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Interaction of Shungite Carbon Nanoparticles with Blood Protein and Cell Components

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Abstract—We have analyzed and compared the effects of aqueous dispersion of shungite carbon nanoparticles, fullerene C_{60}, and nanodiamonds on structure, dynamics, and thermodynamic and redox properties of blood proteins (serum albumin and hemoglobin), proteins of erythrocyte ghost membranes as well as on erythrocyte integrity and aggregation.. All the nanomaterials dispersions have induced similar effects; however, nanodiamonds have not influences the redox properties. Basing on the results, the experimental and theoretical approaches presented can be employed to estimate the effects of biological structures contact with the nanoparticles on the bioreactivity.

Keywords: Shungite nanocarbon, fullerene, nanodiamond, protein, membrane, interaction, stabilization of structure, dynamic complexes, oxidative properties.

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INTRODUCTION

The artificial and natural materials (the examples of latter are biological structures fragments, anthropogenic products of diesel fuel combustion or trash burning) are referred to as "nanoparticles" if there size ranges from ~1 to ~100 nm. The nano-particles are different in nature (metal and polymer quantum dots, ultradispersed SiO_2 or TiO_2 in aerosols or sun cream, asbestos or beryllium in the industrial workplaces, diesel and other pollutant particles) and thus vary in physico-chemical properties and the induced biological effects. The effects induced by nanoparticles cannot be therefore a priori predicted: depending on the particles properties (surface charge, biological reactivity, shape, size, deformability, strength, aggregation, and hydrophobicity) the nanoparticles interaction with the environment and biological systems is unique [1, 2].

Due to difference in the properties of nanoparticles and the analogous bulk materials, the nanosized matter is potentially toxic, unless the safety is proved. The reports on estimation of the risks of nanomaterials application and on the analysis of the nanoparticles effects on human body have been scarce so far. The understanding of nanoparticles interactions in the biological systems, even at the isolated cells level, has been well behind the overall progress in the field of nanotechnologies. The reports available have been dedicated to the complex formation between the nanoparticles and proteins, cells or cell membranes.

Carbon Nanoparticles

At the current stage of nanotechnology development, the carbon-based materials attract much attention; the most well-known of such materials being carbon nanotubes, fullerenes and their derivatives, nanodiamonds, and shungite nanocarbon. The yearly production of fullerenes has reached tons and will be shortly increased to hundreds of tons; other carbon nanomaterials are produced at even larger scale. Therefore, the nanocarbon materials induce larger and larger environmental effects [3, 4]. It is known that some of the non-toxic materials can damage pulmonary tissue when applied in the form of nanoparticles. For example, aggregates of C_{60} fullerene induce the biological reaction at below 1 ppm [5–7]. Some nanomaterials can pass through the natural barriers and be transported to the organs by blood or lymph [8]. Basing on the above-stated, the biocompatibility or toxicity of nanocarbon need accurate confirmation. Moreover, the toxic effects connected with the (pro)oxidative nanocarbon pro-

perties and the formation of singlet oxygen should be characterized depending on the external conditions. In summary, it is necessary to estimate the possible effects of the nanocarbon particles contact with the biological systems and to develop the theoretical approach to predict such effects basing on the molecular mechanism of the nanoparticles interaction with biological systems.

Until now, biomedical technologies have been among the most promising fields of nanocarbon materials applications. The known carbon nanotubes utilization examples include drug, gene material, and other biomacromolecules delivery as well as production of substrates for cell growth, implants, and low-impedance bioelectrodes [9–13]. There are some data on the biological action of the fullerene nanoparticles and their derivatives, including antiviral, neuromodulating, immunomodulating, and antioxidant [14–16]; the conclusions on biological activity of nanocarbon available to date have been largely based on the information of pristine and modified C_{60} fullerenes. Due to the lipophilicity, fullerenes can be localized in the hydrophobic regions of membranes and protein structures. However, with development of water-soluble fullerene derivatives [17–24] some papers have appeared discussing the possible toxic, prooxidant, mutagenic, and carcinogenic effects of fullerenes [5, 25–30]. Possibly, it is the nanomaterials interaction with water that influences their toxicity [31, 32].

Most of carbon nanomaterials are synthetic. However, carbon nanomaterials sources are known in nature as well, one of them being the shungite nanocarbon, a by-product of mining, pro-cessing, and using of shungite, a Precambrian rock. The shungite rocks are complex and heterogeneous material, characterized by high specific surface and peculiar electrophysical properties. The major constituent of shungite is carbon; it contains alumino-silicates and a variety of minor elements as well. Mineral waters known for their medicinal properties are formed in the shungite strata; shungite itself is applied to produce cosmetics, sorbents for water treatment, and as enterosorbent. Nevertheless, these applications are not well scientifically based, as the mechanism of shungite biological action is not well studied. In this regard, the evaluation of the role of carbon released from shungite rocks upon their interaction with water in the shungite biological activity is of particular interest.

All the known mechanisms of biological activity can be roughly divided into three groups: (1) nonspecific (the action is based solely on the general physic-chemical properties of the molecule, such as acid-base, redox, etc), (2) specific (the action is due to complementary interaction of the active molecule with defined target), and (3) membrane-acting (the interaction of the active molecule with cell membrane alters the membrane or membrane proteins properties) [15].

In order to evaluate the shungite carbon biological activity, we planned the study as follows: preparation of shungite aqueous dispersions free of the admixtures, investigation of their stabilization mechanism, sterilization of the dispersions so that they could be applied to living organisms, and comparison of shungite-induced biological effect with that of fullerenes and nanodiamonds. We used the serum proteins and erythrocyte membranes as models. In particular, we aimed to elucidate the effects of nanocarbon dispersions on the structural, dynamic, thermodynamic, hydrodynamic, and redox properties of the biomacromolecules and cell membranes. As a result of the study, we expected to estimate the type of shungite nanocarbon biological activity.

We used a set of precise physico-chemical methods: spin probe and spin label ESR studies, differential scanning microcalorimetry, dynamic light scattering, and scanning electron microscopy.

EXPERIMENTAL

The carbon nanomaterials were used in the form of aqueous dispersions prepared via procedures described in [22] (C_{60} fullerene), [33–35] (shungite nanocarbon), and [36] (nanodiamonds, the method included ultrasonication at 0.18 mg/mL of carbon).

The enthalpies and temperatures of thermally induced denaturation of various groups of membrane proteins and cytoskeleton of ghost erythrocytes in their complexes with nanoparticles were studied by means of differential scanning microcalorimetry (DASM-4 and Nano DSC AT-Instr devices, Institute of Biology, Karelian Scientific Center, RAS).

The segmental mobility of the proteins and the protein-protein interaction in erythrocytes cytoskeleton were studied by spin label ESR. The spin label was immobilized at the spectrin-actin complex of the inner side of the erythrocyte membrane. In order to

investigate the nanocarbon-induced changes in the annular lipid layer we took advantage of the spin probe based on stearic acid. The kinetics of iron(II)-driven radical reactions in the aqueous medium as well as in the erythrocyte membranes in the presence of nanocarbon was studied for the first time by means of spin probe ESR.

Changes of erythrocytes morphology in the presence of nanocarbon were studied by means of scanning electron microscopy. The influence of various forms of nanocarbon was investigated in the concentration range of 3 to 50 μg/mL. The specimens were observed with Tescan scanning electron microscope at magnification of about 2000–2500. To quantify the morphological changes, 200–300 erythrocytes were examined in each specimen. The cells shape was classify following Kozinets et al. [37].

Hydrodynamic properties of nanocarbon dispersions (in particular, the components size distribution) were evaluated by means of DLS (Zetasizer Nano analyzer, Malvern).

Erythrocytes stability with respect to osmotic and thermal lysis was affected by their membrane structural organization; therefore, they could be applied to follow the cell functions in the presence of nanocarbon. Thermally induced lysis was performed at 4, 37, and 56–60ºC at the cells concentration of 5×10^7 mL^{-1}. The amount of hemoglobin released in the course of lysis was determined by measuring the supernatant absorbance at 540 nm after centrifugation [38]. The osmotic resistance of erythrocytes in the presence of nanocarbons was studied following the standard procedure [39].

Hemoglobin auto oxidation in the nanocarbons dispersions was studied following the equilibrium state between the oxy- and met- forms (Fe^{3+} and Fe^{2+}, respectively) by tracking the spectral changes in UV-visible range (SF-256-UVI). Kinetic parameters of the hemoglobin oxy-met transformation were evaluated following the model described in [40], basing on the absorption at 560, 576, and 630 nm.

Physico-Chemical Peculiarities of Shungite Nanoparticles

The prospects of carbon nanoparticles in biomedical applications (in particular, therapy and diagnostics) are revealed by studies of biophysics of interactions between the nanomaterial and biological system. These interactions may result in formation of protein corona, wrapping of the nanoparticles with cell surface, endocytosis, and in-cell biocatalysis. Despite certain success in standardization of preparation of the nanoparticles aqueous dispersions, one of the major issues in the study of nanoparticles interaction with biomacromolecules is still the reproducibility of carbon nanoparticles dispersions preparation and their stability. This is so in the case of shungite nanoparticles as well [35].

Studies of shungite powders have revealed that shungite tends to form globules of about 10 nm. They are readily assembled into aggregates – clusters up to 100 nm in size. These structures are preserved in the course of carbon sedimentation from aqueous dispersions. Recent investigations have shown that it is carbon contained in shungite rocks that determines their specific adsorption and filtration properties as well as the biological activity. Intensive studies of carbon nanoparticles including fullerenes and their derivatives, nanotubes, onion-like structures, and nanodiamonds have concluded that morphology of shungite nanoparticles was the most close to fullerenes.

The hydrophobic nature of carbon contradicts substantial amount of water adsorbed by shungite (2–7 wt %). Till recently, the shungite activity has been ascribed to the presence of small globules (<6 nm) that demonstrate some properties close to these of fullerenes [41]. The globular elements tend to aggregate forming the long-living clusters, thus decreasing shungite activity; on the contrary, their disaggregation is accompanied with enhancement of shungite activity. Among the three-dimensional closed shells (globular structures), the shell fragments and bent graphene layers have been found [34]. Later, it has been shown that such open fragments constitute the major part of shungite carbon. The bent layers (“cups”) are of 0.5–0.7 nm in size and 2–5 nm thick (5 to 4 layers), as revealed by X-ray and electron diffraction. The smallest unit of shungite (0.51 nm) has been registered by means of small-angle X-ray diffraction; it is likely the most mobile and active shungite element. These data along with AFM results point that the above-mentioned structural types of shungite are combinations of the basic units [34, 35].

Due to the non-planar structure, graphenes show nonzero dipole moment, being very important in the shungite interaction with water and stabilizing the aqueous dispersions. Probably, the peculiarities of nanoparticles and protein molecules hydration are

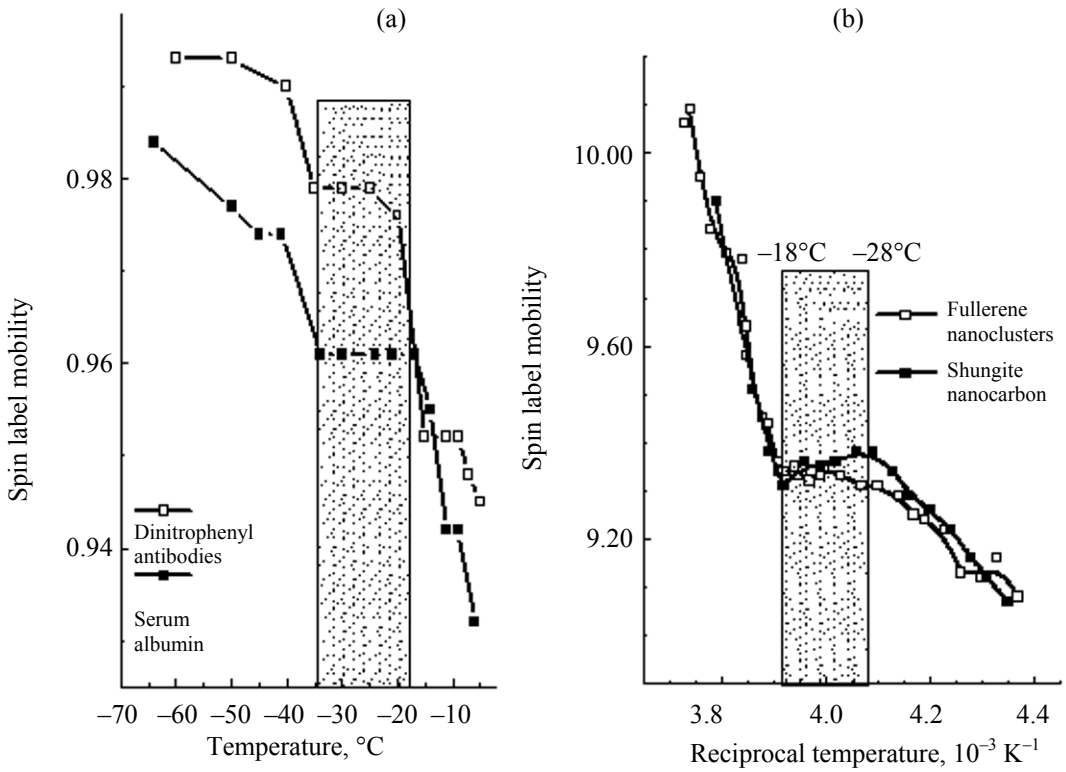

Fig. 1. Relative changes of mobility (rotation frequency): (a) of spin label in the water-protein matrix and (b) of spin probe in the dispersion of shungite carbon nanoparticles and hydrated fullerene clusters, as function of temperature upon cooling the dispersions in the ESR spectrometer cuvette.

responsible for their interactions and dispersions stabilization. The spin probe ESR studies have demonstrated the presence of non-freezing water down to –30°C, interacting with the mosaic regions at the carbon nanoclusters surface, composer of the polar centers and nonpolar groups [33]. The similar effect has been observed in the case of proteins [42] (Fig. 1). Thus, the hydration of the nanoparticles and proteins is to some extend similar. The fraction of hydrating water has been estimated to be of 6%, its self-diffusion coefficient being reduced by an order of magnitude [35]. To conclude, the interaction of carbon nanoclusters with water and biological molecules was majorly determined by the properties of bent surface graphene structures, bearing nonzero dipole moment.

Oxidative Properties of Carbon Nanoparticles

It is known that oxygen solubility and its active forms in the surface (hydrate) water is enhanced; therefore, the developed hydrate shell of the nanoparticles can alter their biological activity, in particular, in redox reactions. Carbon materials have revealed different effects on the oxidative processes in the presence of water and air oxygen [43]. Studies of the fullerenes C_{60} properties in connection with their oxidative activity [44–46] have revealed that C_{60} promotes spontaneous generation of the active oxygen forms due to ultraviolet photoactivation, possibly, due to formation of the electron-hole pairs at the surface defects and inclusions [47, 48]. However, this effect can take place only in the nonpolar organic solvents. Moreover, under such conditions fullerenes can act as electron acceptors. At the same time, in the aqueous fullerenes suspensions neither singlet oxygen nor superoxide radical has been observed [49].

We have studied the effects of fullerenes and shungite nanocarbon suspensions on the reduction and oxidation of stable nitroxyl spin probe in the presence of iron(II) [50, 51]. In particular, in the nanocarbon dispersions the spin probe is rapidly reduced to hydroxylamine in the presence of iron(II), the paramagnetic properties of it being vanished (Fig. 2). However, the oxidation of hydroxylamine has started simultaneously, probably, by oxygen dissolved in the hydrate shells. The paramagnetic properties of the spin probe are therefore restored, the both processes being accelerated with increasing nanocarbon concentration.

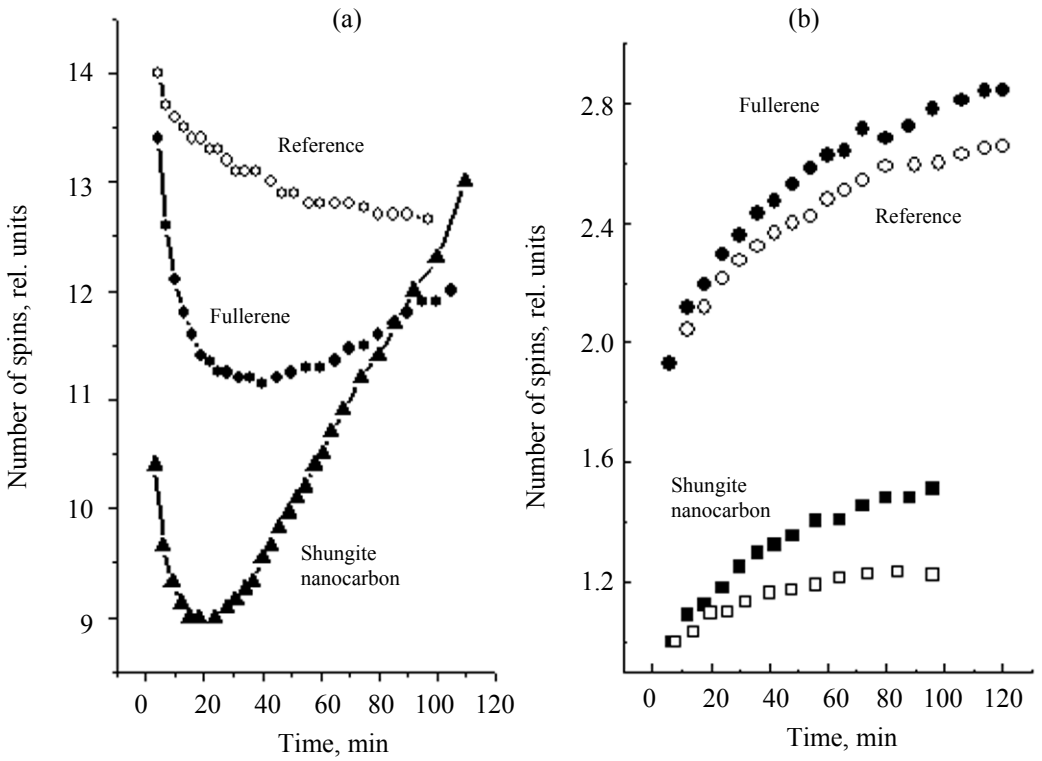

Fig. 2. Kinetics of redox reactions of nitroxyl spin probe induced by $FeSO_4$: (a) in water (reference) and in the dispersions of shungite nanocarbon and C_{60} fullerene; (b) in the erythrocyte membranes suspension in the absence (reference) and in the presence of shungite carbon and C_{60}.

In the absence of nanocarbon, the paramagnetic properties have not been restored, as well as under the conditions of oxygen shortage. Similar phenomena have been observed in the case of suspensions of erythrocyte ghost membranes; however, in this case the rate of the reduction of the spin probe was so high that only the second stage (oxidation of hydroxylamine) has been registered experimentally (Fig. 2).

Extending the above-mentioned studies, we have investigated the oxidative action of nanocarbon dispersions on proteins, using the spontaneous oxidation (known as autooxidation) of hemoglobin Hb in the presence of C_{60} fullerene, nanodiamonds, and shungite carbon [52]. In the presence of hydrated C_{60} nanoclusters (aqu/$n$$C_{60}$) and shungite nanocarbon concentration of oxidized Hb has been significantly increased: Hb has been partially oxidized upon mixing (Fig. 3); this effect has not been observed in the case of nanodiamond dispersions. Moreover, even at high pH (above physiological conditions) the iron(II) oxidation has been significantly accelerated in the presence of aqu/$n$$C_{60}$ and shungite nanocarbon (the autoxidation is extremely slow at so high pH and can be thus neglected) The observed effect has evidenced that both aqu/$n$$C_{60}$ and shungite nanocarbon has acted as prooxidants.

It is known that Hb autooxidation proceeds as iron oxidation (oxyHb is converted to metHb) and oxygen reduction to superoxide anion. It is accompanied by nucleophilic displacement of the superoxide by water molecule and requires imidazole group of distal histidine to be protonated; therefore it is accelerated at lower pH [40]. We have suggested that aqu/$n$$C_{60}$ and shungite nanocarbon promote the protonation of hemoglobin distal histidine in the aqueous dispersion.

Complexes of Proteins with Nanoparticles

The proteins adsorption of carbon nanoclusters (the so called protein corona formation) is a general phenomenon, it has been observed in the cases of nanodiamonds and fullerenes [53–56]; we have revealed similar effect in the case of shungite nanocarbon.

The complexes of serum albumen with shungite carbon have been studied by means of DLS. The complex formed is several times larger than carbon nanoparticles in the protein-free aqueous nanocarbon

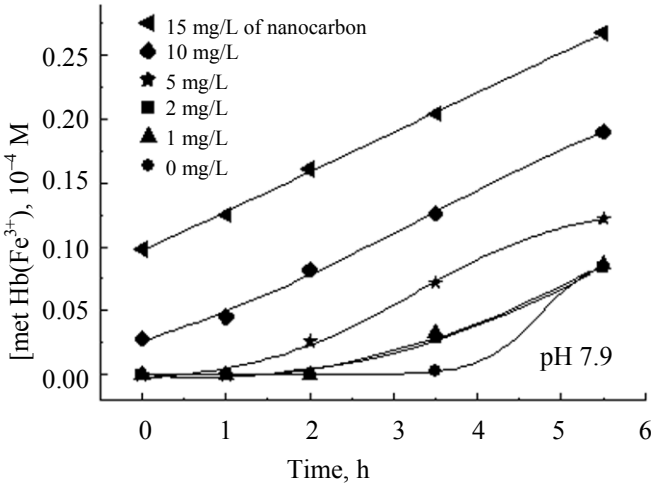

Fig. 3. Kinetics of hemoglobin (1.7 mg/mL) autooxidation at pH 7.9 in the presence of varied concentrations of C_{60} fullerene.

dispersion, and the protein molecules are almost completely bound to the nanoparticles even at quite high protein concentration, up to 10 g/L (Fig. 4). The calculations have revealed that the efficient multilayer adsorption has occurred at the nanoparticles surface. At protein concentration significantly above 10 g/L, the free protein molecules have been detected in the size distribution diagrams, thus allowing the estimation of adsorption capacity and the protein corona size.

Gel-filtration and ultracentrifugation experiments have shown that under physiological conditions the dispersions of the protein–nanocarbon are highly stable towards sedimentation and aggregation, whereas the stability of protein-free nanocarbon is minimal under these conditions. The complexes have also revealed much higher chromatographic mobility (Fig. 5) as compared with the free proteins. On the opposite, shungite nanocarbon has not passed through the column at all, likely due to efficient binding with the gel.

Figure 6 demonstrates the DSC data on serum albumen melting at varied concentrations of the protein and shungite nanocarbon [57]. Certain differences have been revealed; however, in general the proteins are expanded in the complexes as compared to the free proteins. DSC curves deconvolution has demonstrated that the intermolecular interactions between the protein domains are altered by intermolecular interactions with shungite nanoparticles; simultaneously, the domains are partially united. Similar data are available in the cases of nano-

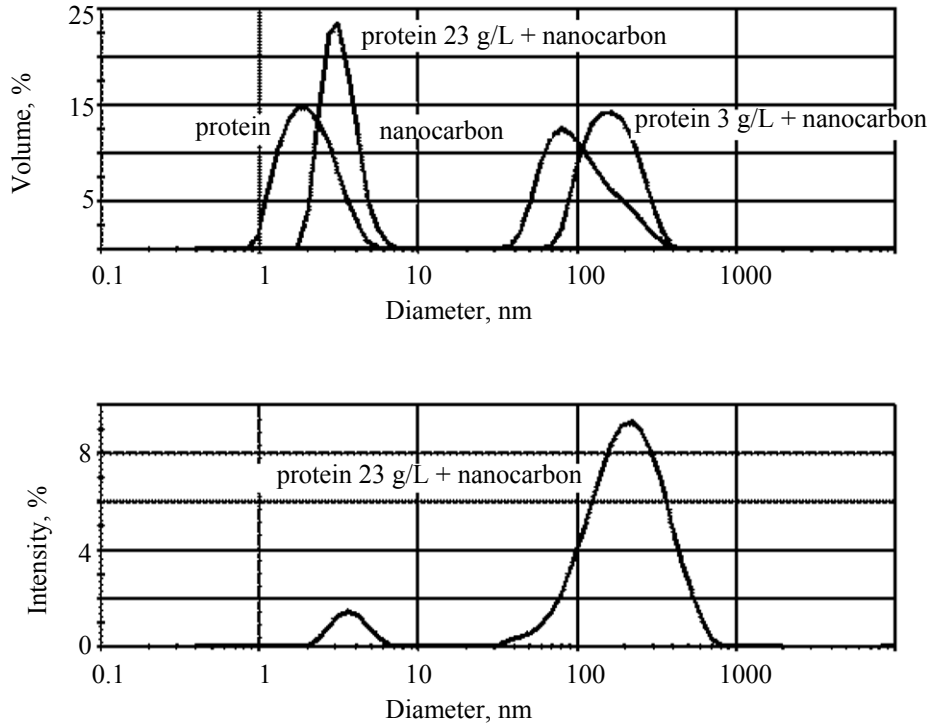

Fig. 4. Size distribution of the particles in the aqueous dispersion of shungite nanocarbon – serum albumin complexes: (top) volume of the particles and (bottom) light scattering intensity.

diamonds and C_{60} fullerenes [57]. The thermograms changes have confirmed that the nanoparticles interaction occurs predominantly via the fatty acids binding site.

The protein structure destabilization upon the complex formation has been additionally confirmed by the spin probe EPR; the spin probe based on the fatty acid is efficiently bound at the corresponding site of the protein. The spectral changes observed are similar to those in the case of proteins denaturation with urea (Fig. 7). Seemingly, the protein is partially unfolded upon interaction with the nanoparticles surface. However, at increasing protein/nanoparticles concentrations ratio the protein structure has been somewhat stabilized, possibly, due to the protein–protein interactions in the course of multilayer adsorption [58].

Not all the proteins show up the so efficient complex formation with the nanoparticles. In particular, microcalorimetry studies of hemoglobin solutions have not revealed any structural differences of the protein structure in the presence of the nanoparticles [58].

To conclude, carbon nanoparticles in general show up the properties essential for efficient specific enterosorbent of the compounds capable of binding with the adsorbed protein (for instance, fatty acids in the case of serum albumen). The adsorption of proteins onto flat surfaces or in the colloid dispersions has been intensively studied [59–64] allowing estimation of the binding rates and the affinity of various proteins towards nanoparticles [65]. The modern methods, such as size-exclusion chromatography, isothermal titration calorimetry, and surface plasmon resonance, allow determination of the major as well as minor proteins bound to the nanoparticles and investigation of the competitive reactions of the binding proteins. The data

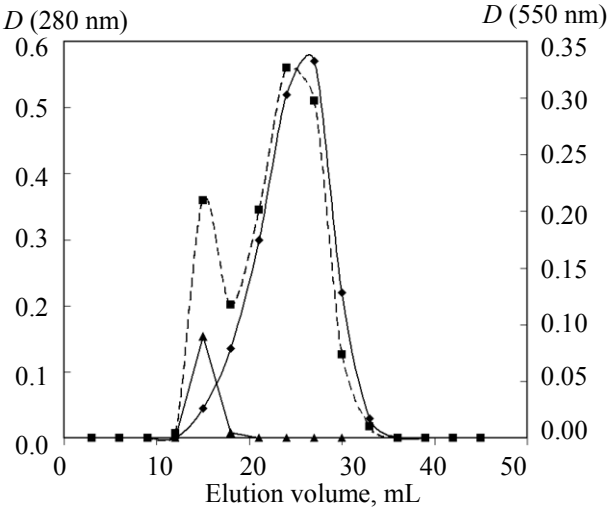

Fig. 5. Elution profiles of the shungite nanocarbon (0.2 g/L) complexes with serum albumin (6.3 g/L), as detected by light absorbance. (■) bioconjugate; (♦) protein; (▲) nanocarbon.

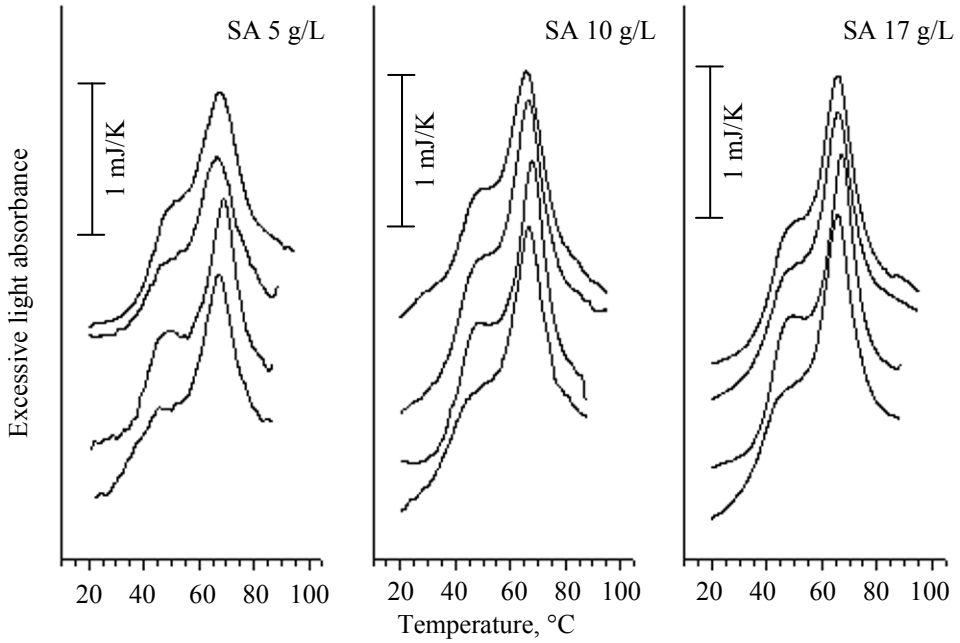

Fig. 6. Microcalorimetry of serum albumin melting in the aqueous solutions (pH 5.5) in the presence of shungite nanoparticles, curves from top to bottom: 0; 0.1; 0.2; and 0.3 g/L of shungite carbon.

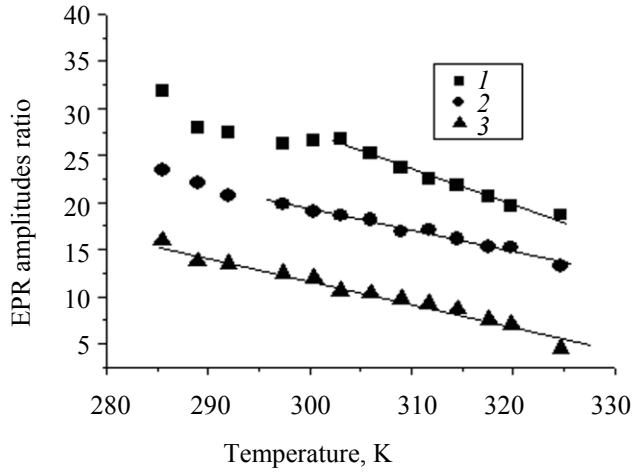

Fig. 7. The ratio of ESR spectrum amplitudes of the signals corresponding to strong and weak immobilization of 5-doxyl stearic acid spin probe in the water-protein matrix of serum albumen, as function of temperature: (*1*) reference, 10 mg/mL of albumin; (*2*) reference, in the presence of 0.1 mg/mL of shungite carbon; (*3*) reference, in the presence of 2 mol/L of urea.

obtained have demonstrated that many proteins form transition complexes with nanoparticles, the association-dissociation parameters being dependent on the protein type and the nanoparticle surface properties. Upon formation of the protein corona, the modified surface shows up the altered properties, this fact being important for subsequent interaction with the cells surface. The nanoparticles can be thus prospective for corona proteins transfer through the cell membranes.

Another type of the nanoparticles interaction with protein molecules can be embedding into the tertiary structure of the latter. In particular, serum proteins molecules form the complexes with water-soluble C_{60} fullerene derivatives that penetrate into the hydrophobic part of the protein macromolecule [66–67]. Computer modeling data as well as results of some experiments have evidenced that albumen can bound the unmodified C_{60} fullerene as well [67–68].

Membrane-Acting Activity of Nanocarbon

The interaction with cell membrane can play a decisive part in the view of nanoparticles cytotoxicity as well as the prospects of their applications for targeted drug delivery. In has been shown that the interaction of nanoparticles with the membranes often differs from that of the microparticles (~10^{-6} m), their chemical nature being the same [69, 70]. The morphology of cell membrane can be modified upon interaction with various nanoparticles. In particular, in the course of efficient nanoparticles adsorption onto the membrane, the latter can be perforated [71–74], the effect being dependent on the size and the charge of nanoparticles.

The experimental results available to date cannot unambiguously conclude on the type and mechanism of fullerenes and nanocarbon particles interaction with lipid bilayers and cell membranes. Some studies have revealed that the water-soluble fullerenes induce the cell death in the cases of eukaryotic [5, 27, 75] as well as prokaryotic [4, 27, 29, 76] cells. However, other results have indicated that water-soluble fullerenes are non-toxic and can even prevent the oxidative damage of lipids [22, 46, 77]. Some works have demonstrated that fullerene aggregates located in the region of lipid tails of the bilayer [78, 79] can disrupt the hydrocarbon chains packing [80, 81] and thus increase the electronic permeability of the lipid bilayers causing the decrease of phase transition temperature [81].

In the recent studies it has been demonstrated that the aqueous suspensions of fullerene aggregates show up significant antibacterial activity towards *Bacillus subtilis,* irrespectively of the suspension preparation method [82]. The suspensions containing smaller particles reveal higher antibacterial activity; the activity enhancement being much more than the increase of the corresponding surface area increase.

AFM studies of water-soluble fullerenes interaction with planar bilayers at mica support have not revealed any bilayer packing disruption in the presence of C_{60}. Probably, the fullerene aggregates can only interact with the lipid heads but cannot penetrate to the lipid hydrocarbon tails. The lipid bilayer thickness, morphology, and the phase transition temperature have not been changed upon interaction with fullerenes.

In our experiments with hydrated fullerenes, nanodiamonds, and shungite nanocarbon we have modified the proteins of actin-spectrin complex of erythrocyte membrane with the spin label [83]. The nanocarbon particles have enhanced thermal stability of the studied complex. The scanning microcalorimetry data have demonstrated the increased thermal stability of the proteins of cytoskeleton erythrocytes complex, whereas their membrane proteins have not been affected [84].

Studies of thermal and osmotic stability of erythrocytes in the shungite nanocarbon dispersions have

revealed that the major effect of the possible interaction is the decrease of hemolytic stability of the cells [85]. From the analysis of SEM pictures, the morphology of erythrocytes in the presence of shungite dispersion has not been significantly changed [86]. The aggregation of the cells has been much enhanced. In the case of human erythrocytes samples, the presence of the nanoparticles induced the discocytes aggregation. Their aggregates, in the shape of bunch, have not revealed any particular structure. Upon separation off the nanoparticles, the cells aggregation has been reversed [87]. Hence, the nanoparticles affected the cells behavior through aggregation of the latter. Likely, the action molecular mechanism consists of the aggregation of the surface proteins of erythrocytes. From the obtained data, carbon nanoparticles can modify the erythrocytes state, the effect being dependent of the nanoparticles concentration as well as on temperature.

CONCLUSIONS

Sterile stable aqueous dispersions of shungite carbon were prepared for the first time taking advantage of the original procedure. We studied the biological activity of the prepared dispersions in the model experiments using blood molecular and cellular components. The *in vitro* experiments revealed the significant biological effects; in particular, the nanoparticles could act as pro-oxidants or anti-oxidants, depending on the conditions. Therefore, care should be taken in planning their applications in contact with visceral liquids containing active oxygen forms; additional studies should be performed in that regard. The ability of nanoparticles to convert hemoglobin into the inactive state (metHb), to induce erythrocytes aggregation, and to decrease the cells resistance to lysis can suppress the oxygen transport functions. At the same time, shungite nanocarbon was shown to form the complexes with some proteins, changing their conformation and local concentration. Hence, that form of nanocarbon can be potentially used for non-specific regulatory purposes, including osmosis- and immune-regulation. Shungite nanocarbon can also be applied in other biotechnology fields due to ability to selectively bind certain proteins and stabilize their structure.

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