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COMPLEXATION OF IBUPROFEN WITH BOVINE SERUM ALBUMIN: SPECTROSCOPIC STUDY AND MOLECULAR SIMULATION

The interaction of ibuprofen with bovine serum albumin (BSA) in the aqueous environment has been studied using both experimental and computer simulation methods. The fluorescence quenching spectra are obtained at a constant BSA concentration of 2 μM, varying ibuprofen concentrations of 0–1.5 μM, and three fixed temperatures of 293, 303, and 313 K. The intensity dependences follow the Stern–Volmer equation and testify to the static quenching mechanism. Together with the temperature-induced growth of the binding constant, this result points to the predominantly hydrophobic nature of the interaction. The obtained binding constants equal lg $K_s = 4.3 \div 5.0$ at the binding stoichiometry close to 1 : 1. The thermodynamic analysis of the complexation showed that $\Delta G < 0$, $\Delta H > 0$, and $\Delta S > 0$, G, H, S which confirms the spontaneous and entropy-driven character of the binding process. Molecular docking simulation using AutoDock 4.2.6 made it possible to identify three main binding sites of ibuprofen with BSA. The most energetically favorable binding modes include van der Waals, hydrogen bonding, hydrophobic, and electrostatic interactions; nevertheless, contacts with hydrophobic residues of BSA prevail. The calculated spatial arrangement of ibuprofen with respect to tryptophan residues corresponds to the experimentally observed fluorescence quenching.

Keywords: bovine serum albumin, ibuprofen, fluorescence quenching, molecular docking.

1. Introduction

In modern pharmacy, protein nanoconjugates and protein-functionalized nanoparticles become of particular importance as transport platforms for drugs, by increasing their bioavailability, stability, and targeted delivery [1–3]. Among the protein components used as a basis for the creation of nanotransport systems, bovine serum albumin (BSA) and leporine serum albumin (LSA) attract special attention. They are globular, water-soluble, consist of three domains (each with two subdomains), and have a high proportion of α-helices (≈67%) [4]. BSA and LSA demonstrate low immunogenicity, high biocompatibility, long half-life, and the ability to bind a wide range of ligands (lipids, hormones, drug compounds) at their hydrophobic sites of subdomains IIA and IIIA [5]. Serum albumin, owing to its relative availability and high affinity for ligands, is widely used as a model protein in spectroscopic (fluorescence quenching due to the presence of tryptophan in the amino acid sequence), calorimetric, and bioinformatic studies of the pharmacokinetics and pharmacodynamics of drugs [6]. In particular, the physicochemical aspects of the BSA interaction with widely used bioactive compounds [7–10], metal nanoparticles [11–15], and their oxides [16–20] demonstrate the importance

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of protein carriers in the control over pharmacokinetics and pharmacodynamics.

Among the drugs, ibuprofen is widely used. This is a weakly acidic, lipophilic, nonsteroidal, anti-inflammatory drug with a high affinity to blood plasma albumins, which determines its pharmacokinetic properties [21–23]. The previous studies of the ibuprofen interaction with albumin were limited to either fluorescence quenching [24, 25] or molecular simulation [26] separately. However, an integrated approach, a combination of spectroscopy and molecular docking, is required. The aim of this work is to analyze the interaction of ibuprofen with BSA using a combined approach of fluorescence spectroscopy and molecular docking, and quantitatively evaluate the binding constants, the mechanism of interaction, and molecular complexation sites, which is necessary for predicting the behavior of the drug *in vivo*. The study of this interaction is challenging for assessing the delivery efficiency, bioavailability, and potential systemic effects of the drug. The research methods included the molecular simulation and an experimental method of fluorescence quenching of aqueous solutions of BSA and ibuprofen.

2. Materials and Methods

When preparing specimens, BSA (Sigma, USA) and ibuprofen (China, supplied by LLC "Bioline") – the quality of the latter met the requirements of the monograph of the State Pharmacopoeia of Ukraine [27] – were used. The powdered substances were dissolved in water (pH = 5.5) with the addition of dimethyl sulfoxide (DMSO) to the required ratio in a volume of 1.5 ml, which was associated with the poor solubility of ibuprofen in aqueous solutions [21]. The percentage of DMSO in the solutions was proportional to the ibuprofen concentration. The BSA concentration was constant in all specimens, $C_{\rm BSA} = 2 \times 10^{-6}$ M, whereas the ibuprofen concentration varied ($C_{ib} = 0$, 2.5×10^{-7}, 5×10^{-7}, 7.5×10^{-7}, 10^{-6}, and 1.5×10^{-6} M).

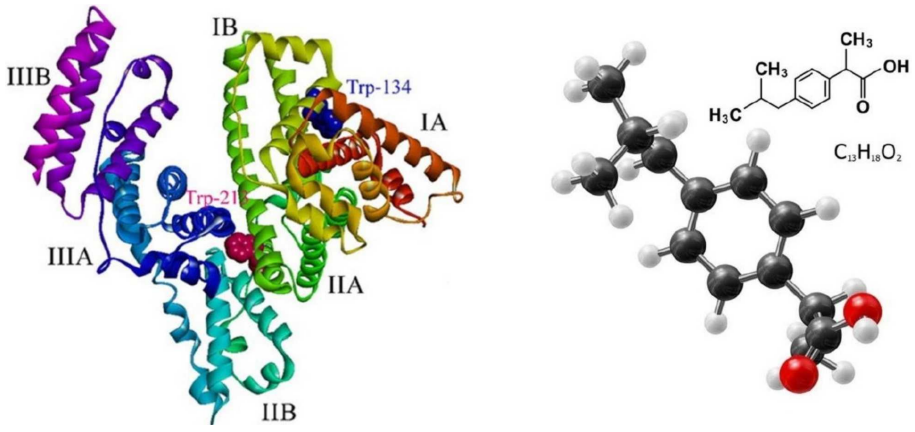
The fluorescence spectra of the aqueous BSA-ibuprofen solutions were registered at three temperatures $T = 293, 303,$ and 313 K using a stationary spectrofluorometer QuantaMaster 40 Intensity Based Spectrofluorometer (PTI, HORIBA Scientific, Canada).

The fluorescence in the aqueous BSA solution is a result of the presence of aromatic amino acids in the BSA protein molecule; mainly, this is tryptophan, which acts as the main internal fluorophore. When excited at about 280 nm, tryptophan emits, with a spectral maximum located at about 340 nm. Provided the indicated pH level, BSA has a positive charge, which allows the aggregation of macromolecules and the spectrum distortion to be avoided [28]. The concentrations of the substances were chosen so that the ligand/protein ratio remained relevant for *in vivo* [28, 29].

Fluorescence spectroscopy is a sensitive and informative method for studying the complexation of proteins with drug molecules because it allows the *in vitro* registration of changes in the microenvironment of the internal fluorophores of the protein macromolecule: amino acid residues of tryptophan (Trp213 and Trp134 in BSA), tyrosine, or phenylalanine. Interaction with a ligand (a drug molecule) can invoke a change of the fluorescence intensity, a shift of the emission maximum, a change in the quantum yield or in the lifetime of the excited state, which are indicators of complexation. In addition, the analysis of fluorescence quenching in the framework of the dynamic and static quenching models (the Stern–Volmer theory) makes it possible to determine the binding constants, the number of binding centers, and the nature of interaction (hydrophobic, electrostatic, hydrogen, and so forth). The method is characterized by a high sensitivity, which allows working with micromolar concentrations [30].

Computer simulation of the interaction between BSA and ibuprofen molecules was carried out using the molecular docking method, which makes it possible to predict the most probable spatial orientation of the ligand molecule (the drug) at the active center of the receptor (the protein) and evaluate the interaction energy in the complex [31]. The essence of the method consists in a systematic or stochastic search for ligand positions with respect to the receptor and a subsequent assessment of the complex stability using a scoring function. Molecular docking was performed using the AutoDock 4 program, which implements a genetic algorithm for generating conformations and calculates the semi-empirical binding free energy (the scoring function) in the following form:

$$\Delta G_{\rm bind} = \Delta G_{vdW} + \Delta G_{\rm elec} + \Delta G_{h\rm bond} + \\ + \Delta G_{\rm desolv} + \Delta G_{\rm tors}, \quad (1)$$



a *b*

Fig. 1. BSA (*a*) and ibuprofen molecules (*b*)

where ΔG_{bind} is the binding energy considered as the difference between the free energy of the complex and the energies of separate molecules. The summands on the right-hand side of this equality correspond to the contributions of the van der Waals interaction, the electrostatic interaction, hydrogen bonds, the influence of the solvent, and the entropy component; the latter describes the loss of the ligand's rotational degrees of freedom, when the ligand binds with the receptor. When searching for molecular complex conformations, the binding modes were ranked according to the binding energy values. The structure of the BSA molecule (Fig. 1) was obtained from the crystallographic database RCSB Protein Data Bank (code 4F5S) [33], and the coordinates of the ibuprofen molecule were taken from PubChem (CID: 3672) [33].

3. Fluorescence Spectroscopic Study

In Fig. 2, the registered fluorescence spectra of the aqueous solutions of BSA (at a fixed concentration of 2 μM) and ibuprofen (with a varying concentration) and, at various temperatures of 293 K (20 °C), 303 K (30 °C) and, 313 K (40 °C), are shown. The indicated values were chosen to study the interaction of BSA with buprofien, because they are physiologically relevant for *in vivo* conditions and, at the same time, remain below the BSA denaturation temperature (about 60 °C) [34]. Several temperatures are necessary for the analysis of the temperature dependence of molecular binding using the thermodynamic parameters of the interaction in order to elucidate the nature of dominant forces in the complexation process [35].

As the drug concentration grows, a monotonic decrease in the BSA fluorescence intensity (fluorescence quenching) is observed at all temperatures. Such a behavior can occur due to several physicochemical mechanisms [30]. First, the dynamic mechanism is possible, which is caused by collisions of the fluorophore (a tryptophan residue) with the ligand, giving rise to non-radiative relaxation of the fluorophore from the excited to the ground state. The static mechanism is also possible, which consists in the formation of a stable non-fluorescent complex between the protein molecule and the ligand, without the fluorophore transition into the excited state. The intensity reduction can also be a consequence of the Förster resonance energy transfer (FRET), if the ligand (acceptor) is located at a small distance (up to 6 nm) from the fluorophore (donor) and receives energy from the latter via the dipole-dipole interaction. In addition, conformational changes of the protein near the fluorophore can change its microenvironment and, accordingly, the fluorescence quantum yield. Charge transfer or intramolecular relaxation processes can also play a role in the intensity decrease. Finally, an important factor is the overabsorption of the excitation or emission light by the ligand (the inner filter effect), when high drug concentrations lead to partial light absorption [36]. Since the studies were carried out at low ibuprofen concentrations ($\leq 1.5 \times 10^{-6}$ M), when the optical density of the solutions remained below the inner filtration effect threshold, the influ-

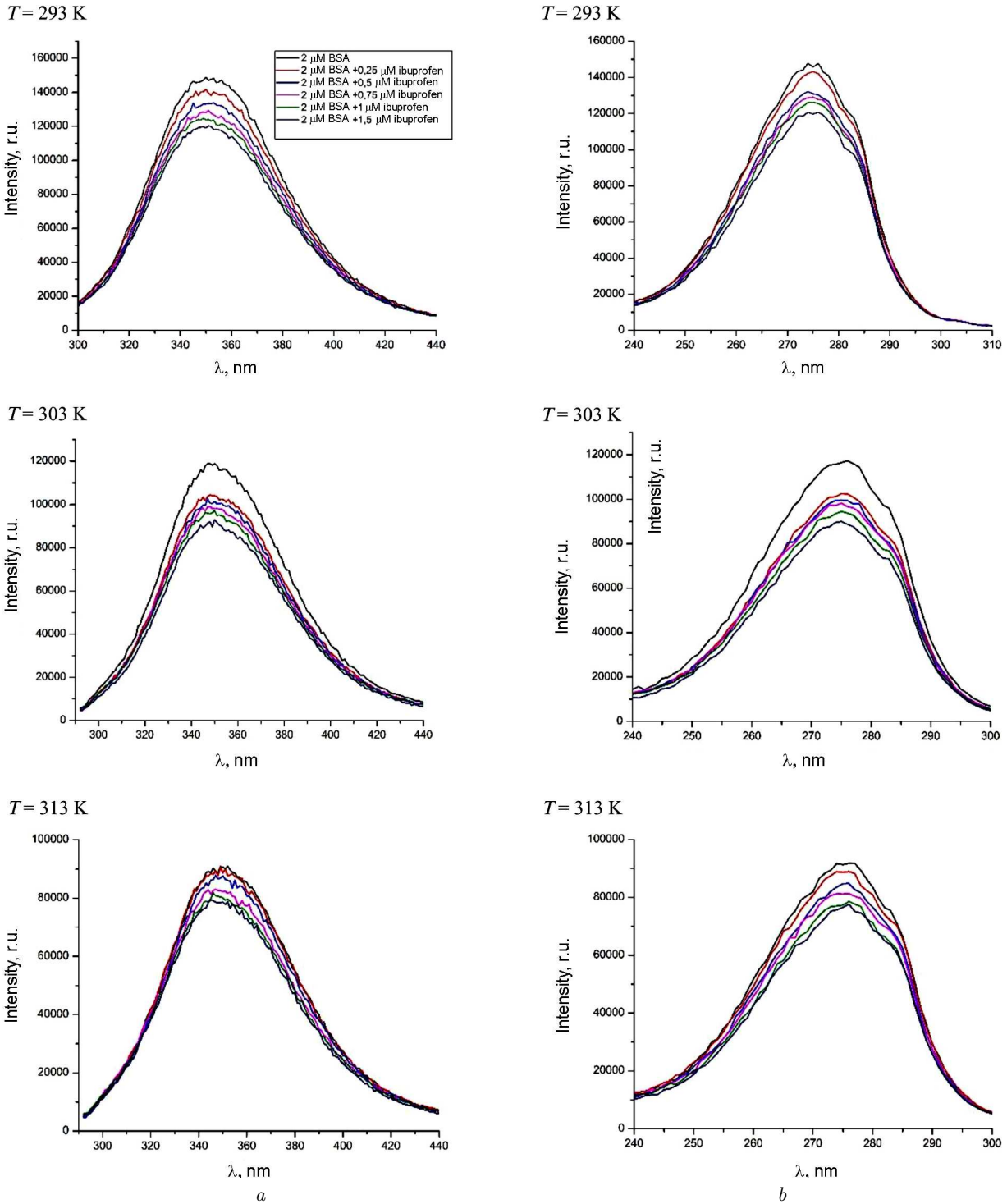
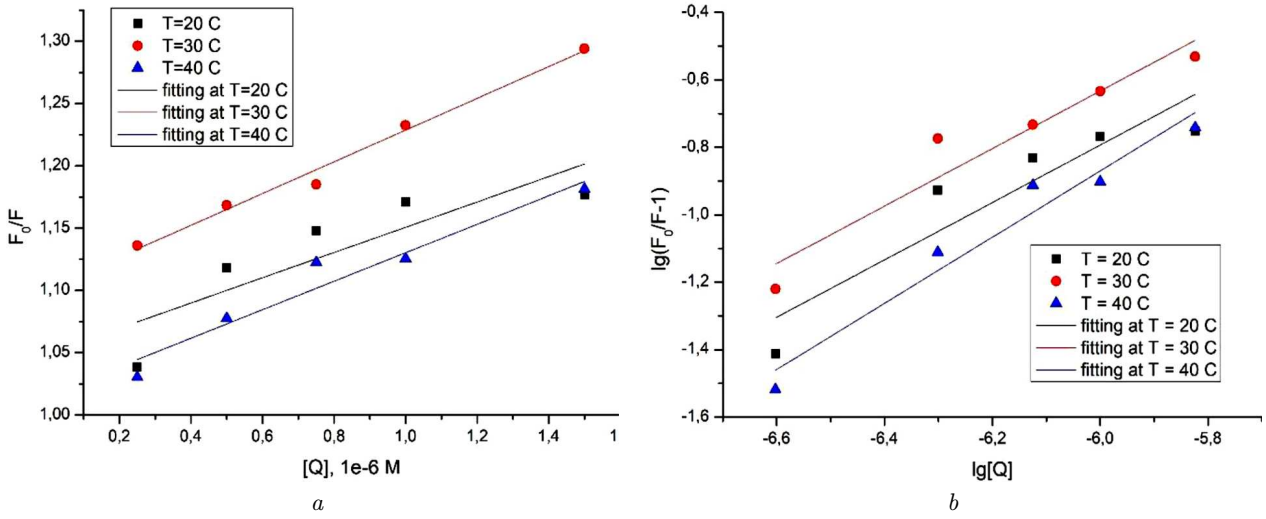


Fig. 2. Emission ($\lambda_{ex} = 353$ nm) (*a*) and excitation ($\lambda_{em} = 275$ nm) fluorescence spectra of the aqueous solutions of BSA and ibuprofen at various temperatures (*b*)



a *b*

Fig. 3. Stern–Volmer (*a*) and Hill (*b*) dependences for describing the dynamic and static mechanisms of fluorescence quenching

ence of this effect on the results of fluorescence analysis can be considered insignificant [36]. Furthermore, the absence of the shifts of the maxima in the emission spectra testifies to the stability of the tryptophan residue microenvironment, which points to the absence of substantial conformational changes in the protein structure, when the protein binds to the ligand [37]. Thus, the observed fluorescence quenching takes place mainly due to specific mechanisms of protein-ligand interaction: dynamic quenching, static complexation, or FRET (if the distances to the fluorophore are sufficiently small).

Dynamic quenching is considered to be a result of contacts (collisions) between the fluorophore and quencher molecules, owing to their diffusional motion, within the lifetime of the energy donor in the excited state. This quenching process is described by the Stern–Volmer equation [30]

$$\frac{F_0}{F} = 1 + K_{\mathrm{SV}}\,[Q] = 1 + k_q \tau_q\,[Q], \qquad (2)$$

where F_0 and F are the fluorescence intensities in the BSA solutions in the quencher absence and presence, respectively. In the Stern–Volmer theory, it is assumed that the ratio F_0/F linearly depends on the quencher concentration $[Q]$ with the following parameters: K_{SV} is the Stern–Volmer quenching constant, and k_q is the biomolecular quenching rate constant (they are fixed at dynamic quenching). These constants are related to each other by the relationship $K_{\mathrm{SV}} = k_q\tau_0$, where τ_0 is the lifetime of the fluorophore in the excited state ($\tau_0 = 10^{-8}$ s). As a result of the linear approximation of the experimental data (Fig. 3, *a*), the obtained values for the quenching parameter k_q (see Table 1) exceed the maximum permissible value of 2×10^{10} $\mathrm{M}^{-1}\mathrm{s}^{-1}$ for the process of mutual diffusion of BSA and ibuprofen molecules [30]. Therefore, another quenching mechanism, static, is realized.

The static mechanism is realized through the formation of the stable molecular complexes of protein with drugs in the ground state with non-excited fluorophores. For this mechanism, the analysis of fluorescence quenching is carried out using the Hill equation [30]

$$\lg \frac{F_0 - F}{F} = \lg\, K_A + n \lg\,[Q]\,, \qquad (3)$$

where K_A is the binding constant of the biomolecular complex, and n is the number of ligand-protein binding sites. This equation represents the linear dependence of fluorescence quenching on the quencher concentration (on the logarithmic scale). The corresponding straight line (Fig. 3, *b*) makes it possible to determine the binding parameters n and K_A.

The obtained values (see Table 2) demonstrate that the interaction becomes stronger as the temperature increases, which is characteristic of the hydrophobic nature of the complexation between the ibuprofen and BSA molecules [38], when binding is accompanied by the removal of water from the hydrophobic protein pockets and the exposure of hydrophobic amino

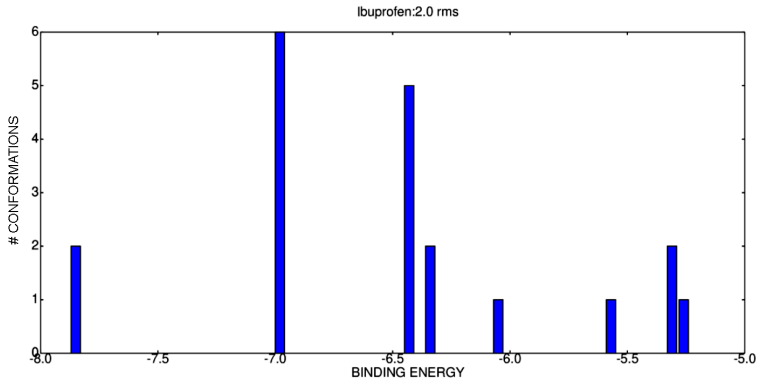


Fig. 4. Кластеризація мод зв'язування БСА з ібупрофеном (енергія вказана в ккал/моль

acid residues (the entropic effect). The magnitudes of those values testify to a moderately strong binding in the complex where one ibuprofen molecule is attached to one BSA molecule. The values of the binding constants determined at various temperatures T allow the changes of the thermodynamic parameters after the complexation to be calculated using the van't Hoff equations

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_A, \qquad (4)$$

where ΔG is the Gibbs free energy, ΔH is the enthalpy, ΔS is the entropy, and $R = 8.314\ \mathrm{J \cdot mol^{-1}K^{-1}}$.

The thermodynamic characteristics obtained by substituting the binding parameters into Eq. (4) are quoted in Table 3. The negative value of the binding energy points to the spontaneous interaction character and the presence of electrostatic interaction. The positive entropy and enthalpy values confirm the dominant hydrophobic nature of complexation.

4. Molecular Docking

Molecular docking simulation was performed with the help of the AutoDock 4.2.6 software package by applying a semi-empirical scoring function and the Lamarckian genetic algorithm to search for the most energetically favorable configuration of the BSA-ibuprofen complex. During the simulation, the structure of the protein macromolecule was rigidly fixed (rigid docking), and the ligand molecule (ibuprofen) was considered flexible due to the presence of five rotational degrees of freedom (Fig. 1). As a result of simulation, 20 modes (see Fig. 5, *a*) of molecular com-

Table 1. **Quenching constants K_{V} and k_q for aqueous BSA–ibuprofen solutions at various temperatures**

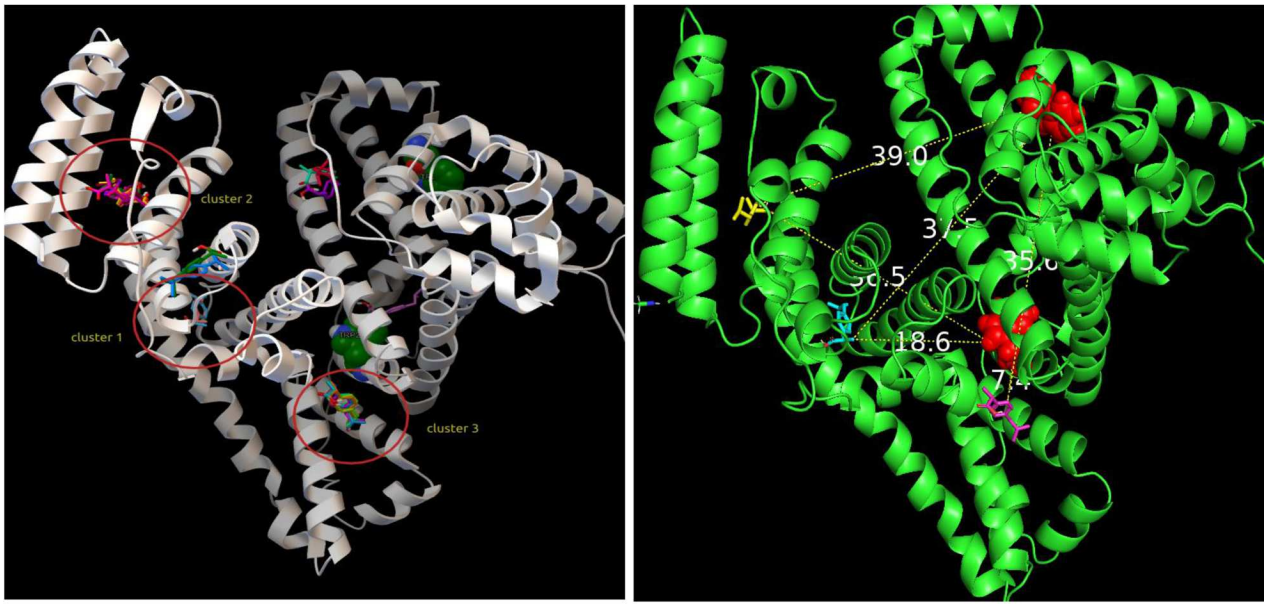
T, K	Quenching parameters	
	$k_q, \times 10^{14}$, $\mathrm{M^{-1}s^{-1}}$	$K_{\mathrm{SV}}, \times 10^6$, $\mathrm{M^{-1}}$
293	0.1 ± 0.033	0.1 ± 0.033
303	0.13 ± 0.008	0.13 ± 0.008
313	0.11 ± 0.016	0.11 ± 0.016

Table 2. **Binding constants K_A and the number of binding sites n for aqueous BSA–ibuprofen solutions at various temperatures**

T, K	Binding parameters	
	$\lg K_A$	n
293	4.3 ± 1.2	0.85 ± 0.2
303	4.5 ± 0.87	0.9 ± 0.14
313	5 ± 0.75	1 ± 0.11

Table 3. **Thermodynamic parameters of aqueous BSA–ibuprofen solutions**

T, K	Thermodynamic parameters		
	$\Delta G \times 10^3$, J/mol	ΔS, $\mathrm{J \cdot mol^{-1} \cdot K^{-1}}$	$\Delta H \times 10^3$, J/mol
293	-24.1 ± 6.7	290 ± 79	61.1 ± 16.4
303	-26.1 ± 5	287 ± 71	
313	-30 ± 4.5	291 ± 68	



a *b*

Fig. 5. (Binding mode clusters *a*) and their most optimal modes; distances to two fluorescent tryptophan amino acid residues are indicated in angstrom units (*b*)

plexes were determined, which can be grouped into several clusters by their close arrangement and binding energy values (Fig. 4).

The most numerous and energetically favorable clusters are highlighted in Fig. 5, *a*. Only one cluster contains ligand conformations located in close proximity to the tryptophan amino acid residue Trp213 in subdomain IIA. The other clusters are located in subdomain IIIA, and they are distant from the tryptophan residues. The arrangement of the clusters with respect to the BSA molecule and the tryptophan amino acid residues is shown in Fig. 5, *b*. The distances to the residues from the most optimal modes of each of the three clusters are given in Table 4. The average distances to the tryptophan residues for each mode are noticeable as compared to the characteristic

Table 4. **Distances from ibuprofen molecules to tryptophan residues (in angstrom units)**

Tryptophan residue	Mode 1 (cluster 1)	Mode 3 (cluster 2)	Mode 9 (cluster 3)
Trp134	37.5	35.6	39
Trp213	18.6	7.4	36.5

size of the BSA molecule, so the fluorescence quenching is not substantial (which, in fact, is observed experimentally; see Fig. 1), but possible because the distance values are smaller than the threshold value (6 nm) for the Förster mechanism of non-radiative energy transfer from the donor (tryptophan) to the acceptor (ibuprofen).

The binding energies of molecules in the complex, which were determined for the three clusters from docking simulation, correspond to the values obtained from fluorescence quenching spectral data. The considerable negative value of ΔG testifies to the formation of stable complexes and, therefore, confirms the static quenching mechanism involving the FRET. The interaction energy of molecules in the complexes includes contributions from van der Waals and hydrophobic interactions, hydrogen bonds, and electrostatic forces (see Table 5). The first two clusters include 8 of the 20 modes, which are characterized by a substantial electrostatic component, so their realization is more probable due to long-range electrostatic forces. The electrostatic interaction is stronger for the 2nd cluster, whereas van der Waals forces, hydrogen bonds, and the hydrophobic effect are more intense for the 1st cluster. The dominant role of a particular interaction type is determined by the type

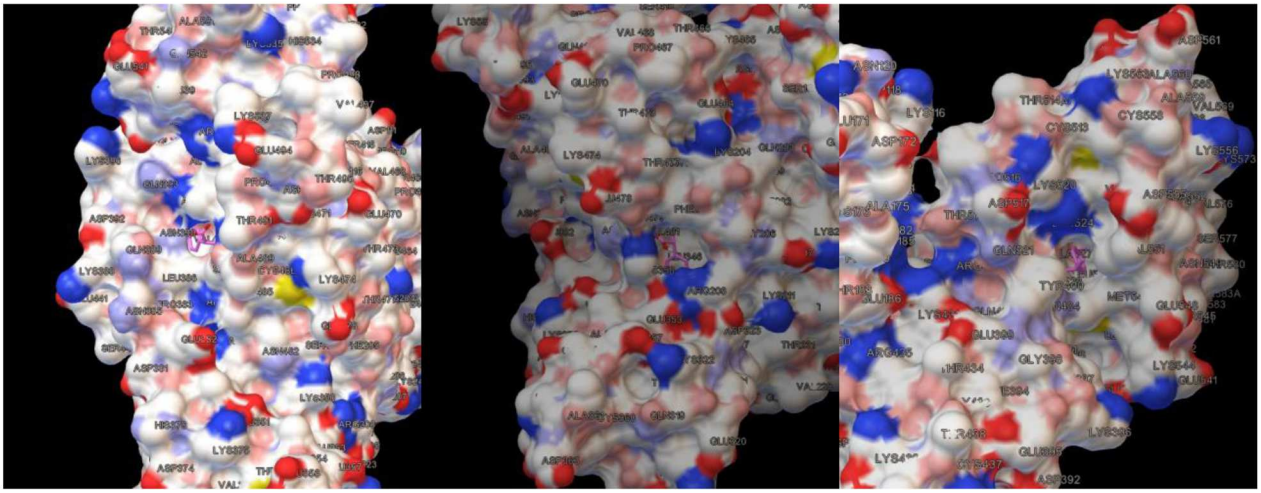
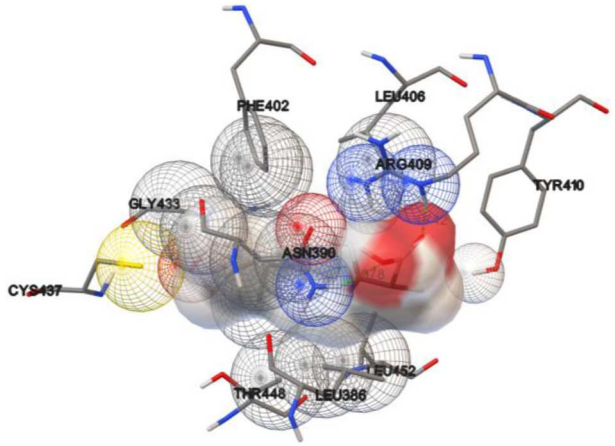


Fig. 6. Binding sites of the ibuprofen molecule with the BSA molecule in the most optimal modes for three docking simulation clusters. Negatively charged, positively charged, and hydrophobic amino acid residues are marked in red, blue, and yellow, respectively



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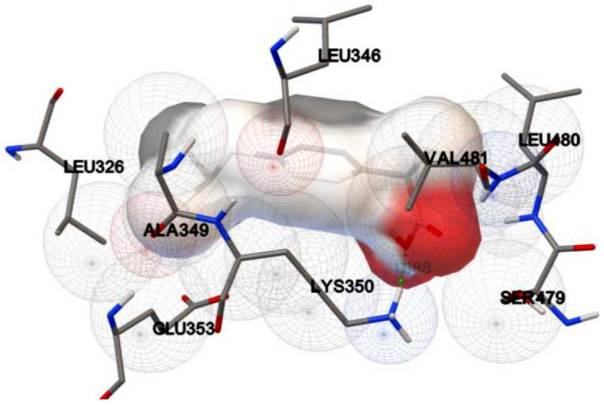

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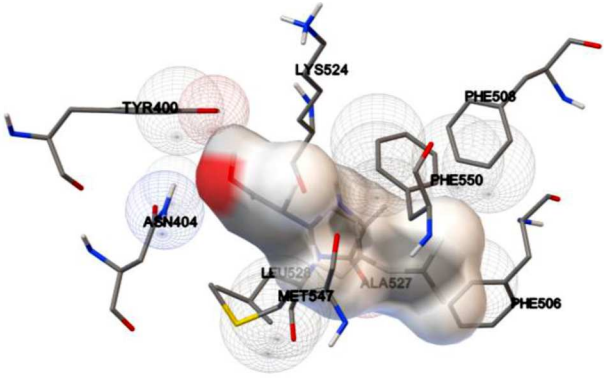

c

Fig. 7. The nearest amino acid environment of ibuprofen in three most optimal modes. (*a*) Mode 1 of the first cluster surrounded by positively charged Arg409, polar uncharged Asn390, Cys437, Thr448, Tyr410, and hydrophobic Gly433, Leu406, Leu452, Leu386, Phe402 with hydrogen bonds formed with Arg409 and Asn390. (*b*) Mode 3 of the second cluster surrounded by positively charged Lys350, negatively charged Glu353, polar uncharged Ser479, and hydrophobic Leu326, Leu346, Leu480, Val481, Ala349 with hydrogen bond formed with Lys350. (*c*) Mode 9 of the third cluster surrounded by positively charged Lys524, polar uncharged Asn404, Tyr400, and hydrophobic Phe508, Phe550, Phe506, Ala527, Met547 and Leu528

Table 5. **Interaction energies of molecules in the most optimal clusters (in kcal/mol units)**

Cluster No.	Mode No.	VdW/hb/ desolvation, kcal/mol	Elec. stat., kcal/mol	Torsional, kcal/mol	Binding energy, kcal/mol
1	1	–8.0	–1.34	1,49	–7.85
	2	–7.37	–1.29	1.49	–7.16
2	3	–6.75	–1.71	1.49	–6.98
	4	–6.74	–1.72	1.49	–6.96
	5	–6.72	–1.73	1.49	–6.96
	6	–6.46	–1.93	1.49	–6.9
	7	–6.57	–1.74	1.49	–6.82
	8	–6.52	–1.68	1.49	–6.71
3	9	–7.84	–0.08	1.49	–6.43
	10	–7.8	–0.07	1.49	–6.38
	11	–7.87	–0.16	1.49	–6.38
	12	–7.7	–0.15	1.49	–6.36
	13	–7.57	–0.1	1.49	–6.18

of amino acid residues (hydrophobic, charged, and polar uncharged) in the closest environment of the ibuprofen molecule at the binding site.

Figure 6 illustrates the binding sites of ibuprofen molecules (the main cluster modes) with a BSA molecule. It can be seen that the modes of the 1st and 2nd clusters are surrounded by charged amino acid residues, which enables the existence of a stable molecular complex. The mode of the 3rd cluster is located in the hydrophobic pocket near Trp213.

The closest amino acid environment (up to 6 Å) with the formed hydrogen bonds is shown in Fig. 7. According to the type of the closest amino acid residues, a conclusion can be drawn that the interaction of ibuprofen with BSA at the binding site is mainly hydrophobic. Note, however, that all binding modes allow various types of complex-forming forces to manifest themselves. In particular, the van der Waals interaction with polar residues is most pronounced in mode 1, hydrogen bonds in modes 1 and 3, and hydrophobic interaction in modes 3 and 9. Electrostatic forces, as was mentioned above, manifest themselves in modes 1 and 3 when interacting with charged amino acid residues.

5. Conclusions

In this study, it has been shown that ibuprofen forms stable complexes with bovine serum albumin (BSA) in aqueous media, which is confirmed by the results of fluorescence spectroscopy and molecular docking simulation. The spectroscopic studies point to the mainly static mechanism of fluorescence quenching, with the quenching constants exceeding the diffusion threshold, and an energetically favorable and moderately strong ($\Delta G = (-24 \div -28)$ kJ/mol) but reversible binding between the BSA and ibuprofen molecules in the complexes. The stoichiometric ratio of about 1 : 1, which is typical of a specific saturable interaction, demonstrates ibuprofen's ability to compete for ligands during its binding to albumin. On the other hand, such complexes dissociate relatively easily, which is important for the controllable drug release. The temperature growth is accompanied by an increase of the binding constants and by positive enthalpy and entropy values, which means the mainly hydrophobic nature of interaction.

Molecular simulation confirmed the availability of several energetically favorable binding sites for ibuprofen on the BSA surface, mainly in subdomains IIA and IIIA. During the binding process, various types of interactions with the amino acid environment are realized: van der Waals, hydrophobic, hydrogen, and electrostatic; however, bonds with hydrophobic amino acid residues dominate. This fact testifies to a high drug bioavailability, the stability of complexes in blood plasma, and the drug ability to accumulate in hydrophobic environments (membranes, lipid matrices). The arrangement of the ibuprofen molecule with respect to tryptophan amino acid residues Trp213 and Trp134 agrees with the static mechanism of fluorescence quenching and enables non-radiative resonance energy transfer with the transition of the complex into an unexcited state without radiation emission.

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КОМПЛЕКСОУТВОРЕННЯ ІБУПРОФЕНУ З БИЧАЧИМ СИРОВАТКОВИМ АЛЬБУМІНОМ: СПЕКТРОСКОПІЧНЕ ДОСЛІДЖЕННЯ ТА МОЛЕКУЛЯРНЕ МОДЕЛЮВАННЯ

У даній роботі досліджено взаємодію ібупрофену з бичачим сироватковим альбуміном (БСА) у водному середовищі з використанням експериментальних та комп'ютерних методів моделювання. Спектри гасіння флуоресценції отримано за сталої концентрації БСА (2 мкМ) та змінної концентрації ібупрофену (0–1,5 мкМ) для трьох значень температури (293, 303 і 313 К). Залежності інтенсивностей відповідають рівнянню Штерна–Фольмера та вказують на статичний механізм гасіння зі зростанням константи зв'язування з ростом температури, що свідчить про переважальний гідрофобний характер взаємодії. Отримані значення констант зв'язування становлять $\lg K_s = 4{,}3$–$5{,}0$ при стехіометрії зв'язування, близькій до $1:1$. Термодинамічний аналіз комплексоутворення показав $\Delta G < 0$, $\Delta H > 0$, $\Delta S > 0$, що підтверджує спонтанний та ентропійно зумовлений характер зв'язування. Молекулярне докінг-моделювання в AutoDock 4.2.6 виявило три основні сайти зв'язування молекули ібупрофену з БСА. Найбільш енергетично вигідні моди зв'язування містять ван-дер-ваальсівські, водневі, гідрофобні та електростатичні взаємодії, однак переважальними є контакти з гідрофобними залишками БСА. Розташування ібупрофену відносно триптофанових залишків відповідає експериментально спостережуваному гасінню флуоресценції.

Ключові слова: бичачий сироватковий альбумін, ібупрофен, гасіння флуоресценції, молекулярний докінг.