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Chapter

Study of the Influence of Humic Acid Macromolecules on the Structure of Erythrocytes of Some Animals by the Method of Absorption

Nikolay L. Lavrik and Tatiana N. Ilyitcheva

Abstract

Erythrocyte absorption spectra were obtained from fresh chicken, goose, and guinea pig blood in solutions with humic acids, isolated from brown coal, to study interactions between erythrocytes and humic acids (HA). It has been established that the addition of HA to erythrocytes leads to the differently directed shifts of Soret band maxima in the erythrocyte absorption spectrum. Thus, for a solution [guinea pig erythrocyte (1.5×10^{12} particle/l) + HA №1], this difference was $\Delta\lambda = +3.3$ nm (shortwave shift); for a solution [chicken erythrocyte (2×10^{12} particle/l) + HA №1], $\Delta\lambda = -1.5$ nm (longwave shift); and for a solution [goose erythrocyte (6×10^{11} particle/l) + HA №1], $\Delta\lambda = +4.3$ nm (shortwave shift). A comparison of the absorption spectra of guinea pig oxyhemoglobin with 2 HA samples indicates that at any erythrocyte concentrations, the positions of the Soret band maxima for various HA samples differ. The data obtained testify to the individual character of the interaction between erythrocyte membranes and HA macromolecules. Two hypotheses were proposed to account for the results obtained. (1) "Structural hypothesis." In the framework of this hypothesis, the molecules of membrane-bound oxyhemoglobin are in erythrocyte volume and can undergo noticeable, structural changes due to the deformation of erythrocyte membrane. (2) "Complexing hypothesis." In terms of this hypothesis, the observed shifts of the position of the Soret band maxima can be explained by the possible penetration of light HA fragments through erythrocyte membrane into the inner erythrocyte region. This can cause the formation of complexes (oxyhemoglobin-HA). In this case, the complex formation can involve both the free oxyhemoglobin molecules (HbO_2) and the membrane-bound ones.

Keywords: chicken, goose, guinea pig blood erythrocytes, humic acids, absorption spectra, Soret band

1. Introduction

Recently, an increase has been observed in the interest to the application of humic acids (HA) in medicine. These are the examples of HA effect on some vitally important properties of human organism [1]:

1. Antioxidant properties. A humic complex manifests an explicit ability to support chemical balance in organism. Depending on the situation, humic acid can behave itself either as donor or electron acceptor. This makes humic acid a powerful, natural antioxidant, the trap of free radicals that damage protein structures and DNA molecules of cells, break their genetic code, and, in particular, promote the development of oncological diseases.

2. Antiviral activity. Humic acids exhibit a high antiviral activity. A humic acid molecule covers a virus as a "coat" to block its escape into the bulk and prevents its reproduction. In this case, humic acid sends a signal to immune system about the appearance of an invader. This pushes the immune system to fight the virus which is in a vulnerable position (bound to a humic acid molecule). As a result, the number of viruses decreases, and the immune system successfully fights the disease.

3. Detoxicant and hepatoprotector. Humic acids are a powerful means of complexing. They bind and remove heavy metals (lead, copper, mercury, cadmium, cobalt, zinc, etc.) from the body. At a certain concentration, these cause severe poisoning and cell mutations. Heavy metals are not removed independently without special therapeutic measures. Humic acids participate actively in liver metabolism and act as a filter for heavy metals. They capture and immobilize toxic substances, preventing them from taking part in chemical reactions. Thereafter, toxin is readily removed from the body.

4. Influence on blood properties. Humic acids, in the amount of 100–300 mg per kg, have no effect on the time of bleeding, the time of blood cluttering, thrombin time, or platelet aggregation. Red cells and hemoglobin remain at normal levels. In this case, in the presence of humic acids, erythrocytes carry a higher percentage of oxygen to tissues.

5. Antibacterial activity. Humic acids have a pronounced antibacterial action on the following pathogenic microorganisms: *C. albicans*, *P. vulgaris*, *P. aeruginosa*, *S. typhimurium*, *S. aureus*, *S. epidermidis*, and *S. pyogenes*. They substantially accelerate bacterium metabolism which leads to a strong destruction of bacterial cells. In an intestinal tract, humic acids neutralize a pathogenic microflora. The bound bacteria and toxins are excreted naturally.

6. Immune system. One of the most pronounced HA effects is strengthening of the general immune response. Humic compounds regulate the number of glycoproteins that affect the balance of T- and B-lymphocytes. In addition, these activate the synthesis of interleukins 1 and 2 and the production of endogenous interferon and gamma-globulins which activates the oppressed functions of immune system. A series of clinical investigations indicate that humic acids can manifest the anticancer properties by inhibiting tumor growth and suppressing the action of viruses that can cause the development of cancer. Fulvic acid decreases protease activity which allows one to decrease the metastatic activity of cancer cells.

7. Anti-inflammatory properties. Humic acids have an anti-inflammatory action. These accelerate the healing of wounds and ulcerative defect by strengthening the processes of fibroblast proliferation, acceleration of water, protein, and lipid exchange. They also inhibit the synthesis of inflammation mediators—prostaglandins. The tissue hyaluronidase, which accelerates wound healing, is

activated locally. Humic acids were established to inhibit proteolytic enzymes that damage the walls of vessels and skin.

8. Antiatherosclerotic effect. Since humic acids can distinguish and bind substances, present in excess in the body, they form and remove the complexes with cholesterol and lipoproteins of low density which makes them efficient in their fight against atherosclerosis and its effects.

9. Antiallergic effect. Humic acids decrease organism sensibilization by actively binding and removing allergens from organisms. In this case, the symptoms of allergy vanish, the number of eosinophils in blood is normalized, and stable remission is attained.

10. Anti-stress effect. Humic acids regulate the action of stress hormones produced by adrenals (adrenaline, noradrenaline). The high level of adrenaline and noradrenaline indicates an increased level of anxiety. Excess hormones are blocked by humic acids and fail to reach their receptors in a cell. In addition, the ability of humic acids to affect the saturation of red blood cells with oxygen improves overall health and causes a surge of strength.

On humic acid structure, humic acid macromolecules are the polymer of variable molecular size and composition [2, 3]. At present, there is no full clarity in the understanding of a concrete structure of HA macromolecules, and only the general structural peculiarities are available. In terms of the generally accepted concepts, chemically, humic acids are the highly molecular nitrogen-containing organic acids whose molecules include aromatic groupings. A general pattern of the structure of HA macromolecule is as follows. There is a nucleus (aromatic carbon skeleton) and a periphery (polysaccharide-polypeptide chains) ([4]; **Figure 1**). It is assumed then that the molecular fragments of the nucleus and periphery of one HA macromolecule are bound by chemical bonds. The condensed aromatic nuclei, bound by the chains with a fair conjugation of carbon-carbon and other bonds, are the carriers of the specific properties of humic acids. Peripheral, irregular, structural elements (peripheral chains) are the variable components. As a result, the structure of HA macromolecules is unstable and subjected to statistic fluctuations. As a whole, the

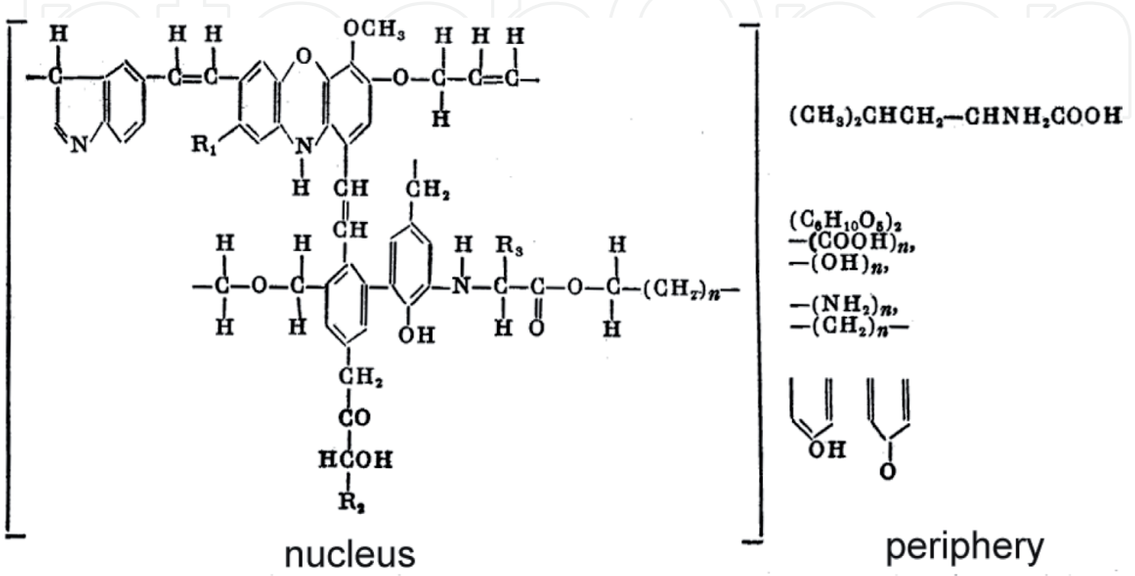

Figure 1.
Model of macromolecule structure of humic acid according to Felbeck [4].

HA macromolecules are characterized by statistically continuous range of various structural units. Thus, a characteristic feature of HA macromolecules is their polydispersion.

Recently, along with the traditional viewpoint on the structure of HA macromolecules, there appeared the alternative one [5]. In the framework of the alternative concept, the HA macromolecule structure is the supramolecular self-organizing ensemble of heterogeneous and relatively small molecules, arising from a dead biological material, rather than a single molecule in which the various structural fragments are bound by covalent bonds. The most important property of such a humic, supramolecular structure is that it is stabilized not by covalent bonds but by weak dispersion forces (van der Waals, π-π interactions, and CH-π interactions) and by H-bonds. The efficiency of HA macromolecule complexing with various simple organic and inorganic compounds is known, at present, in detail [6–16]. It is worth noting that all the works on the study of HA complexing were performed without the method of absorption and information on HA interaction with erythrocytes is unavailable at all.

It is concluded then that by now, there are numerous data on the effect of HA on the vitally essential functions of human organisms. However, the medical HA-based preparations (HA preparations) are not at present widely used in medicine.

One of the reasons, constraining a broad development and implementation of HA preparations, is the absence of a systematic study and, thus, the absence of fundamental knowledge of the mechanism of interaction between humic substance and cell at the molecular level. In particular, there are no data on the interaction of HA macromolecules with such an important cell, contained in blood, as erythrocyte. Information about the HA-erythrocyte interaction can be extracted by observing a supramolecular effect, i.e., the presence of hemagglutination.

It is known that humic substances can agglutinate erythrocytes. As verified by our preliminary experiments, the HA components, obtained from brown coal, selectively agglutinate human and animal erythrocytes: some components agglutinate human and chicken erythrocytes but fail to agglutinate the goose and guinea pig ones. On the contrary, the other types of humic acids agglutinated the goose and guinea pig erythrocytes and failed in the case of chicken and human erythrocytes. Finally, some types of HA agglutinated all erythrocytes used in experiments, whereas the other types of humic acids could not agglutinate erythrocytes at all. In particular, the samples of commercial, artificial fulvic and humic acids did not agglutinate erythrocytes. These results indicate that upon agglutination, the specific HA-cell interactions occur that allow one to distinguish their binding sites; i.e., the interaction efficiency is individual for a concrete HA-erythrocyte pair. Thus, of interest is the study on the efficiency of HA-erythrocyte interaction at the molecular level.

The goal of this work was to determine the efficiency of the interaction between erythrocytes of some animals and HA macromolecules by the method of absorption. Information on the efficiency of HA-erythrocyte interaction can be extracted from the experiments on the change in the parameters of Soret absorption band, that, as shown in [17], are sensitive to the change in the state of oxyhemoglobin molecule, HbO_2, contained in erythrocytes. The visible region of HbO_2 absorption spectra exhibits the three strongest characteristic bands with maxima at $\lambda \sim 415$ nm (Soret band), $\lambda \sim 545$ nm (β band), and $\lambda \sim 580$ nm (α band) [18, 19]. The origin of HbO_2 absorption spectra has been established quite reliably. It is defined by the electron properties of hemes that are the prosthetic groups in the structure of hemoglobins [20]. In metalloporphyrins, the Soret band (heme molecules in erythrocytes) is determined by both the electronic $\pi \rightarrow \pi^*$ transition $2^1E_a \leftarrow 1^1A_g$, when the upper state is twice degenerated (D_{4h} symmetry), and the electronic

$\pi \rightarrow \pi^*$ transitions $2^1B_{3u} \leftarrow 1^1A_g$ and $2^1B_{2u} \leftarrow 1^1A_g$ in the absence of degeneracy (D_{2h} symmetry) [19, 20]. Experimentally, the Soret spectrum of oxyhemoglobin in erythrocytes exhibits one band which allows us to assign the origin of the spectrum observed to the electron transition $2^1E_a \leftarrow 1^1A_g$.

On the possibility to observe spectral changes in the Cope band spectrum of oxyhemoglobin molecule upon HA addition.

The changes in the HbO_2 absorption spectrum upon both HA addition and conservation of initial erythrocyte can be a priori expected due to the following. In the initial erythrocytes, about 10% of HbO_2 can be bound to the inner surface of the membrane [21–24], i.e., the membrane-bound oxyhemoglobin results from the interaction between HbO_2 molecule and membrane components (band 3 protein, spectrin, glycophorin, membrane lipids). The form of erythrocytes can vary after addition of HA and upon hemagglutination which can have effect on the structure of the membrane-bound HbO_2 molecules, i.e., cause changes in heme structure. Thus, the structural rearrangement of the membrane-bound HbO_2 molecules can lead to the changes in their spectral parameters. As a result, the absorption spectra of initial erythrocyte samples and of those that can agglutinate will be different. The position of absorption band maxima depends on the state of the electron orbitals of porphyrin rings, contained in the heme. The sensitivity of electron transitions between orbitals to the structural changes in porphyrin rings, which form a heme, has been established quite reliably [19, 20]. The Soret band is determined by the electron $\pi \rightarrow \pi^*$ transition which is highly sensitive to the change in structure and environment [25]. In particular, as has been established, upon binding of porphyrins to DNA, the Soret band undergoes a long-wave shift of up to 20 nm upon internal intercalation and of 8 nm upon the external one [26]. Besides, the addition of protein causes a bathochromic shift of 10 nm in the chlorine absorption spectrum, contained in porphyrin rings [27]. At last, the effect of erythrocyte lysis on the position of the Soret band maxima is reported in [17]. Thus, the spectral parameters of the oxyhemoglobin molecule absorption spectrum are assumed to change upon hemagglutination due to the deformation of erythrocyte structure.

The goose, chicken, and guinea pig erythrocytes of different geometric parameters and the HA, isolated from brown coal, were used as the samples for solving the problem stated. A preliminary work indicates that the HA preparation agglutinates the chicken and guinea pig erythrocytes and does not agglutinate the goose ones.

2. Experimental

Isolation of humic acids. In this work, we used humic acids isolated from brown coal by two different techniques. The first one [28] includes the treatment of coal with alkaline reagents with subsequent oxidation in a cathode chamber of a diaphragm electrolyzer at room temperature and atmospheric pressure. In the second technique [29], brown coal was treated with an aqueous ammonia solution of 5–25% upon mixing. Cyclohexanol, or its analog, was then added in the amount of 0.1–1.0% of the initial coal mass. Hydrogen peroxide was added in small portions by stirring until the maximal dissolution of brown coal. The concentration of NaCl in HA solutions amounted to 0.9% (a standard physiological solution (0.9% NaCl "Fluka") was used). pH of working solutions was 7.2 (NaOH).

Production of erythrocytes. To prepare erythrocytes, the samples of fresh chicken, goose, or guinea pig blood with 5–10 units/ml of heparin were used and then filtered through a sterile cotton gauze cloth (Fisher, cat. № 22-415-469) into a conical 500 ml tube which was then carefully filled with cold 0.01 M phosphate-saline buffer (PSB, pH = 7.2), capped and stirred slightly while turning the tube. Centrifugation was performed at 1200 rpm/min for 10 min at +4°C. The supernatant was then removed

using a 10 ml pipette. This was followed by a cold phosphate-saline buffered double wash. The remaining supernatant was removed with a micropipette. Compacted erythrocytes were stored on ice. The erythrocyte suspension (1%) was prepared by adding 2.5 ml of compacted erythrocytes to 247.5 ml of cold PSB in a 50 ml glass flask and stirred via rotation. The concentrations of erythrocytes from guinea pig, chicken, and goose blood were 30×10^{12}, 40×10^{12}, and 12×10^{12} particle/l, respectively.

Preparation of solutions of erythrocytes with humic acids. The concentrations of erythrocyte solutions in HA were prepared as follows. The initial solutions were diluted 10 times. 0.3, 0.5, 1, and 1.5 ml were poured into a tube, and the HA solution was added to obtain 10 ml.

Obtaining absorption spectra. The absorption spectra were recorded on a Hewlett Packard 6041 spectrophotometer. Standard 1 cm quartz cuvettes were used.

Determination of absorption band maximum. The position of the absorption band maxima was determined by taking the first absorption spectrum derivative. The value of the abscissa at which the first derivative was zero was taken as the position of a maximum. The absorption spectrum derivative was determined using a standard method, contained in the program "Origin 7."

3. Results and discussion

Figure 2 shows the absorption spectra of erythrocyte samples of various concentrations in HA solution, isolated from the guinea pig (a), chicken (b), and goose (c) blood. In the HA solutions, the obtained spectra of the initial erythrocyte samples also coincide with the available data on oxyhemoglobin, whose spectra exhibit the bands at λ ~ 415 nm, λ ~ 545 nm, and λ ~580 nm [18, 19]. The presence of characteristic bands at λ ~ 545 nm and at λ~580 nm in the spectra of erythrocyte samples with HA indicates that the HA solutions contain just the oxyhemoglobin molecules because this doublet is not recorded for other hemoglobin derivatives (deoxyhemoglobin, methemoglobin, carboxyhemoglobin, and hemichrome) [18, 19, 30].

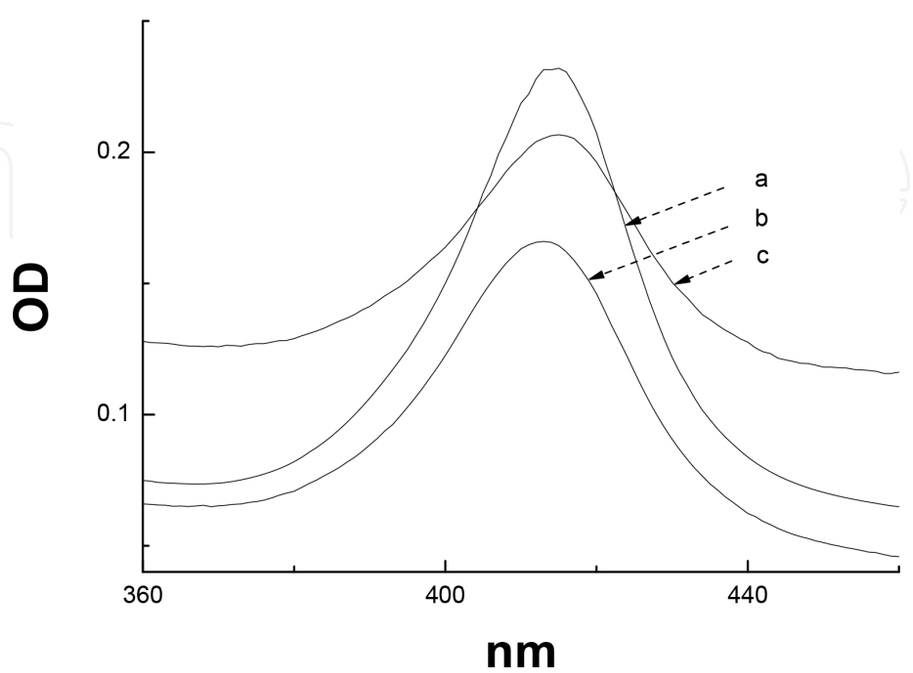

Figure 2.
Absorption spectra of erythrocyte samples. (a) guinea pig, [Erythr] = 3 × 10^{12} particles/k; (b) chicken, [Erythr] = 4 × 10^{12} particles/l; and (c) goose, [Erythr] = 1.2 × 10^{12} particles/l.

Figure 3 demonstrates the absorption spectra of oxyhemoglobin of different concentrations in HA solutions. According to the data presented, as the concentration of erythrocytes decreases, the position of the Soret band maximum undergoes a noticeable long-wave shift for goose and chicken erythrocytes, and in the case of the guinea pig ones, the shift is very weak. Thus, the interaction between erythrocytes and HA causes the maximal spectral changes in goose erythrocytes, and the minimal ones are observed in the chicken erythrocytes.

The highest effect of the difference in the position of the Soret $\Delta\lambda$ band maximum is observed by comparing the absorption spectra of the initial sample erythrocytes with the sample of {erythrocyte + HA} solution. Thus, for a solution [guinea pig erythrocyte (1.5×10^{12} particle/l) + HA №1], this difference was $\Delta\lambda = +3.3$ nm (shortwave

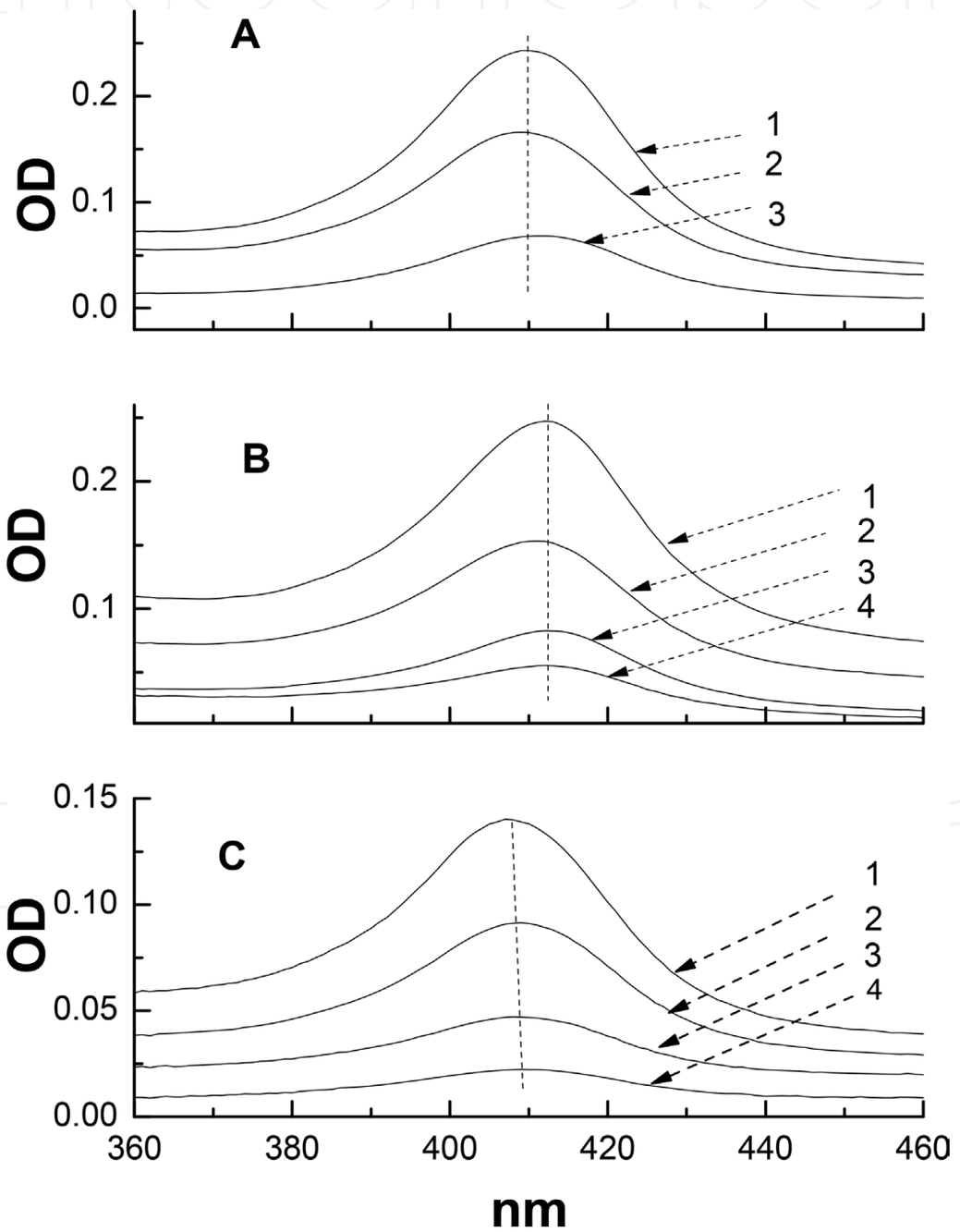

Figure 3.
Absorption spectra of erythrocytes of different concentrations in HA solutions. (A) guinea pig: (1) [Erythr] = 4.5×10^{11} particles/l, (2) [Erythr] = 3×10^{11} particles/l, and (3) [Erythr] = [1.5×10^{11} particles/l; (B) chicken: (1) [Erythr] = 6×10^{11} particles/l, (2) [Erythr] = 4×10^{11} particles/l, (3) [Erythr] = 2×10^{11} particles/l, and (4) [Erythr] = 1.2×10^{11} particles/l; and (C) goose, (1) [Erythr] = 1.8×10^{12} particles/l., (2) [Erythr] = 1.2×10^{11} particles/l, (3) [Erythr] = 0.6×10^{12} particles/l, and (4) [Erythr] = 0.36×10^{11} particles/l.

shift); for a solution [chicken erythrocyte (2×10^{12} particle/l) + HA №1], $\Delta\lambda = -1.5$ nm (longwave shift); and for a solution [goose erythrocyte (6×10^{11} particle/l) + HA№1], $\Delta\lambda = +4.3$ nm (shortwave shift). Thus, there is a difference in the change in the position of the Soret band maxima by both the absolute value and the direction.

However, a conclusion can be now drawn on the individual character of the effect of HA interaction with the erythrocytes of the animals studied, observed at the molecular level, which correlates with the previous information on the individual character of the hemagglutination of the erythrocytes of these animals.

Figure 4 shows the absorption spectra of guinea pig oxyhemoglobin of various concentrations with HA samples №1 and №2. As follows from the figure, at any erythrocyte concentration, the positions of the Soret band maxima for HA sample №1 are always in a redder region of the spectrum. Thus, the efficiency of the interaction between the guinea pig erythrocytes and HA samples №1 and №2 is different. This effect is, probably, due to the difference in the structural properties of HA samples (**Figure 5**).

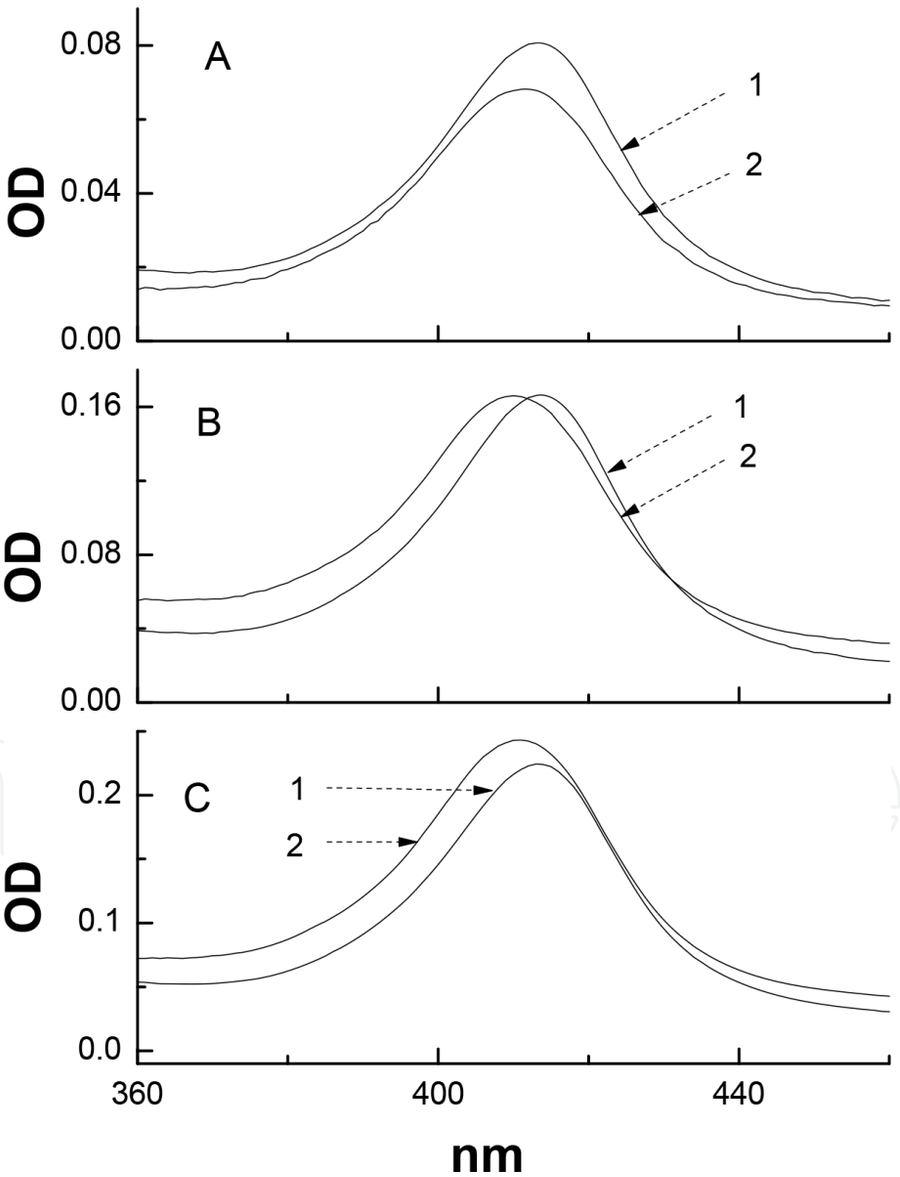

Figure 4.
Absorption spectra of guinea pig oxyhemoglobin of different concentrations with HA samples №1 and №2. (A) [Erythr] = 1.5 × 10¹¹ particle/l, (B) [Erythr] = 3 × 10¹¹ particle/l, and (C) [Erythr] = 4.5 × 10¹¹ particle/l. (1) HA №2 and (2) HA №1.

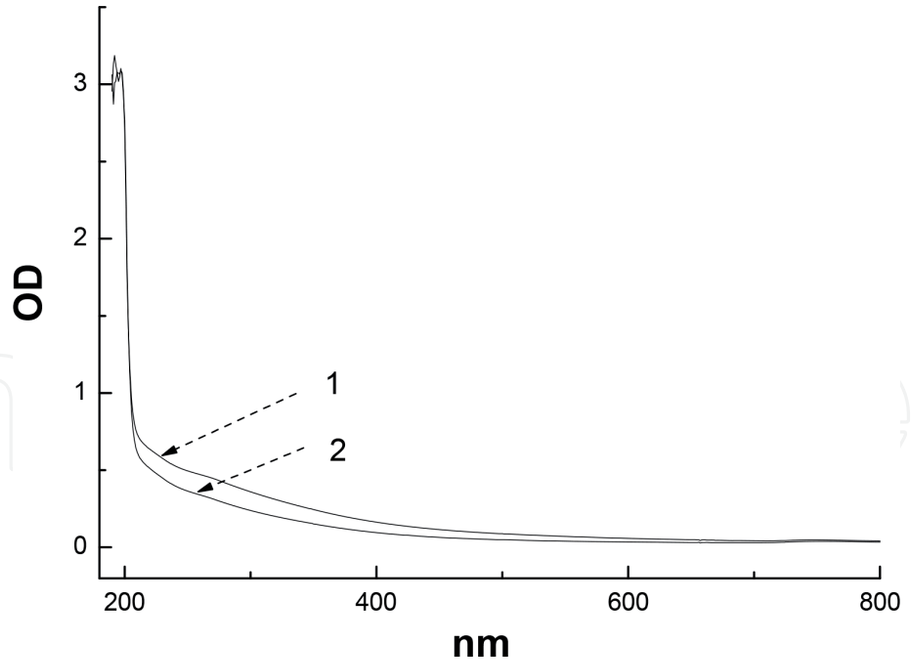

Figure 5.
HA absorption spectra [(2.5 mg/l)]. (1) №1 and (2) №2.

4. Discussion of results

1. "Structural hypothesis." As mentioned in Introduction, the addition of HA and the presence of hemagglutination cause changes in erythrocyte form which then can have an effect on the structure of the membrane-bound HbO_2 molecules, i.e., finally, can lead to the change in heme structure. Thus, the spectral parameters of the membrane-bound HbO_2 molecules can change due to their structural rearrangement. As a result, the absorption spectra of the initial erythrocyte samples without addition of HA and erythrocytes that agglutinate will differ. It is readily seen that in the framework of this hypothesis, the molecules of the membrane-bound oxyhemoglobin must undergo noticeable structural changes due to the deformation of erythrocyte membrane. The possibility of the deformation of erythrocyte structure upon interaction with other molecules was reported, e.g., in [31]. In this work, the method of atomic force microscopy was used to verify that hemin has a specific effect on the nanostructure of erythrocyte membranes by forming domains on its surface.

2. "Complexing hypothesis." The shift observed in the position of the Soret band maximum can be differently explained by a feasible penetration of light HA fragments through erythrocyte membrane into the inner erythrocyte region. (This mechanism can be similar to the penetration of virus into a cell. The possibility of this process was verified by studies on the interaction between human erythrocytes and carnosine molecule [32]. In any sample of HA, due to the property of polydispersity of HA [2], light fragments are always present. Direct evidence of the presence of light fractions in the "Aldrich" sample was shown in [33]). The penetration of HA fragments into erythrocyte can lead to the formation of oxyhemoglobin-HA complexes. In this case, this process can involve both the free oxyhemoglobin molecules and the membrane-bound ones. However,

this hypothesis fails to account for the experimentally observed shortwave shift of the Soret band with increasing erythrocyte concentration, Soret band origin and $\pi^* \rightarrow \pi$ transitions, and the process of complexing must cause a long-wave shift [25].

It is impossible now to give preference to one of these hypotheses.

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Author details

Nikolay L. Lavrik[1*] and Tatiana N. Ilyitcheva[2]

1 Voevodsky Institute of Chemical Kinetics and Combustion, SB RAS, Novosibirsk, Russia

2 State Scientific Center of Virology and Biotechnology "Vektor", Koltzovo, Novosibirsk, Russia

*Address all correspondence to: lavrik@kinetics.nsc.ru

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