MOLECULAR BIOPHYSICS

Hydratation of erythrocyte membranes and their interaction with ultrafine silica

V. V. Turov, N. P. Galagan, I. V. Grytsenko, A. A. Chuiko

The Institute of Surface Chemistry, NAS of Ukraine
17, General Naumov Str., Kyiv-164, Ukraine, 03680

E. mail: v_turov@ipnet.kiev.ua

The interaction of ultrafine silica (UFS) with membrane preparations of donor erythrocytes (ghosts) was investigated by the method of ^1H NMR spectroscopy. The values of their hydratation in contact with silica, interphase energy, concentrations of weakly and strongly bound water were measured and calculated. Possible mechanisms of UFS effect on cell hemolysis are discussed.

Key words: *NMR spectroscopy, ultrafine silica, erythrocytes, ghosts (membrane), hydratation.*

Introduction. A specific feature of the recent decade is a rapid development of the nanotechnology which proposed the usage of ultrafine materials with the particles size smaller than 100 nm. Regarding their size, nanostructures occupy the intermediate position between molecular (with C order size) and microscopic (with micrometer order size) objects [1]. Their distinctive feature is possessing certain physical, chemical or biophysical characteristics, different from those of molecular and microscopic objects. The usage of nanomaterials in biology and medicine is supposed not only to increase research efficiency of physical and chemical properties of the cells and biomacromolecules, but also to develop new methods of severe diseases treatment and diagnostics, based on the application of nanosized carriers for medicine delivery which do not cause the rejection reaction and interact with the affected tissues in a specific way.

In creating nanomaterials, special attention is paid to ultrafine oxides of silicon, titanium, aluminum, zirconium, etc. The presence of a large quantity of chemically active hydroxyl groups on their surface provides the surface modification with different functional groups [2]. As a result, the materials with the required hydrophobic\hydrophilic properties or specific to definite chemical groups in biological objects can be synthesized [3].

Medical and biological properties of ultrafine silica (UFS), (the technical name is Aerosile) which is widely used in pharmaceutics as adjunct in producing medicines, are mostly studied among the ultrafine materials [4, 5]. It is a synthetic amorphous silica dioxide consisting of nanosized initial particles, the size of which is from 4 to 40 nm, which are easy to form aggregates and agglomerates of the micron size in water suspension. The UFS particles possess high specific surface (up to 400 m^2/gr), which provides its high adsorption properties towards many types of organic molecules, proteins, biopolymers, and some synthetic polymers. At present, the methods of synthesis of

pharmaceutical and biologically active substances immobilized on UFS surface have been developed. These composite nanomaterials are a sort of substances depot, reversibly set on the particle surface, which allows using them as medications of durable action. At the same time, the therapeutic medication dose is decreased and its concentration is optimized in the organism [3]. A great potential of nanomaterials application for medical purposes is supported by a number of publications on this topic [5-7].

Ultrafine silica (UFS) forms are widely used not only in pharmaceutics but also in experimental medicine for the treatment of purulent infections, different kinds of toxicosis, diarrhea, and some other diseases [3, 8]. Surface properties of these nanoparticles allow prognosing these materials usage in hematology as well. It was established [9, 10] that during the UFS interaction with erythrocytes, the reaction of cell membranes depends on nanomaterial concentration in biological liquid. It was also noted that at UFS quantity increase in suspension containing erythrocytes, there is a certain concentration limit under which the plasma membrane rupture is observed, that causes cell hemolysis. In the presence of UFS with protein bound to its surface this process decreases rapidly [10]. Since protein molecules are irreversibly adsorbed on the UFS surface [3], it is possible to suppose that hemolysis takes place only at direct contact of membranes with silica surface, and protein molecules adsorbed on its surface screen the cells from direct contact with the active centers of the UFS surface. Nevertheless, the erythrocytes hemolysis mechanism induced by UFS is weakly characterized. Based on the earlier researches on UFS binding strength with some components of animal reproductive cells surface [11], it is possible to suppose that nanoparticles will interact not with the membrane itself, but with so called "surface proteins" and oligosaccharide parts of glicoproteins or glycolipids which compose receptor system of the cell. Hence, two reasons of the cells death in the presence of UFS at concentrations, which cause erythrocytes hemolysis, can be considered: i) cell homeostasis disorder due to the interaction of structural elements of cell receptors, the most distant from the membrane, with the silica surface; ii) irreversible sorption of membrane proteins on the silica surface, and later of membrane phospholipids. As the surface of the cells and of UFS particles is hydrated greatly [12], their interaction may be expected to result in significant dehydration of the "cell-surface" system, since some part of bound water (up to 50%) ought to be eliminated out of space between cell and particle for their direct contact. If we suppose that the particles structure (cells, UFS particles) is not changed as the result of interparticle interaction, then the parameters of their interaction can be defined according to the change in the bound water concentration [12, 13]. If the ultrafine particles do not interact, then interphase energy value, maximum possible for this system, is observed. The particles get closer at the interaction and the interphase energy is decreased by the value equal to the value of interparticle interaction energy.

It is worth mentioning that a part of intercellular water does not freeze in cellular objects, due to the presence of low-molecular substances in the cytoplasm. The mentioned "bulk" unfreezable water may also contribute to the interphase energy value being measured. In studying "cell-particle" interactions, the principal interest is paid to studying the interaction of external cell surface with UFS particles. Therefore, the purpose of the following work was to determine the parameters of UFS particles interaction with cell membranes (erythrocyte ghosts). Thus, intracellular water, biopolymers, and structural cell elements were excluded from consideration.

Materials and Methods. The erythrocytes were obtained from red blood cells of the donor's blood (ABO), supplied by Kyiv Blood Transfusion Centre in standard Glyugitsyr blood stabilizer, used for erythrocytes storing. Erythrocyte ghosts were obtained in accordance with a slightly modified Dodge et al. method [14]. Their preparations were kept frozen at -15°C. To determine erythrocytes ghosts concentration, 5mM Na-phosphate buffer pH 7.6 buffer was added to the precipitate, and the aliquot of 0.5-1.0ml was taken into the Petri dish, dried at 22°C and weighed. 1ml of erythrocytes ghosts made 7.5 mg of the dry substance. Microphotograph of erythrocytes ghosts is shown in Fig.1.

UFS, Aerosile A-300 with the specific surface area of $300 m^2/gr$ (Kalush, Ukraine) was synthesized by high-temperature hydrolysis of $SiCl_4$ in the flame of hydrogen torch [16] in accordance with general scheme:

$$SiCl_4 + 2H_2 + O_2 \text{ ч } SiO_2 + 4HCl$$

The silica powder was ignited at 400EC for 2 hours to eliminate HCl and atmospheric water. UFS suspensions were prepared by slurrying dry powder in distilled water, stirring intensively.

NMR spectra were obtained with NMR high resolution spectrometer Bruker WP-100 SY, working frequency 100 MHz and band width of 50 kHz. The temperature was controlled with the accuracy of ±1°C, using Bruker VT-1000 device. The signal intensity was defined with the accuracy of ±10%. To prevent supercooling, the measurements of amount of unfreezable water concentrations were conducted at heating of samples, preliminary cooled down to the temperature of 210 K.

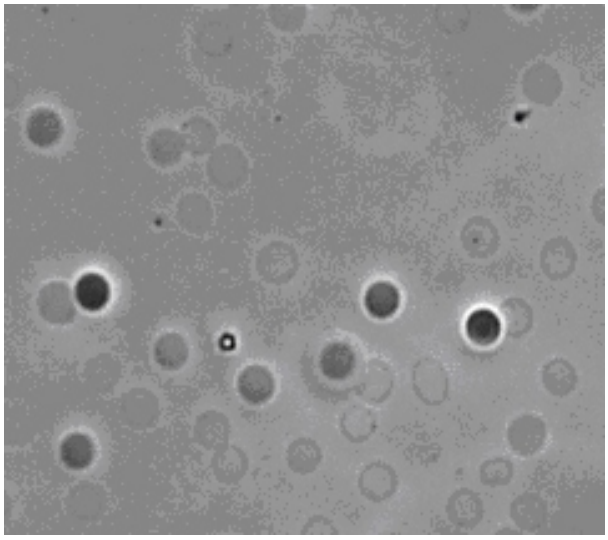

Fig.1 Micro photos of membranes (shadows) of erythrocytes in Na-phosphate buffer (? 900)

The parameters of interphase water layers were defined using the method of layered freezing of liquid phase with ^{1}H NMR registration of the unfreezable water signal, like in[12, 13]. The method is based on the fact, that at the absence of low-molecule substances in the solution, the condition of water freezing at the interphase "nanoparticle-water" is the equality of free energies of molecules of adsorbed water and ice. The interphase water freezes at the temperature lower than 273 K. The decrease of the freezing-point for interphase water (273 K) is caused with the decrease of free energy of water, due to adsorptive interactions. As a result, the decrease of free water energy in adsorption layer equals to the difference between free energies of ice at the temperature of measurement and 273K ($G = G_o - G$, where G_o is free energy of ice at T = 273 K). The thermodynamic functions of the ice are tabulated with high accuracy in the wide range of temperatures [16]. According to these results, free ice energy at the decrease of temperature is changing according to the linear law:

$$G = 0.036(T - 273) \quad (1)$$

If interphase energy of the "dispersed phase/water" system (r_s) is defined as summarized decrease of the system free energy, caused by the existence of interface, it can be calculated as a square, restricted by the curve of the dependence of Gibbs differential energy on the concentration of unfreezable water $G(C_{uw})$:

$$s \quad k \int_0^{c_{uw}^{max}} G dC_{uw} \quad (2)$$

In this expression C_{uw}^{max} – is the thickness of unfreezable water layer at T=273 K, and **k** is a dimension factor. When the interface area is known, and interparticle interactions are insignificant, the value of r_s equals to the surface energy, usually measured in mJ/m^2. In this case **k**=55.6/S (where S is the specific surface area of the dispersed phase). The value of r_s for biological objects is related to the unit of the dispersed phase mass and measured in J/g, the constant **k** = 18^{-1}. As the value of r_s is defined in isobaric process of freezing-melting its dependence on temperature should be neglected. Nevertheless, the comparison of interphase energies r_s and wetting heat, measured using the microcalorimetry method, for a number of modified silicas, showed practically complete coincidence of the measured values [17]. The error of measuring r_s value is defined by the accuracy of integrating, and it is usually not higher than 15%.

The layer thickness of strongly and weakly bound water can be calculated by the dependences of G on C_{uw}. The weakly bound water is understood as that part of unfreezable water, the free energy of which is only decreased a little at adsorptive interactions with the surface of adsorbents or biopolymers. It freezes at the temperature close to 273 K. Vice versa, strongly bound water may not freeze even at supercooling of the system [13]. The layers thickness of each water type (C_{uw}^{s} and C_{uw}^{w} for strongly and weakly bound water) and maximum values of decrease of water free energy (G^s and G^w), corresponding to them, can be obtained extrapolating corresponding parts of dependences to abscisses and ordinates axes.

The maximum (true) value of interphase energy is registered for infinitely deluted colloid systems, when there is no overlapping of hydrate shells of dispersed phase particles. At concentrating there is a possibility of interparticle interactions, the level of which can be evaluated upon the concentration change of bound water in the system. According to the equation 2, the interphase energy defines the summarized decrease of free energy in the system "particle (cell)-water in the whole layer of interphase water", which is under perturbation action by interfaces. It follows from thermodynamic equilibrium at the approaching of particles as a result of suspension concentration, that the decrease in interaction level of the "particle-water" type must be compensated by the interaction of "particle-particle" type. The decrease in interphase energy as a result of interparticle interactions is equal to the energy of interparticle interactions. A natural restriction of such an approach is the reversibility of concentration process (the structure of aggregates should not depend on concentration).

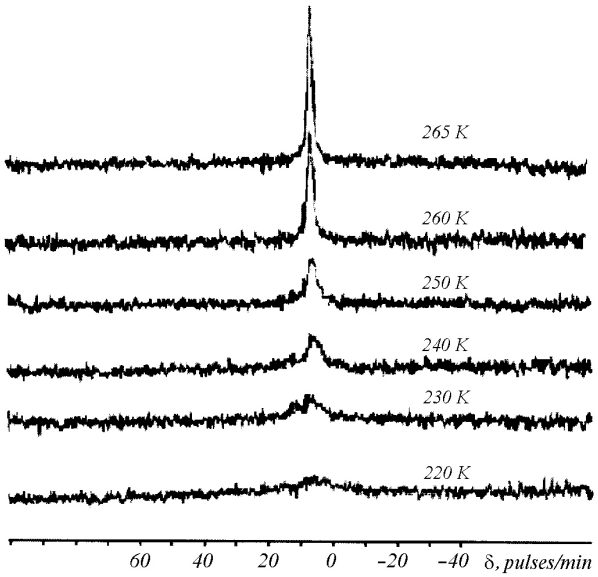

Fig.2 Temperature dependence of ^{1}H NMR spectral shape of unfreezable water.

Results and Discussion. The temperature dependence of the ^{1}H NMR spectral shape for unfreezable water at the temperature of 220 K < T < 265 K is shown in Fig.2. The signal of unfreezable water is a singlet, the half-width of which increases from 0.5ppm to 10ppm at the temperature decrease. The average value of the chemical shift is 4.5ppm which corresponds to the chemical shift for liquid water. As some quantity of dissolved low-molecule substances is in the solution besides erythrocyte ghosts (NaCl, buffer components), unfreezable water is originate not only from hydrated shell membranes of biomacromolecules, but also from water in microcavities, filled with the solution, the concentration of which is defined with the Raul's law [17]. Nevertheless, as it was shown in [19], while freezing solutions in the presence of biopolymers, the dissolved substances are concentrated easily in their hydrate shells, especially if the concentration of dissolved substances does not exceed 1-2%. Thus, it is possible to think that the bulk water does not contribute significantly to the measured concentration value of unfreezable water in case of frozen erythrocytes ghosts. A large signal width for unfreezable water is in favour of this assumption. Fig.3 shows the temperature dependences of unfreezable water concentrations (A) and built on their basis according to the formula (1) the dependences of Gibbs differential energy on unfreezable water concentrations (B) for erythrocytes ghosts suspensions, containing 1.5% of cellular membranes recounted to dry substance (curve 1), as well as corresponding dependences for 6% SiO_2 gel with additions of different quantities of erythrocyte ghosts (curves 2-5). Two regions may be marked on the $G(C_{uw})$ dependences, one of which is characterized by a weak change of G value in the wide range of C_{uw} changes. It is this part of bound water the free energy of which is decreased only a little due to interactions

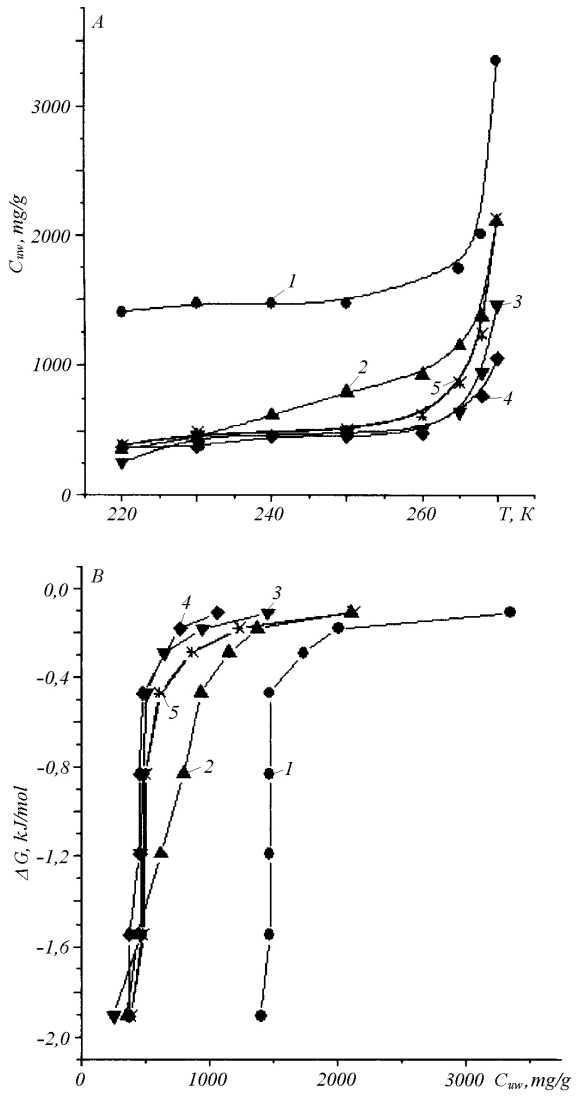

Fig.3 Temperature dependences of concentration of unfreezable water (A) and Gibbs differential energy dependence on unfreezable water concentration (B) for erythrocyte ghosts suspension.

The characteristics of bound water layers in water suspensions of erythrocyte ghosts, containing 6% of silica.

Sample	Cshadows%	Cwmg/g	Csmg/g	?GwкJ/	?GsкДж/моль	?SДж/г
Ghosts	1.5	1870	1480	0.3	-3	218
SiO2	0	1100	1000	0.3	-3	98
Shadows + + SiO2	0.19	980	500	0.4	-3	67
	0.38	500	500	0.3	-3	66
	0.75	1600	500	0.3	-3	83
	1.5	1600	500	0.4	-3	85

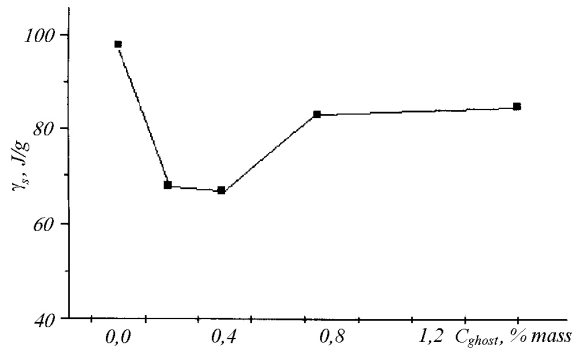

Fig. 4 The influence of membrane erythrocytes concentration in suspension on interphase energy in the system "plasmatic membrane - water", containing 6% of silica.

with the interface (weakly bound water). The second part is characterized by the considerable changing of G value in the relatively narrow range of C_{uw} changes. Such water corresponds to the features of strongly bound water [13]. The characteristics of different types of bound water as well as interphase energy values calculated according to the formula (2) are shown in Table 1. The results obtained correlate well with the previous data on yeast cells [20]. However, higher concentration values of strongly and weakly bound water, obtained in the experiments with erythrocytes, may be explained by the difference in the components structure of cellular surface and the difference in specific surface area of yeast cells and erythrocytes.

As it follows from the data of the Table, erythrocyte membranes bind a big quantity of water on their surface. It is even more hydrated than silica is in 6% gels. At the same, time concentrations of strongly and weakly bound water for 1.5% suspension of erythrocyte ghosts are close. As the specific surface area of cells is relatively small, less than $10 m^2/g$ [20] the bound water seems to form a polymolecular layer on the cell surface. Probably, it fills up completely the space between the glycoprotein molecules, exposed over the surface of the cell membrane.

The ingress of cellular membranes into the concentrated SiO_2 gel results in sharp decrease in unfreezable water concentration in the system (Table 1). It indicates intensive interaction between erythrocytes surface and silica. According to the approach, presented in details in [13], dehydratation of the surface of nanosized particles in the process of their interaction is caused by partial removal of bound water from interparticle space. Thus, it is possible to determine the interaction energy of "cell-SiO_2 surface" system upon the decrease in r_s value while adding the third component to the system (in our case, this is erythrocyte ghosts).

The dependence of $r_s(C_{shadows})$ at the addition of cellular material to silica gel is shown in Fig.4. As it is seen from the Figure, at $C_{ghosts} < 0.45\%$, at the ghosts concentration increase interphase energy of "silica – water" decreases from 98 to 66 J/g. The change of r_s in case of adding silica into the "shadows - water" system is even bigger. According to the data in Table 1, the r_s value for 1.5% erythrocyte suspension at adding silica up to mass 6% increases more than twice. The relative increase of the r_s value at the further increase of cells concentration (Fig.5) is connected with the increase of the total concentration of bound water in the system. The minimum of $r_s(C_{shadows})$ dependence corresponds to the most complete binding of erythrocyte ghosts with silica surface.

The maximum interaction of silica with cellular membranes is viewed at the comparison of shadows corpuscles masses and silica, equal to 1:20. Probably, at the same time equal distribution of cellular membranes takes place in the volume of silica gel. With the increase of ghosts concentration the possibility increased for intercellular interactions for which dehydratation of cells surface is smaller, than in case of "cell-silica" interaction. Assuming that the area of erythrocyte ghost membrane is $140 мм^2$ [21], and the mass

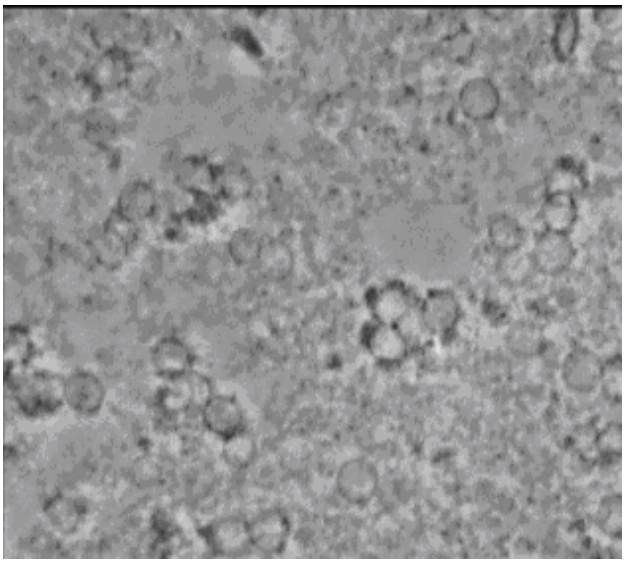

Fig.5 Micro photo of suspension of erythrocyte ghosts, containing 1% of silica in 0.14 M NaCl (? 900).

is 10^{-12}g, the specific surface area is considered to be $S=140 m^2/g$. Subsequently the cell surface energy can be calculated for erythrocyte ghost on the basis of data in Table 1. As shown in [7], to obtain the value of surface energy in mJ/m^2, the constant **k** in the equation (2) has to be taken as 55.6/S. In this case r_s of erythrocyte ghosts is equal 1560 mJ/g. This value is one order higher than the one for water suspensions of UFS [13]. A bigger value of r_s indicates the existence of a thick layer of bound water on the surface of erythrocytes, the average thickness of which exceeds 40 diameters of water molecules. Probably, the bound water fills up all the space between glycoproteins of cellular receptors.

As the size of erythrocytes is 5-7 мм [22], several UFS particles can interact with each cell. The interaction is accompanied by the rapid decrease in concentration of strongly bound water (Table 1). It is found, that already at the 1% concentration of silica in the suspension of erythrocyte ghosts (Fig.5) the membranes shape and their assembly with UFS particles changed. Probably, as a result of "cell-UFS surface" interaction there is irreversible adsorption of glycoproteins, included in the structure of cellular receptors, on the surface of silica. Obviously, this process is essential for the interaction of living cells with UFS surface as well. If the contact area of "cell-particle" is too large, irreversible changes are possible in the structure of cellular receptors, resulting in the loss of the integrity of cellular membranes and rapture of cells.

В.В. Туров, Н.П. Галаган, І.В. Грищенко, О.О. Чуйко

Гидратация мембран эритроцитов и их взаимодействие с высокодисперсным кремнеземом

Резюме

Методом ^{1}H ЯМР – спектроскопії досліджено взаємодію високодисперсного кремнезему (ВДК) з препаратами мембран (тіней) еритроцитів крові донорів. Визначені і розраховані величини їх гідратації при контакті з кремнеземом, міжфазна енергія, концентрації сильно- і слабозв'язаної води. На основі отриманих даних обговорюються можливі механізми дії ВДК, що зумовлюють гемоліз еритроцитів.

Ключові слова: *ЯМР – спектроскопія, високодисперсний кремнезем, еритроцити, тіні (мембрани), гідратація.*

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