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Structural–Morphological and Biological Properties of Selenium Nanoparticles Stabilized by Bovine Serum Albumin

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Abstract—Nanostructures formed during the reduction of ionic selenium in the selenite–ascorbate redox system in an aqueous solution of bovine serum albumin (BSA) were studied using static and dynamic light scattering and flow birefringence. It was established that this process results in the formation of stable aggregates of selenium nanoparticles that adsorb BSA molecules. It was found that highly-ordered superhigh-molecular-weight spherical nanostructures with high density and unique morphology are formed. Experiments with a cell culture of promyelocytic leukemia HL-60 showed that BSA adsorbed on selenium nanoparticles can inhibit the growth of tumor cells and deactivate free radicals with an efficiency comparable with that of sodium selenite.

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The discovery that selenium is highly important for human health [1] gave impetus to the development of preventive and therapeutic drugs based on inorganic and organic selenium compounds. The distinctions between the vital, therapeutic, and toxic doses of these forms of selenium are extremely small. The toxicity of selenium decreases in going from its ionic forms to organic compounds, especially containing zero-oxidation-state selenium. The assessment of the specific biological activity of zero-valence selenium nanoparticles obtained by chemical reduction of its ionic forms in the absence of stabilizers [2] and by biotechnological methods involving bacteria capable of accumulating and reducing ionic selenium [3] showed that the activity of these nanoparticles does not exceed 2% of the activity of sodium selenite, a medicinal agent widely used in medicine. In [4], however, it was established that addition of bovine serum albumin to the sodium selenite—glutathione redox system results in the formation of red amorphous nanoselenium (a-nano-Se^0), which is seven times less toxic than sodium selenite but retains the profile and level of the biological activity of sodium selenite. Hence, it was supposed that selenium is bound to albumin by means of relatively weak noncovalent interactions, the nature of which was not examined by the authors of [4]. The assessment of the biological activity of selenium resuspended after centrifuging the precipitate [4] showed that, in the absence of albumin, selenium does not exhibit the biological activity characteristic of the selenium–albumin composite.

The aim of this work is to synthesize nanoparticles of zero-valence selenium by reduction of selenious acid with ascorbic acid in the presence of globular BSA, to discuss the nature of its interactions with nanoparticles, to determine the morphology of BSA–Se^0 nanostructures formed in aqueous solutions, and to assess in vitro the potentialities of these nanocomposites as antitumor and antioxidant agent in comparison with sodium selenite.

EXPERIMENTAL

We used selenious and ascorbic acids with main substance content not less than 99.99%. Before experiments, BSA (Calbiochem, fraction V, grade B) was additionally purified by gel-filtration on a Sefadex G-100 (the eluent was distilled water).

The reduction of ionic selenium was performed at a selenium concentration in its aqueous solution of 0.01% and a Se : BSA mass ratio of $\nu = 0.1$, conditions corresponding to a complete saturation of the adsorption capacity of nanoparticles in the poly(vinylpyrrolidone)–selenium–water system [5].

The kinetic measurements were performed at 20°C and pH 3.5 on a Specord M 40 spectrophotometer. The time evolution of the optical density of the solution during the reduction of selenous acid was recorded at $\lambda = 320$ nm.

The method of static light scattering [6] was used to determine the molecular masses (MMs) (M_w and M_w^* for BSA and nanostructures, respectively) and root-mean-square inertia radii R_g^* of the nanostructure and its affinity for water (determined from the second virial coefficient A_2^*). The number of BSA macromolecules (N^*) adsorbed on the surface of Se nanoparticles was

† Deceased.

determined from the ratio between the MMs of BSA and the nanostructure formed. To measure the reduced scattering intensity of the solutions R_θ, we used a Fica photogoniodiffusometer. The wavelength of vertically polarized incident light was 546.1 nm. Measurements were performed for angles of scattering within $\theta = 30°$–150°. The refractive index increment dn/dc_{BSA} (c_{BSA} is the BSA concentration) was determined from refractometric measurements performed on an IRF-23 instrument.

The experimental data on light scattering for the nanocomposite solutions were processed in accordance with the Zimm procedure, by double extrapolation (to $c_{BSA} = 0$, $\theta = 0$) of the Kc_{BSA}/R_θ versus $\sin^2(\theta/2) + kc_{BSA}$ dependence (K and k are the calibration and numerical constants, respectively).

The method of dynamic light scattering [7] was used to determine the average hydrodynamic size R_h^* of the nanostructure at $c_{BSA} \longrightarrow 0$. The ratio between the experimental values R_g^* and R_h^* was used to determine the parameter $\rho^* = R_g^*/R_h^*$, which characterizes the conformation of the nanostructure [8].

The optical equipment of the setup used for measuring the dynamic light scattering consisted of an ALV-SP goniometer (Germany) and a helium–neon (He–Ne) laser (Spectra-Physics), as a light source ($\lambda = 632.8$ nm, $W \sim 20$ mW). The correlation function of the scattered light intensity was measured on a Photo Cor-FS (Anteks, Russia) correlator with 288 channels. The correlation function was analyzed using the Dynals software package (Gelios, Russia).

The mean density of the spherical nanostructure was calculated from M_w^* and the root-mean-square radius of inertia by the formula

$$\Phi^* = 3M_w^*/4\pi N_a R_{sph}^3, \quad (1)$$

where $R_{sph} = 1.29R_g^*$ [9].

The molecular dispersity of the test solutions of nanostructures was determined by the flow birefringence method [6] from the dependences of the birefringence Δn on the velocity gradient g and the BSA concentration c. We used a titanium dynamooptimeter with a $h = 4.0$ cm and a rotor–stator gap of $\Delta r = 0.03$. The flow birefringence measurements were performed with thermostatted solution (at 21°C) to avoid viscosity variations and optical distortion caused by temperature gradients. The setup was calibrated with phenylethyl alcohol which has a significant birefringence increment ($\Delta n/g = 1.7 \times 10^{11}$), and with polystyrene–bromoform solution. The error in determination of the characteristic birefringence, $[n] = \lim_{g \to 0, c \to 0} (\Delta n/gc_{BSA}\eta_0]$ (where η_0 is the solvent viscosity) did not exceed 10%. The measurements were performed at $g < g_k$, where g_k is the velocity gradient of onset of flow turbulence.

In the general case, at $dn/dc \neq 0$ (dn/dc is the refractive index increment of the solution, 0.146 for the BSA–Se^0–H_2O system), the experimental value of $[n]$ is the sum of three contributions: $[n] = [n]_e + [n]_{fs} + [n]_f$, where $[n]_e$ is the intrinsic anisotropy, $[n]_{fs}$ is the microform effect, and $[n]_f$ is the macroform effect [6]. The quantity $[n]_f$ depends on the particle shape asymmetry p as

$$[n]_f = ((n_s^2 + 2)/3)^2 (M_w^*(dn/dc)^2 f(p))/30\pi RTn_s = \text{const} \times M_w^*(dn/dc)^2 f(p), \quad (2)$$

where n_s is the refractive index of the solvent, T is the absolute temperature, R is the universal gas constant, and $f(p)$ is the tabulated function of the ratio between the lengths of the axes of the solid ellipsoid that modeled the particle [6].

The biological tests were performed with cells of promyelocytic leukemia HL-60 from the bank of cell cultures at the Institute of Cytology of the Russian Academy of Sciences. The cells were cultured in an incubator (5% CO_2, 37°C) on plastic Petri dishes in an RPMI medium (Biolot, St. Petersburg) containing 15% of cattle embryo blood serum (Gibco). To exclude microbial contamination of the cultivation medium, we introduced antibiotic gentamycin in a concentration of 80 μg/ml. The cells were preliminary planted in plastic cups 35 mm in diameter or in 24-socket plates.

HL-60 cells were treated 24 h after transplantation by adding the corresponding agents to the cultivation medium at certain time intervals (24 h to 3 days).

The cell distribution over the DNA content was determined using flow cytofluorometry. To enhance the membrane permeability, we treated the cells with X-100 triton at a final concentration of 0.01% for 0.5 h at room temperature (~20°C), added propidium iodide (10 μg/ml, Sigma, USA), incubated the cells for 15 min at 37°C, and analyzed them on a ATC 300 flow cytofluorometer (Bruker) at a flow rate of 20 μl/min for 3 min.

To determine the content of active oxygen species (AOS) in the cells, the cultivation medium was treated with dihydroethidium bromide (30 min before the analysis) so as to attain a final concentration of 5 μmol/l. The cells were washed twice with a PBS solution (20 mmol/l phosphate buffer, pH 7.4, 0.1 mol/l NaCl). The fluorescence of ethidium bromide was analyzed on an ATC 300 flow cytofluorometer (Bruker).

The AOS yield in the cells was stimulated by adriamycin at a concentration of 10 ng/ml.

RESULTS AND DISCUSSION

The reduction of ionic selenium was found to be a first-order reaction in the selenious acid concentration, with rate constants k^* of 2.5×10^{-3} s^{-1} (Table 1) and 1.7×10^{-3} s^{-1} in the presence and absence of BSA, respectively. Thus, the protein accelerates the reaction.

Table 1. Rate constant of the first-order reaction in selenious acid in the presence of BSA and the structura–morphological characteristics of the selenium-containing BSA-based nanostructures under study

$k^* \times 10^3$, s^{-1}	$M_w \times 10^{-6}$	N^*	R_g^*, nm	R_h^*, nm	ρ^*	$A_2^* \times 10^4$, $cm^3\ mol/g^2$	p	Φ^*, g/cm^3
2.5	70	1000	50	50	1.0	0.2	1.2	0.1

According to the static light scattering data, the molecular mass M_w of BSA is 7.0×10^4 while that of the nanostructure formed, 7.0×10^7, i.e., 1000 times higher (Table 1).

For free BSA macromolecules in an aqueous solution, the mean hydrodynamic radius R_h was found to be ~ 15 nm, approximately equal to the value reported in [10, 11]. The value of R_h^* for selenium nanoparticles formed in the absence of the protein stabilizer was estimated as 170 nm [5]. The size of the BSA–Se^0 nanostructure determined by dynamic and static light scattering is smaller (the root-mean-square radius of inertia and the mean hydrodynamic radius are equal: 50 nm, Table 1). The ratio $\rho^* = R_g^*/R_h^* = 1$ suggests that the nanostructure formed is spherical [8].

The second virial coefficient A_2^* for the BSA–Se^0–H_2O system was found to be 2×10^{-5} $cm^3\ mol/g^2$ (Table 1), a value typical of ideal solutions. This value is considerably lower than that for BSA in water or in other solvents [10].

Note that the molecular-dispersed state of solutions of the BSA–Se^0–H_2O system remained unchanged over the entire concentration range covered (c_{BSA}), as can be seen from the character of the $\Delta n = f(g)$ dependence: at $g < g_k$, they are closely approximated by straight lines passing through the origin of the coordinates at all c_{BSA} values. From the concentration dependence of the reduced optical anisotropy $\Delta n/gc_{BSA}\eta_0$, we determined the characteristic birefringence $[n] = 5 \times 10^{-7}$ $cm^4\ s^2/g^2$.

The value of the parameter p For the BSA–Se^0–H_2O system calculated in the approximation of $[n] \sim [n]_f$ suggest that the nanostructure is almost spherical: p = 1.2 (Table 1), as is the case with the BSA–H_2O system (at pH 6.0, p = 1.0 [12]).

The parameter Φ^* was evaluated from the calculated density of the BSA–Se^0 nanostructure: 0.1 g/cm^3 (Table 1, Eq. (1)). This value substantially exceeds the density of free BSA macromolecules in water [10].

A comparison of the hydrodynamic radius of the free BSA macromolecule (R_h = 15 nm) with the radius of the nanostructure (R_h^* = 50 nm) showed that $R_h \ll R_h^*$. Thus, the BSA–Se^0 superhigh-molecular-weight nanosystem can be thought of as a spherical dense polynuclear nanostructure or a mononuclear dense sphere composed of a selenium nucleus and multilayer protein shell comprised of about 1000 BSA macromolecules.

The formation of a BSA–Se^0 nanostructure and stabilization of selenium nanoparticles are determined by the adsorption processes caused by hydrophobic interactions of the nonpolar areas of macromolecules with the high-energy surface of nanoparticles [5] and by intermolecular electrostatic interactions of polyampholytes of protein nature.

It is well-known that sodium selenite exhibits an antitumor effect, in particular, with respect to leukemia [13, 14]. In this work, by the example of promyelocytic leukemia HL-60 cells, we demonstrated that sodium selenite and the BSA–Se^0 nanocomposite reduce the number of cancer cells in the G_1-phase of the cellular cycle (a diploid 2n DNA set) and increase the number of them in the G_2-phase (a 4n DNA set).

During the first 24 h, selenium nanocomposite and sodium selenite inhibit the growth of HL-60 cells proportionally to the increase in their concentration. The addition of sodium selenite and BSA–Se^0 at concentrations of 10 and 1–50 μmol/l, respectively, resulted in enhancement of the cell proliferation on the second day of incubation (Table 2). The addition of sodium selenite and selenium nanocomposite so as to obtain a final concentration of 200 μmol/l resulted in cancer cells death on the third day. Thus, nanoparticles of zero-valence selenium entering into the composition of BSA–Se^0 nanocomposite retain the profile of biological activity of ionic selenium, an observation indicative of the inter-

Table 2. Dynamics of changes in the number of live cells of human promyelocytic leukemia in the absence and in the presence of selenium compounds during the first (I), second (II), and third (III) days

Additives to the culture medium	I	II	III
Reference run (no additives)	499	780	1342
BSA	350	758	1170
Sodium selenium, 10 μmol/l	442	955	1313
Sodium selenium, 100 μmol/l	48	24	18
Sodium selenium, 200 μmol/l	36	22	0
BSA–Se^0, 1 μmol/l	250	702	1011
BSA–Se^0, 50 μmol/l	250	276	383
BSA–Se^0, 100 μmol/l	250	168	221
BSA–Se^0, 200 μmol/l	40	18	0

Table 3. AOS level in HL-60 cells as compared to the reference one: (A) in the absence and (B) presence of adriamycin

Cells, %	Control	Na_2SeO_3 10 μmol/l	Na_2SeO_3 100 μmol/l	BSA–Se^0 10 μmol/l	BSA–Se^0 100 μmol/l
A	100	92	92	87	87
B	150	100	150	150	100

Table 4. Content of live cells (LC) (A) in the absence and (B) presence of adriamycin, %

LC	Control	Na_2SeO_3 10 μmol/l	Na_2SeO_3 100 μmol/l	BSA–Se^0 10 μmol/l	BSA–Se^0 100 μmol/l
A	90	85	85	85	85
B	85	88	65	88	72

Note: For notations, see Table 3.

crossing of the metabolic ways of sodium selenite and the BSA–Se^0 nanosystem and of the effect of their common metabolite.

One disadvantages of adriamycin, an anthracycline-series antibiotic that is widely used in chemotherapy of tumors, is the generation of AOS [15]. It damages DNA or causes lipid peroxidation. The AOS level is normalized by selenoproteins, the activity of which depends on the selenium concentration. Therefore, we supposed that adriamycin-generated AOS may be neutralized by selenium.

To verify this assumption, we stimulated the cells by adding adriamycin in a concentration of 10 ng/ml, i.e., within the range of its concentrations in blood 1 h after an injection and later until the next injection [15]; this is a typical effective therapeutic concentration. Note that this concentration causes almost no damages in DNA; the effect concerns only AOS [16].

At a sodium selenite concentration of 10 μmol/l, the AOS level associated with the induction of free radicals by adriamycin corresponds to the control one in the absence of adriamycin. At a concentration of 100 μmol/l, the AOS level did not vary as compared with that for pure adriamycin (Table 3). For the nano-Se^0–BSA system, the addition of the complex at the concentration 10 μmol/l did not change the AOS level as compared with adriamycin; for 100 μmol/l, this level decreased to the control value (in the absence of adriamycin) but the number of live cells decreases (Table 4).

Thus, a conclusion may be drawn that selenium in the composition of the Se^0–BSA nanosystem decreases the AOS level in HL-60 cells.

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