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Synthesis, Properties, and in vivo Testing of Biogenic Ferrihydrite Nanoparticles

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Abstract—A sol containing biogenic ferrihydrite nanoparticles is obtained by cultivating *Klebsiella oxytoca* microorganisms. Data on the physical properties of the biogenic ferrihydrite and its effect on the organism of laboratory animals are obtained using a model of experimental hemolytic anemia, according to indicators of the functional activity of erythrocytes and morphological descriptions of organs.

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INTRODUCTION

In contrast to Fe^{3+} hydroxides and oxides, ferrihydrite $Fe_2O_3 \cdot nH_2O$ or Fe^{3+} oxyhydroxide is a compound with high metastability. Ferrihydrite therefore plays a huge role in metabolism of living organisms. It forms in the nucleus of the protein complex ferritin, a capsule of the protein apoferritin. The size of ferrihydrite nanoparticles ranges from 2 to 8 nm. Although ferrihydrite is characterized by an antiferromagnetic order with a Néel temperature of ~350 K, the presence of defects caused by its nanoscale state produces an uncompensated magnetic moment in an antiferromagnetic particle [1–5]. The latter opens up prospects for using ferrihydrite nanoparticles in various environmental [6, 7] and biomedical applications [8, 9]. Polysaccharide-coated iron-containing complexes are used in treating iron deficiency anemia. Pharmaceutical polysaccharide iron complexes consist of iron hydroxide particles several nanometers in size. The ferrihydrite nanoparticles synthesized by *Klebsiella oxytoca* are embedded in a polysaccharide matrix, so they can also be a promising drug for treating iron deficiency anemia. The prospect of applying ferrihydrite nanoparticles requires that we study mechanisms of the interaction between nanoparticles and cells, along with ways of transforming and eliminating them together with possible toxic effects.

The aim of this work was to obtain biogenic ferrihydrite nanoparticles by cultivating *Klebsiella oxytoca* microorganisms and investigating their physical properties, along with the effect a ferrihydrite sol has on the organisms of laboratory animals in simulations of hemolytic anemia.

EXPERIMENTAL

The investigated *Klebsiella oxytoca* microorganisms were extracted from the sapropel of Lake Borovoe (Krasnoyarsk krai). Microorganisms of the *Klebsiella oxytoca* culture were seeded on a Lovley medium on iron citrate. To extract ferrihydrite from the precipitate and obtain a sol, the bacterial biomass was separated from the supernatant and the bacterial cells were destroyed with ultrasound. The resulting precipitate was washed with distilled water.

Magnetic resonance spectra were measured on a Bruker ELEXSYS 560 spectrometer operating in the X range (characteristic frequency of microwave radiation, ~9.4 GHz) in the temperature interval of 100–300 K. Nanoparticle size was determined via dynamic light scattering (DLS) on the Zetasizer Nano instrument (Malvern Instruments Ltd; HeNe laser; λ = 632.8 nm) at the Krasnoyarsk Scientific Center's regional shared resource center.

We used a ferrihydrite sol with an iron concentration of 2.4 g/L to study the effect the nanoparticles had on the organisms of laboratory animals. The iron concentration was measured on the Perkin Elmer A Analyst 400 atomic absorption spectrometer at the same

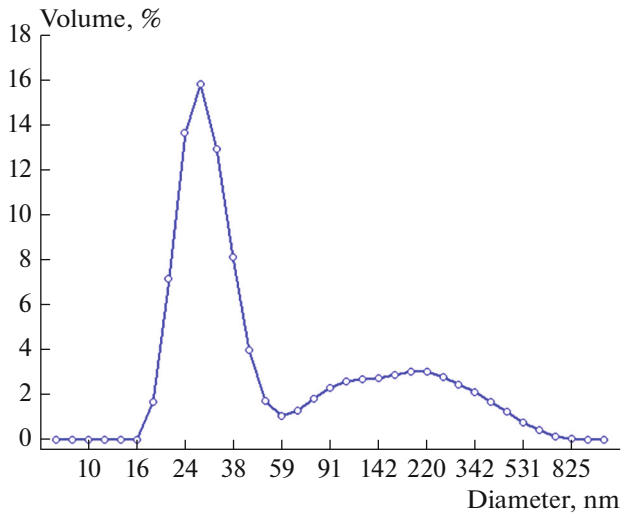

Fig. 1. Distribution of the hydrodynamic diameter of ferrihydrite sol aggregates.

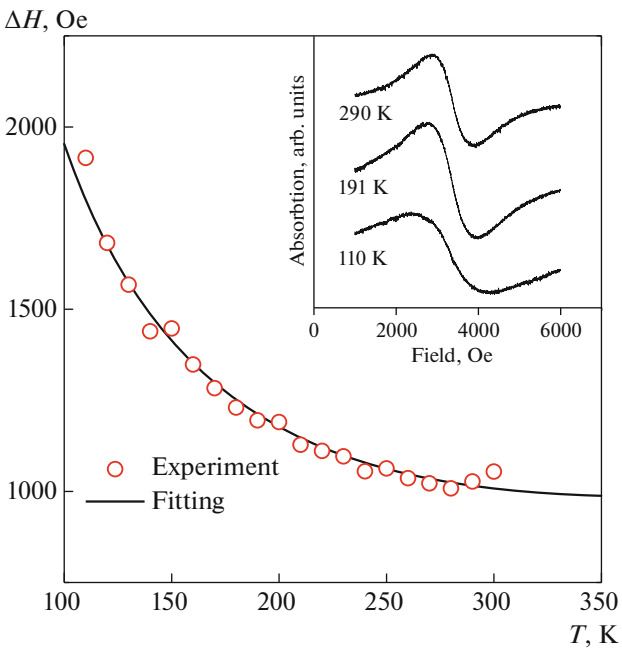

Fig. 2. Temperature dependence of the ferromagnetic resonance linewidth for ferrihydrite nanoparticles. The inset shows the FMR spectra at three temperatures.

shared resource center. The doses of the stabilized ferrihydrite suspension were chosen on the basis of a therapeutic dose of the commercial Ferranimal 75 drug (OOO Firma A-BIO, Russia), a colloidal solution of ferric hydroxide complex in dextran.

The effect of the drugs was studied on sexually mature male Wistar rats weighing 220 ± 40 g, divided in 3 groups: Group 1 (8 rats) of untreated animals; Group 2 (8 rats) of animals after repeated doses of ferranimal-75; and Group 3 (8 rats) of animals after repeated doses of the stabilized ferrihydrite suspension. Toxic hemolytic anemia was induced in each animal via intraperitoneal administration of phenylhydrozine hydrochloric acid in doses of 6 mg/100 g of body weight on the first day after the start of the experiment.

Exposure to the drug every 8 days (in single intramuscular doses of 75 mg/kg Fe(III), injected into the posterior femoral muscle group) began upon the onset of anemia (the fourth day) and lasted for 30 days. The functional parameters of the erythrocytes, including the saturation of hemoglobin with oxygen and the ability of blood to carry oxygen, were determined on days 1, 4, 8, 15 and 30 using an ABL-800 FLEX device (Radiometr, Denmark).

All of the animals were euthanized at the end of the experiment. The materials for histological examination were liver and spleen samples, used for histological preparations via conventional staining with hematoxylin and eosin for general purposes, and according to Perls to determine the presence of iron [III] compounds.

RESULTS AND DISCUSSION

The results from determining the hydrodynamic radii of nanoparticles in the sols are presented in Fig. 1. The nanoparticle distribution is polymodal with modal values of 28.2, 105.7, and 220.2 nm. The diameters of ferrihydrite nanoparticles, determined via transmission electron microscopy, are actually 2–7 nm. The nanoparticles detected via dynamic scattering were aggregates. The aggregation of magnetic nanoparticles in colloidal solutions was due to electro- and magnetostatic interactions. The fraction of nanosized aggregates with hydrodynamic diameters of ~28 nm is 65%, and the fraction of coarse formations is 35%.

Figure 2 presents the temperature dependence of ferromagnetic resonance linewidth $\Delta H(T)$ for dried nanoparticle sol. The inset in Fig. 2 shows the resonance absorption curves for three temperatures. The intensity of the FMR signal falls almost linearly over the range of measured temperatures, indicating the nanoparticles were in the unblocked superparamagnetic (SP) state.

According to the results presented in [10], the width of absorption lines in powders of randomly oriented ferromagnetic and ferrite particles is a nonmonotonic function of temperature: $\Delta H(T) = \Delta H_s(T) + \Delta H_u(T)$, where $\Delta H_s(T)$ is the contribution from superparamagnetism to broadening, and $\Delta H_u(T)$ is the contribution from the spread of the directions of particle anisotropy fields to (nonuniform) broadening. $\Delta H_s(T)$ and $\Delta H_u(T)$ are functions of Langevin parameter $x = MV\omega/\gamma kT$ (where M is magnetization, V is particle volume, k is the Boltzmann constant, T is temperature, ω is frequency, and γ is the gyromagnetic ratio). $\Delta H_s(T) =$

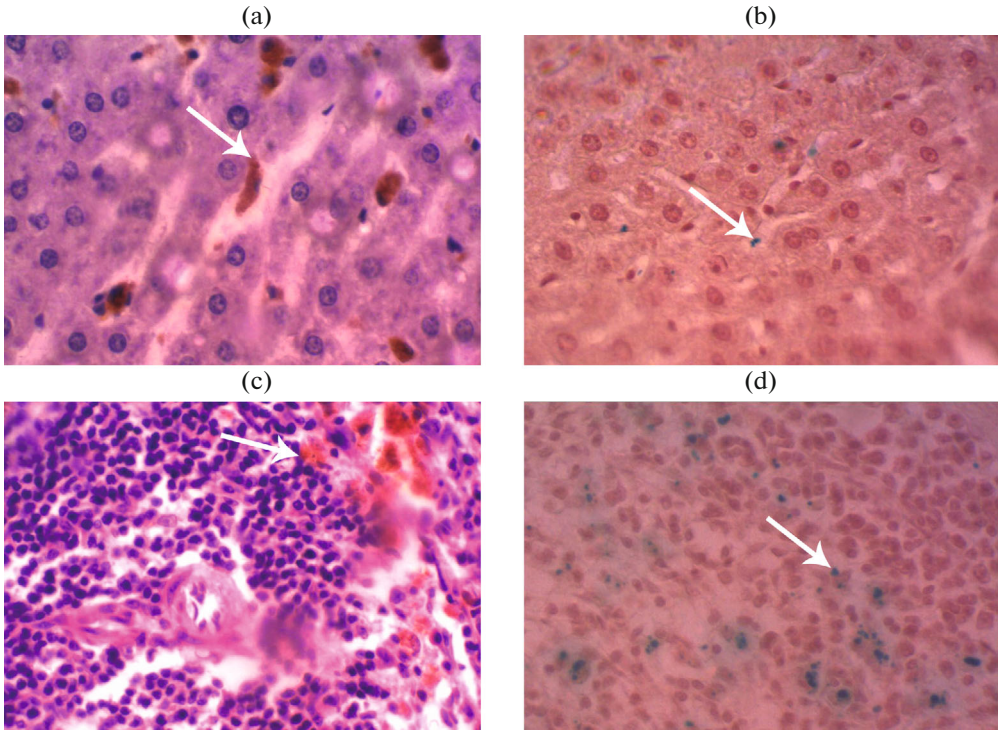

Fig. 3. Histologic specimens of rat organs (10× eyepiece and 40× objective). (a) Group 2 rat liver. Deposition of hemosiderin is noted. Staining with hematoxylin and eosin. (b) Group 3 rat liver. Single particles colored blue can be seen. Perls staining. (c) Group 2 rat spleen. Deposition of hemosiderin in the red and white pulp is noted. Staining with hematoxylin and eosin. (d) Group 3 rat liver. Ferrihydrite nanoparticles colored blue in the red and white pulp of the organ can be seen.

$\omega\alpha(x - L_1)/(\sqrt{3}x\gamma L_1)$, and $\Delta H_u(T) = 3\omega\varepsilon L_2/\gamma L_1$ ($\alpha = 0.01$) is a damping factor; $\varepsilon = K\gamma/M\omega$ (where K is the constant of anisotropy and $L_{1,2}$ is the Langevin function). The curve plotted in Fig. 2 is characterized by two fitting parameters: KV and MV. In this case, $KV = 1.25 \times 10^{-14}$ erg and $MV = 2.37 \times 10^{-17}$ emu. The obtained anisotropy agrees with the KV values for ferrihydrite of different origins. For ferritin in particular, $KV = 2.5 \times 10^{-14}$ erg [11]. For ferrihydrite obtained via chemical deposition, $KV = 2 \times 10^{-14}$ erg [12].

Examination of the effect of ferrihydrite sol in the experimental modeling of hemolytic anemia in rats showed its higher efficiency relative to the commercial Ferranimal-75 drug. In Groups 2 and 3 in particular, the examined parameters fell relative to the control group by a factor of 1.65 ($p = 0.036$) on the fourth day after the administration of phenylhydrozine. On the 15th day after injecting the ferrihydrite sol and the commercial drug, the indicators for Group 2 stabilized and returned to the control range, while the considered indicators for the Group 3 rats were higher than the control value ($p = 0.04$) by a factor of 1.2.

The morphological variations found in studying the livers of Group 1 rats included hemodynamic disturbances and the development of alternative processes in the parenchymal cells of the organ. The beam structure of the organs was preserved, but the hepatocytes were in a granular state of degeneration. The Perls staining was negative.

In studying the livers of Group 2 animals, we noted dystrophic processes and the deposition of hemosiderin in the parenchyma of the organs (Fig. 3a). The beam structure of the livers was preserved, but the hepatocytes were in a granular state of degeneration. The Perls staining was positive.

The beam structure of the organs was preserved in the livers of Group 3 rats. The hepatocytes were in a moderate state of granular degeneration. The Perls staining was positive and showed a partial distribution of iron nanoparticles in the organ parenchyma (Fig. 3b).

The spleen micromorphology in Group 1 rats was not disturbed. The parenchyma of the organ was in the form of well-defined red and white pulp. The Perls staining was negative.

In studying the spleen of experimental Group 2, the main structural elements of the organs were clearly distinguishable in the form of white and red pulp. The follicles were medium to large in size. Hemosiderin was deposited inside the red and white pulp in the form of grains (Fig. 3c) that reacted positively with iron upon Perls staining.

In the spleens of rats of Group 3, the spread of nanoparticles was noted in both the parenchyma and in stroma of the organs, which produced a positive

reaction upon Perls staining (Fig. 3d). The area of the red pulp in the spleen grew relative to the white pulp. The follicles were of different sizes.

CONCLUSIONS

We investigated biogenic ferrihydrite nanoparticles synthesized by cultivating *Klebsiella oxytoca* bacteria. The quantities characterizing the anisotropy and magnetization were determined using the temperature dependences of the FMR linewidth: $KV = 1.25 \times 10^{-14}$ erg and $MV = 2.37 \times 10^{-17}$ emu, respectively.

Our study of the functional activity of erythrocytes showed rapid recovery of the control parameters upon exposure to ferrihydrite sol, in contrast to the commercial drug Ferranimal-75.

Histological examinations revealed different accumulations of iron. The commercial drug displayed strong toxicity and was characterized by the accumulation of iron in the form of hemosiderin in livers and spleens. Partial deposition of hemosiderin in livers was noted when ferrihydrite sol was introduced into the bodies of rats, testifying to its rapid elimination from those organs and deposition in their spleens, accompanied by a compensatory increase in hematopoiesis.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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