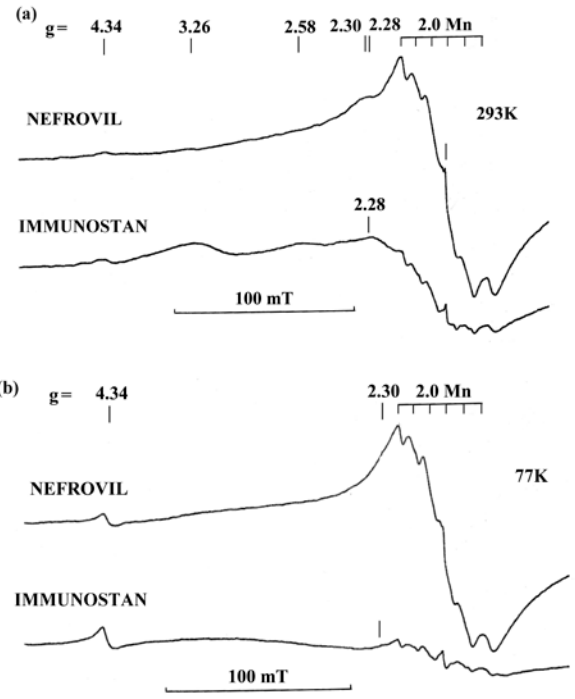

Fig. 10. EPR spectra of Nefrovil and Immunostan measured at the temperatures: (a) 293 K and (b) 77 K.

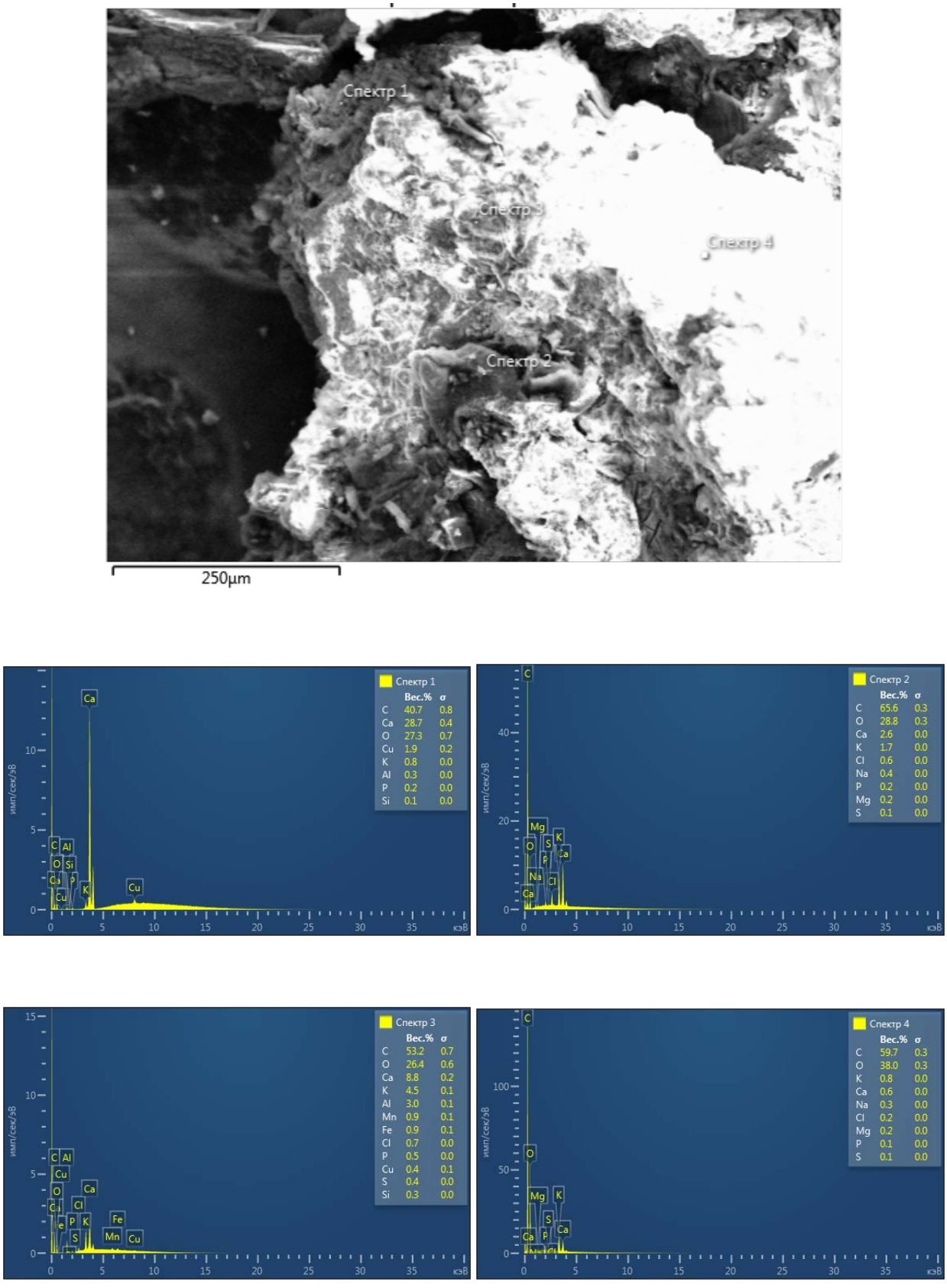

Fig. 11. SEM image and EDX analysis data for the Nefrovil sample.

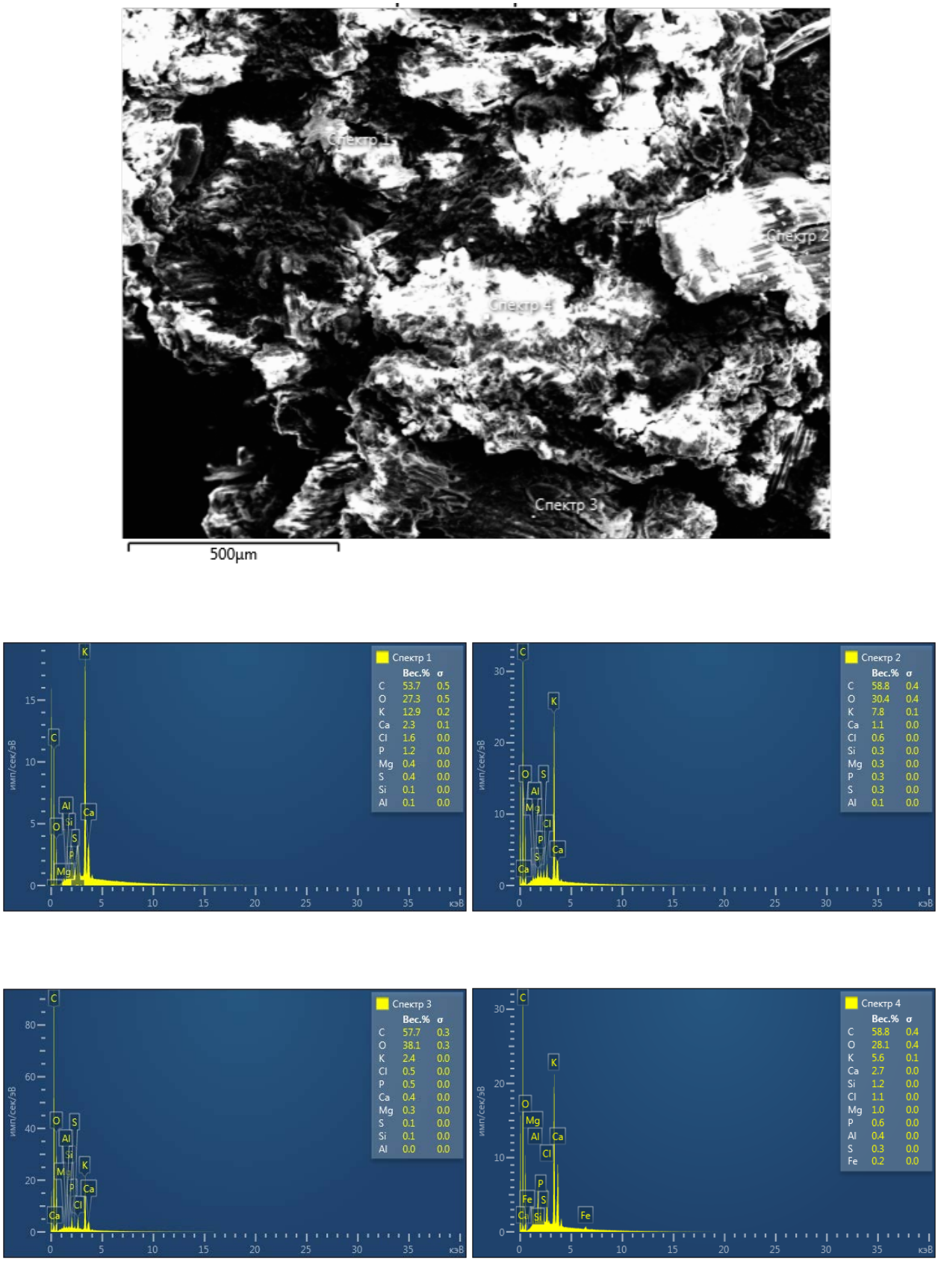

Fig. 12. SEM image and EDX analysis data for the Immunostan sample.

Similarly to the case of JC seeds (Fig. 4), the test performed for Nefrovil drug indicated that the intensity of the EPR signals from Mn-containing compounds is independent of the sample orientation as illustrated in Fig. 6. At the same time, the intensity of the room-temperature EPR signals from Mn-containing compounds typically depends on the microwave power as demonstrated in Fig. 7.

The bioactivity of Immunostan drug was also confirmed to improve the immune system of human organism as shown in Fig. 8. Indeed, SPIONs (g = 2.28) and iron aggregates (g = 2.58 and 3.26) have the dominant contributions to the EPR spectra as compared to Mn-containing compounds (g = 2.0). Moreover, behaviour of the EPR signals of Immunostan measured at 293 and 77 K is consistent with the results obtained for JC materials. However, the EPR results showed that the effect of sample orientation was less pronounced in the case of Immunostan as compared to that of JC shell as illustrated in Fig. 9. In particular, the orientation effect was rather significant for iron aggregates (g = 2.58) than for SPIONs (g = 2.28). In contrast, Fig. 10 shows the EPR spectra of Nefrovil and Immunostan measured at 293 and 77 K. The dominant roles of MnSOD for Nefrovil and SPIONs for Immunostan are plausibly confirmed. Moreover, it was ascertained that the EPR signals from SPIONs at g = 2.28 and Fe^{3+} ions at g = 4.34 in Immunostan are fully correlated (Figs. 8 to 10). This result may be considered as an additional evidence of bio-mineralization effect arising in the studied biomaterials under physical stress. This effect is coupled with electron-transport chain of photosynthesis leading to decreasing the Fenton reaction due to transformation of ferric ions (Fe^{3+}) into magnetite (Fe_3O_4) SPIONs. Therefore, SPIONs in the drugs based on JC shell and the antioxidant activity of JC seeds detected by MnSOD can be applied for pharmaceutical and biomedical purposes.

To summarize the obtained EPR results, SEM experiments were carried out on the examined Nefrovil and Immunostan drugs. The SEM images and EDX analysis data for the Nefrovil and Immunostan samples are shown in Figs. 11 and 12, respectively. Two components related to Mn and Fe atoms in the Nefrovil sample are observed, while only one component related to Fe atoms is found in the Immunostan sample under the same conditions (Fig. 11, spectrum 3, and Fig. 12, spectrum 4). Other components related to Ca, K, Cu, Cl, Na, P, Mg, S, Si and Al atoms in EPR spectra were not analyzed here. Therefore, presence of both Mn- and Fe-related components in Nefrovil correlates well with the EPR measurement results, showing that the signals from both Mn-containing compounds and SPIONs in Nefrovil drug stem from the mixture of JC shell and seeds used for drug preparation.

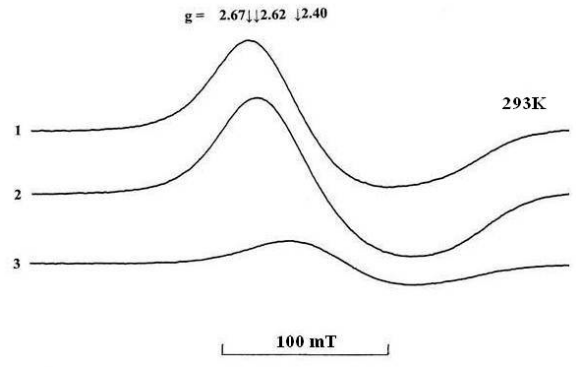

Fig. 13. EPR spectra of Fe_3O_4 SPIONs incorporated into polyethylene glycol (PEG) measured at 293 K by using three samples: *1* – (Fe_3O_4 + PEG), 15 ml, 9.4 nm; *2* – (Fe_3O_4 + PEG), 10 ml, 9.4 nm; *3* – (Fe_3O_4 + PEG), 5 ml, 9.4 nm (Adapted from [3]).

It should be noted that quantitative estimation of the dependence of the intensity of EPR signals on power (*i.e.*, EPR intensity *vs* $\sqrt{P}$) is difficult, which is caused by superposition of signals. Estimation of the integrated number of paramagnetic centers in JC seeds (the area under the integral curve) showed an increase in the area with increasing the power. The main contribution to the increase of intensity of EPR signal was made by paramagnetic centers with g = 2.26...2.30.

In summary, an innovative identification method for bio-functionality of natural bioactive additives is proposed using the example of *Juniperus communis* from Carpathian region of Ukraine as a model object. The effect of bio-mineralization under stress was studied using EPR spectroscopy. Nefrovil and Immunostan drugs, prepared to increase the antioxidant activity and to improve the immune system of human organism, respectively, were chosen as the model objects as well. The influence of temperature and microwave power on the intensity of EPR signals from JC, Nefrovil and Immunostan was examined. The EPR signals from SPIONs and Fe^{3+} ions in Immunostan are fully correlated, providing an additional experimental evidence of bio-mineralization effect under stress. SEM-EDX experiments confirmed the presence of Mn- and Fe-related components in the investigated biomaterials. The obtained g-factor values of EPR signals from JC shell, JC seeds, Nefrovil and Immunostan with their respective identifications are listed in Table 1.

Table 1. The values of g-factor corresponding to EPR signals from JC shell, JC seeds, Nefrovil and Immunostan with their respective identifications.

Samples	g-factor	Identification
JC shell	2.17, 2.2, 2.23, 2.26, 2.28, 2.38, 2.54, 2.58, 2.60	Fe_3O_4 SPIONs
	3.22, 3.26, 3.3, 3.69, 3.87, 3.94	Fe aggregates
	4.30, 4.34	Fe^{3+}
JC seeds	2.0, 2.01	Mn
	4.3	Fe^{3+}
Nefrovil	2.0	Mn
	2.28, 2.3	Fe_3O_4 SPIONs
	4.34	Fe^{3+}
Immunostan	2.0	Mn
	2.28, 2.58	Fe_3O_4 SPIONs
	3.26	Fe aggregates
	4.34	Fe^{3+}

Table 2. Spectral characteristics of the EPR signals observed for (Fe_3O_4 + PEG) samples measured at 293 K.

Samples	Concentration of paramagnetic particles (a.u.)	*g*-factor	Δ*H* (G)
1 – (Fe_3O_4 + PEG), 15 ml, 9.4 nm	1.063e8	2.67	930
2 – (Fe_3O_4 + PEG), 10 ml, 9.4 nm	1.19e8	2.62	840
3 – (Fe_3O_4 + PEG), 5 ml, 9.4 nm	0.541	2.40	750

In order to support the conclusion presented above, a comparative analysis with the results of the EPR study of Fe_3O_4 SPIONs incorporated into the polyethylene glycol (PEG) matrix described in [3] was carried out. Three (Fe_3O_4 + PEG) samples with the NP sizes of 9.4 nm and the concentrations of 5, 10, and 15 ml were prepared and analyzed by EPR technique. The obtained results are adduced in Fig. 13 and Table 2. It has been found that the values of the *g*-factor of 2.40, 2.62 and 2.67 for Fe_3O_4 SPIONs in PEG agree well with the values of g = 2.28...2.30 and g = 2.58...2.60 for the studied biomaterials.

4. Conclusions

Antioxidant activity system represented by MnSOD and other enzymes and formation of SPIONs in *Juniperus communis* based biomaterials (JC shell and seeds, Nefrovil and Immunostan drugs) were studied using EPR spectroscopy. SPIONs were detected only in JC shell, while Mn-containing enzymes were found only in JC seeds. The same features were observed for Nefrovil (prevalent MnSOD) and Immunostan (prevalent SPIONs), when JC-based Nefrovil and Immunostan drugs, both produced by Roslyna Karpat Co., Ltd (Ukraine), were screened in order to increase the antioxidant activity and to improve the immune system of human organism, respectively. It has been shown that the intensity of EPR signals from JC and related biomaterials in SPIONs and iron aggregates (containing SPIONs) is sensitive to the temperature, microwave power and sample orientation relative to the magnetic field. In its turn, the intensity of the EPR signal from Mn-containing enzymes is sensitive only to the temperature and microwave power. The intensity of the weak EPR signal of Fe^{3+} at g = 4.34, which was mainly detected at 77 K, was also almost independent of the sample orientation. It has been found out that the EPR signals from SPIONs and Fe^{3+} ions in Immunostan are fully correlated, which is an additional experimental evidence of the bio-mineralization effect under stress (*i.e.*, transformation of Fe^{3+} ions to Fe_3O_4 SPIONs). The EPR study of Fe_3O_4 SPIONs incorporated into the polymer matrix supports the conclusion of the self-organization of magnetic nanoparticles in the investigated biomaterials.

Acknowledgments

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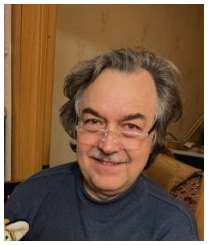

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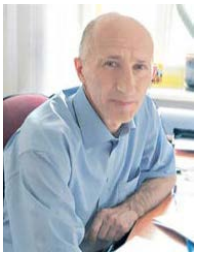

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ЕПР дослідження самоорганізованих магнітних наночастинок у біоматеріалах

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Анотація. Інноваційний підхід, оснований на ефекті біомінералізації як реакції клітин на зменшення їх пошкодження при стресі, застосовано до ялівцю звичайного – *Juniperus communis* (JC) – та біоматеріалів на основі JC (препарати Нефровіл (Nefrovil) та Імуностан (Immunostan)), що мають властивості підвищувати антиоксидантну активність та поліпшувати імунну систему організму людини відповідно. Спектроскопія електронного парамагнітного резонансу (ЕПР), яку також називають електронним спіновим резонансом (ЕСР), використовується як основний експериментальний інструмент для виявлення парамагнітних центрів у результаті існування системи антиоксидантної активності, представленої супероксиддисмутазою з марганцем, каталазою та ін., та утворення суперпарамагнітних наночастинок оксиду заліза (SPIONs). Досліджено вплив температури та потужності мікрохвиль на інтенсивність сигналів ЕПР, виявлених у JC, Nefrovil та Immunostan. Отримані значення g-фактора сигналів ЕПР, що спостерігалися в шкаралупі та насінні ялівцю, а також у Нефровілі та Імуностані, були віднесені до парамагнітних центрів Mn (g = 2,0), Fe_3O_4 SPIONs (g = 2,17…2,60), агрегатів Fe (g = 3,22…3,94) та іонів Fe^{3+} (g = 4,3). Установлено, що сигнали ЕПР від SPIONs та іонів Fe^{3+} в Імуностані повністю корелюють між собою, демонструючи додаткові експериментальні докази ефекту біомінералізації (тобто трансформацію іонів Fe^{3+} у Fe_3O_4 SPIONs). Для порівняльного аналізу наведено результати ЕПР досліджень Fe_3O_4 SPIONs, включених у полімерну матрицю. Отримані результати добре підтверджують даний висновок щодо самоорганізованих магнітних наночастинок у досліджуваних біоматеріалах.

Ключові слова: ЕПР, самоорганізація, суперпарамагнітні наночастинки оксиду заліза, біоматеріали.