Specific features of sorption of bovine serum albumin on hydroxyapatite doped with zinc ions, and the possibilities of its use as an intermediate binding protein for heterocyclic ligands

E. S. Shalamova,[a] A. V. Severin,[a] T. P. Trofimova,[a,b] S. S. Belyshev,[c] and M. A. Orlova[a,d]★

[a]*Lomonosov Moscow State University, Department of Chemistry, Build. 3, 1 Leninskie Gory, 119991 Moscow, Russian Federation. E-mail: orlova.radiochem@mail.ru*
[b]*Institute of Physiologically Active Compounds, Russian Academy of Sciences, 1 Severnyi proezd, 142432 Chernogolovka, Moscow Region, Russian Federation*
[c]*Lomonosov Moscow State University, Skobeltsyn Research Institute of Nuclear Physics, Build. 21, 1 Leninskie Gory, 119991 Moscow, Russian Federation*
[d]*Pirogov Russian National Research Medical University, 1 ul. Ostrovityanova, 117997 Moscow, Russian Federation*

In order to characterize hydroxyapatite (HAP) as a possible carrier of medicinal drugs and radionuclides, the possibility of using HAP modified with zinc ions (HAP-Zn) for the sorption of albumin, in particular, bovine serum albumin (BSA), was considered, using the latter as an intermediate binding protein in complex with a heterocyclic ligand of medical purpose. The kinetics of sorption of albumin correspond to the pseudo-second order, and the parameters of the sorption isotherm can be described using calculations based on the Langmuir theory of monolayer adsorption. The results of experiments on the sequential and joint sorption of a heterocyclic ligand on HAP-Zn in the presence of BSA are ambiguous about the sorption binding of this ligand to the sorbent, however, an interaction (binding) between the starting BSA and the ligand was present.

Key words: hydroxyapatite, sorption, bovine serum albumin, zinc.

Hydroxyapatite (HAP) nanoparticles are of interest as transporters of medicinal drugs and radionuclides[1] because they are biodegradable and able to be incorporated into bone tissue. These qualities result from both their calcium-phosphate composition, which is similar to the chemical composition of the mineral part of human bone, and the unique sorption properties related to the morphology of these particles.[2,3] Varying the methods of obtaining and processing HAP nanoparticles makes it possible to change their sorption ability in relation to metal ions, as well as organic ligands and their complexes.[4—6] However, many cyclic and heterocyclic ligands sorb poorly on HAP (see Ref. 7), or conditions under which HAP particles dissolve are required. Metal organic complexes have the same properties, and, in this case, the ligand also undergoes transchelation with calcium ions.[8] In the body, albumins, namely, human serum albumin (HSA) and bovine serum albumin (BSA), have a transport function, therefore, they can probably serve as an intermediate binding protein between organic ligands and carriers, which can be modified HAP nanoparticles, for example, HAP pre-doped with zinc ions (HAP-Zn).[9] This modification of the sorbent helps to create additional coordination centers on the surface of nanoparticles and, at the same time, assists the preliminary replacement of mobile calcium ions with zinc ions to prevent (or slow down) overchelation. The specific features of sorption on HAP, which indicate the possibility of a change in the conformation of albumins in this process, were presented in the work.[10] The goal of this work is to study the mechanism of sorption—desorption variants of bovine serum albumin on HAP-Zn and to assess the suitability of BSA as a binding component for heterocyclic ligands.

Experimental

Synthesis of nano-hydroxyapatite doped with zinc ions (15 mol.% Zn of the number of moles of Ca). An 87% aqueous solution of H_3PO_4 (20—22 mL) was added to zinc oxide (1.475 g), until its complete dissolution (~20 min). The resulting solution was added with stirring to a suspension of calcium oxide (6.8 g of calcium oxide calcined at 1100 °C for 3 h, 200 mL of water). A 30% aqueous solution of H_3PO_4 was added dropwise to the suspension, reaching pH 7. The solid phase content in the resulting suspension was determined gravimetrically by drying a portion of the suspension to constant weigh; this content was found to be 5.25%, the density of the suspension was 1.05 g mL^{-1}. Thus,

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samples of HAP doped with zinc ions (HAP-Zn) were obtained. This technique is described in more detail in Ref. 9.

Stability of BSA solutions at 20—25 °C. The stability of a 12 mg mL^{-1} aqueous solution of BSA (MP Biomedicals, fraction V, reagent grade) in distilled water over time was studied spectrophotometrically (Shimadzu UV-1280 spectrophotometer, Japan) in the range of wavelengths 190—360 nm, the measurements were carried out in a 1-cm quartz cell (λ_{max} = 280 nm).

Kinetics of sorption of BSA on HAP-Zn. The kinetic regularities of sorption of BSA on HAP-Zn were investigated spectrophotometrically by measuring the residual BSA in the solution after sorption. For this purpose, distilled water (7 mL), a suspension of HAP-Zn (1 mL), and a 12 mg mL^{-1} aqueous solution of BSA (2 mL) were added to ten polyethylene test tubes (10 mL). The resulting solutions were mixed on a shaker (Multi Bio RS-24, Latvia) for various periods of time, after which the contents of the test tubes were centrifuged for 2 min (3000 *g*, MLW T.51.1, GDR), the supernatant was removed, diluted 3 times with distilled water, and the absorption spectrum was recorded (Shimadzu UV-1280, Japan). The absorption at 280 nm was compared to a calibration curve for BSA solutions (Fig. 1, *b*). The absorption coefficient for BSA is $\varepsilon = 0.5945$ mL mg^{-1} cm^{-1}.

Isotherm of sorption of BSA on HAP-Zn. A suspension of HAP-Zn (1 mL) and a 12 mg mL^{-1} aqueous solution of BSA (0.1—6 mL) were added to eight polyethylene test tubes, the volume of the suspension was increased to 10 mL using distilled water, the result was stirred for 3 h, centrifuged (3000 *g*) for 6 min, the supernatant was removed, and the concentration of BSA was determined spectrophotometrically as described above.

Desorption of BSA from HAP-Zn. A 12 mg mL^{-1} aqueous solution of BSA (1 mL), a suspension of HAP-Zn (0.5 mL), and distilled water (3.5 mL) were added to seven polyethylene test tubes (10 mL). The test tubes were placed on a shaker (Multi Bio RS-24, Latvia) for 188 min, after which they were centrifuged (3000 *g*) for 6 min. Aliquots (4.5 mL) of the solution was taken from each test tube, distilled water (4.5 mL) was added, and mixed for various periods of time. After that, an aliquot (2 mL) was taken using an automatic dispenser, transferred into a polyethylene Eppendorf tube (Sarstedt, Germany), and centrifuged for 4 min in an Elmi CM-50M centrifuge (15000 *g*, Latvia). The supernatant was removed and the concentration of BSA was determined spectrophotometrically.

Sequential sorption of *N*-(5,6-dihydro-4*H*-1,3-thiazin-2-yl) benzamide hydrobromide (L • HBr) on HAP-Zn-BSA. Five samples were prepared, each containing a suspension of HAP-Zn (0.5 mL), a 12 mg mL^{-1} solution of BSA (0.25 mL), and distilled water (4.25 mL). After preliminary sorption and phase separation by centrifugation (3000 *g*), the supernatant (4.4 mL) was removed, and the precipitate was washed with distilled water (5 mL). Then, distilled water (3.4 mL) and a 30 μg mL^{-1} solution of L • HBr (1 mL) were added to the resulting precipitate. The test tubes were mixed on a Multi Bio RS-24 shaker for various periods of time. Then, the phases were separated by centrifugation (3000 *g*, 6 min), the supernatants were removed, diluted 3 times with distilled water, and the absorption spectra of the resulting solutions were registered.

Joint sorption of L • HBr and BSA on HAP-Zn. Two parallel experiments were carried out to study the joint sorption of BSA and L. In the first experiment, sorption of BSA and L on HAP-Zn was carried out simultaneously, while in the second one, sorption of BSA with the same concentration on HAP-Zn was carried out in the absence of the ligand. In the first case, a 30 μg mL^{-1} solution of L • HBr (1 mL), a 12 mg mL^{-1} solution of BSA (0.25 mL), distilled water (3.25 mL), and a suspension of HAP-Zn (0.5 mL) were mixed in six polyethylene test tubes. The samples were mixed on a Multi Bio RS-24 shaker and centrifuged after specific periods of time (3000 *g*, 6 min), the supernatants were removed, and their absorption spectra were registered. In the second experiment, sorption of albumin was carried out as described in the previous section. The registration of time began simultaneously with the first experiment. Phase separation and sampling of the supernatant for analysis were performed in parallel. For comparison, two "starting" solutions were prepared: 30 μg mL^{-1} L • HBr and 12 mg mL^{-1} BSA. The optical density of these solutions was measured both immediately after their preparation and simultaneously with the registration of the spectra of the samples obtained in the first and second experiments.

Binding of BSA with *N*-(5,6-dihydro-4*H*-1,3-thiazin-2-yl)-benzamide hydrochloride (L • HCl). Dialysis was used to assess the possibility of binding L • HCl to BSA (Fig. 2). Dialysis bags were used (regenerated cellulose, Scienova GmbH, Germany). Eppendorf tubes (1.5 mL) containing water (1.2 mL, solution No. 1) and a 3 mg mL^{-1} solution of BSA (1.2 mL, solution No. 2) were placed in two scintillation glass vials (20 mL, Sarstedt, Germany). The test tube caps had openings and were connected to dialysis bags with a pore size corresponding to a retention of substances with a molecular weight of more than 12 kDa (MWCO). After placing the Eppendorf tubes inside the vials upside down, a 30 μg mL^{-1} solution of L • HCl was added to the vials, completely covering the tubes. The amount of solution

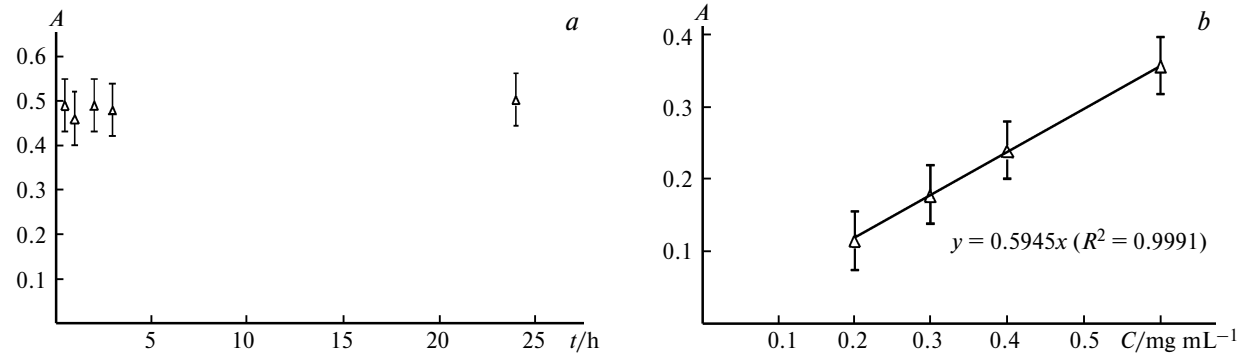

Fig. 1. (*a*) Kinetics of changes in the optical density (*A*) of a BSA solution at room temperature (λ = 280 nm); (*b*) concentration calibration curve for BSA; measurement error is 10—12%.

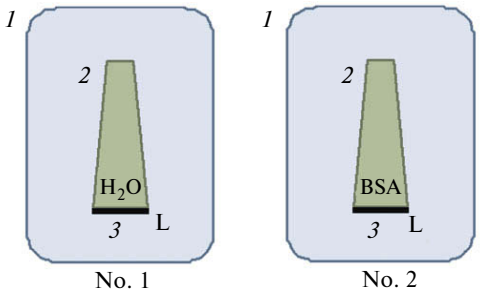

Fig. 2. Dialysis scheme; glass vial (*1*); Eppendorf tube with dialysis bag (*2*) inserted into the cap (*3*).

added was determined from the weight of the vials before and after the addition of the solution. The vials were capped and mixed on a Multi Bio RS-24 shaker. After specific periods of time, the external solution of ligand L·HCl was sampled from both vials, and the relative optical density of the solutions was measured on a Shimadzu UV-1280 spectrophotometer to determine the concentration of the ligand in the external solution in the two vials. The amount of ligand bound to BSA was calculated from the difference in the concentration of the ligand in the vial with BSA and without it. A calibration curve for L·HCl was obtained preliminarily.

Results and Discussion

Figure 1, *a* shows that the concentration of BSA in the solution in the test tubes at room temperature does not change over 24 h. This makes it possible to study the sorption of BSA on HAP-Zn. The kinetics of sorption of BSA on HAP-Zn can be described by a smooth curve (Fig. 3), which reaches saturation (216 mg BSA (g HAP-Zn)$^{-1}$) in about 8—9 hours. Desorption was not observed for at least 24 h after saturation.

Processing the kinetic curve using pseudo-first and pseudo-second order models showed that the experimental results are described more fittingly using the pseudo-second order model (Table 1). Using the Langmuir model, the following parameters of the isotherm of BSA sorption on HAP-Zn were obtained: $\Gamma_{max} = 285.7$ mg BSA (g HAP-Zn)$^{-1}$, K_L = 2.19 mL (mg BSA)$^{-1}$, R^2 = 0.9911. Using the Freundlich model, the following values were obtained: $k = 157.4$; $n = 0.4565$; $R^2 = 0.8453$. The isotherm of BSA sorption on HAP-Zn and the results of experimental data processing using the Langmuir monolayer adsorption model and the Freundlich empirical model are shown in Fig. 4. It can be seen that the experimental isotherm

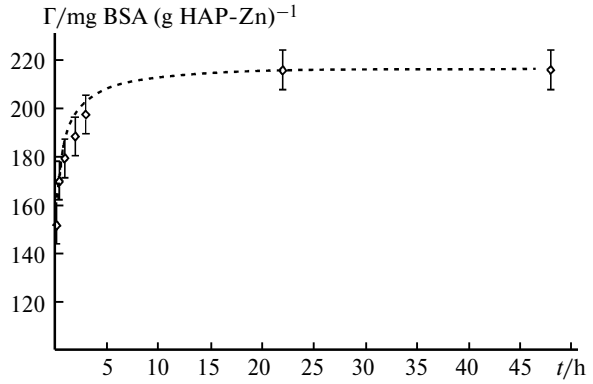

Fig. 3. Kinetics of sorption of BSA on HAP-Zn.

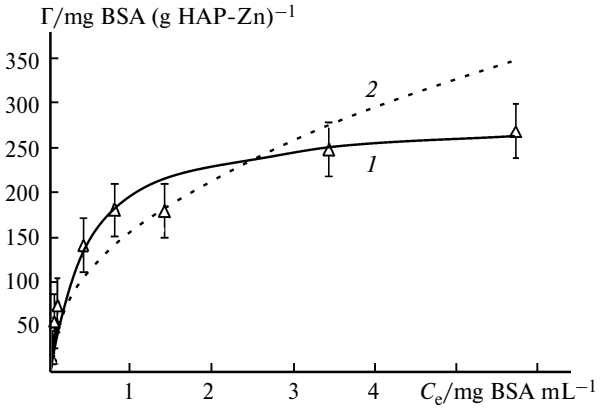

Fig. 4. Comparison of experimental and theoretical models of the isotherm of sorption of BSA on HAP-Zn: experiment (triangles), calculations using the Langmuir-type model (*1*) and the Freundlich-type model (*2*); C_e is the equilibrium concentration of BSA in the mother liquor after sorption.

of sorption is described better by the Langmuir-type model.

When comparing the kinetics of sorption of BSA on ordinary HAP studied in Ref. 9 and on HAP-Zn, it was determined that the period of time required to reach the plateau at comparable concentrations of the starting solution of BSA is considerably longer in the latter case (60 and ~300 min, respectively), the sorption capacity also increases considerably (37 and 217 mg BSA (g HAP)$^{-1}$, respectively). This is probably resultant from the use of an aqueous suspension of modified HAP-Zn as a sorbent, rather than HAP powder as in Ref. 9. In addition, the presence of zinc in the HAP-Zn sample affects the mechanism and kinet-

Table 1. Kinetic parameters of sorption of BSA on HAP-Zn calculated using pseudo-first and pseudo-second order models (*W*)

W	*k*	Γ_e/mg BSA (g HAP-Zn)$^{-1}$	R^2
1	$6.11 \cdot 10^{-3}$ min^{-1}	216	0.99894
2	$3.86 \cdot 10^{-4}$ g HAP-Zn (mg BSA)$^{-1}$ min^{-1}	217	0.99998

ics of sorption, because the zinc ions themselves are capable of forming complexes with BSA and, therefore, will serve as additional coordination centers. A comparison of sorption isotherms shows that they are in good agreement with each other in comparable ranges of equilibrium concentrations of BSA. Our experimental results correspond to the first stage of the two-stage sorption isotherm[10] and can be formally described by the models we have chosen.

The results of the experiment on the sequential sorption of L on HAP-Zn-BSA are shown in Fig. 5, *a*. It can be seen that the starting concentration of the ligand is lower than its concentration in residual solutions after sorption, which is calculated taking into account the optical density of the maxima in the spectra. Most likely, a complex interaction occurs between the ligand and BSA during the sorption of L on the sorbent. In particular, it can include desorption of BSA from HAP, formation of a complex between BSA and the ligand, and subsequent sorption of the complex. Since the peaks in the absorption spectra of the ligand and BSA overlap, it is likely that the sum spectra of the ligand and BSA or the complex were recorded in this experiment. Therefore, it is difficult to reliably estimate the probability of this interaction, since the concentrations of the ligand in residual solutions calculated based on the spectra are the same as the starting concentrations of the ligand taking into account the measurement error. Thus, these results indicate rather the absence of sorption of this ligand in the sequential mode of sorption on HAP-Zn-BSA.

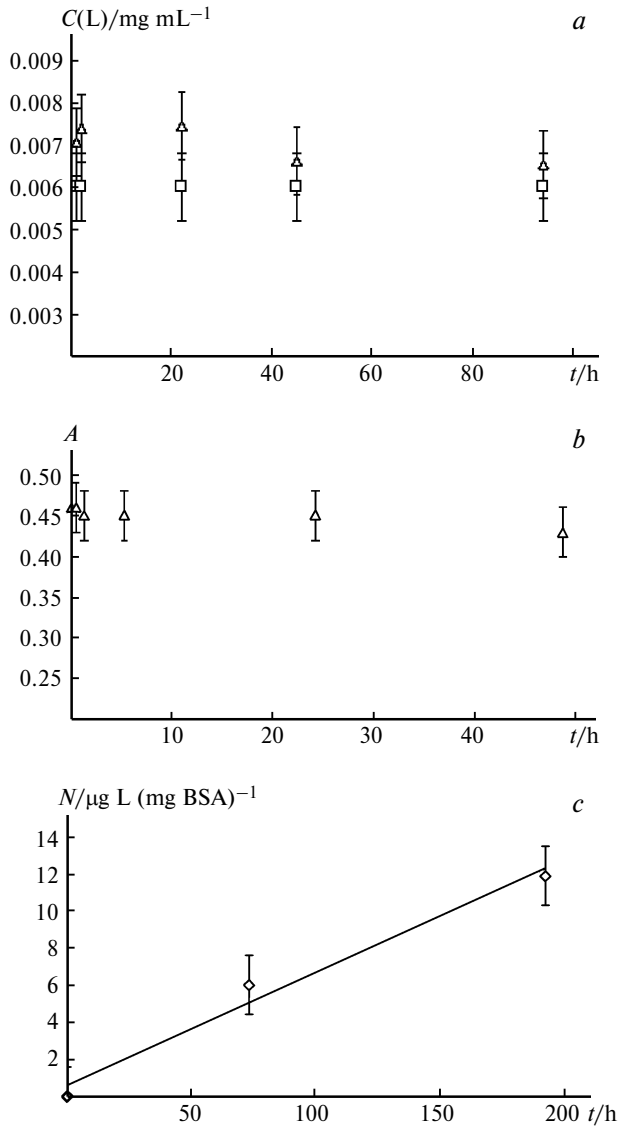

Fig. 5. Interaction between the components of the investigated system: (*a*) kinetics of sorption of L on HAP-Zn-BSA (squares is pure solution of ligand L; triangles is content of ligand L in mother liquor after sorption); (*b*) kinetics of changes in optical density of L in residual solutions after joint sorption of L and BSA on HAP-Zn; (*c*) binding of BSA with ligand L (determined by dialysis); *N* is amount of ligand L which passed through the dialysis bag and became bound to albumin.

The results of the study of joint sorption of ligand L and BSA on HAP-Zn are shown in Fig. 5, *b*. The optical density of the residual solution upon sorption of pure BSA was subtracted from the optical density of the residual solution after joint sorption (because of the overlap of the maxima in BSA and L spectra). The Figure indicates that the concentration of ligand L in residual solutions does not change within the measurement error. Therefore, it can be inferred that the use of BSA as an intermediate binding protein does not lead to the formation of a sorption complex of ligand L with modified HAP-Zn in sequential or in joint sorption. The reason for this may be the formation of a stronger complex between the protein and the ligand in the solution. A dialysis experiment was carried out to test this assumption. It was found that ligand L can bind with BSA during dialysis, which can be seen in Fig. 5, *c*. The question of how this binding influences the structure of the protein and its ability toward sorption binding with HAP requires additional study.

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This paper does not contain descriptions of studies on animals or humans.

The authors declare no competing interests.

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