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Formation of a 'protein corona' on the Fe_3O_4@SiO_2 nanoparticles surface

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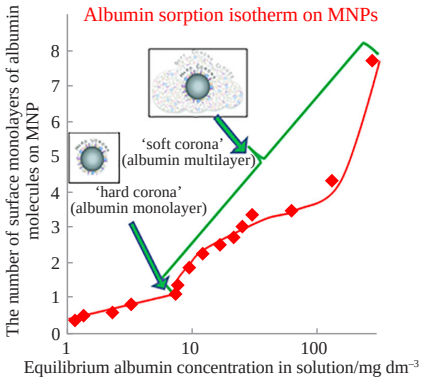
The patterns of interaction of the nanoparticles surface with protein components of biosystems have been studied on the example of the interaction of magnetic nanoparticles of the core–shell structure with albumin. Data on particle sizes and their spectra in the visible region in the presence of different quantity of protein have been obtained and analyzed by sorption, spectral and dynamic light scattering methods and albumin sorption isotherms in various media. The formation of the ordered protein monolayer (so-called 'hard corona') with subsequent forming of the multilayer of a different structure ('soft corona') has been experimentally shown; it demonstrates that it is possible to computationally distinguish between mono- and multi-layer using data from sorption experiments.



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The key area of modern medical science is theranostics – an approach to the development of fundamentally new medical materials capable of providing both diagnosis and targeted therapy of diseases. This approach can significantly reduce the toxic effect and at the same time increase the performance of the drug.

Magnetic surface-modified nanoparticles (NPs) of the core–shell structure are in-demand tools for using the principles of theranostics in biomedical practice. They can combine the ability to detect pathologically altered cells of the body, *in vivo* imaging of NPs, targeted drug therapy, radionuclide therapy and/or tumor hyperthermia and the possibility of controlled NPs delivery to the target organ under the effect of an external magnetic field. There are numerous examples of such developments;[1–4] a number of such nanomaterials are at the stage of preclinical and clinical trials or are already approved for use in medicine.[5,6] However, many tested variants of NPs have shown high efficiency only *in vitro*, while in living organisms they significantly lose it and cause the side effects. The reasons for this should be sought in regularities of interactions of NPs with *in vivo* biological objects (dissolved proteins, cell membranes, antibodies, *etc*.).

In blood flow NPs are quickly covered with the so-called protein corona (PC), a shell of proteins usually present in the blood plasma.[7] The qualitative and quantitative composition of PC strongly depends on the parameters of the NPs and on the conditions of interaction. Meanwhile, it is the specific PC composition and structure that largely determine the biochemical behavior and therapeutic effect *in vivo*, as a result of the PC formation the properties of the NPs surface radically change.[8] Thus, analytical and physico-chemical information about PC is extremely important for the final assessment of the certain NPs effectiveness as a theranostic agent.[9–12] Due to the complexity of living biosystems, PC studies *in vivo* can give ambiguous results, therefore, *in vitro* modeling of biosystems from simple to more complex is needed. The purpose of this work has been to investigate the interaction of proteins with the surface on magnetic NPs Fe_3O_4@SiO_2 in model experiments with the involvement of a comprehensive analysis of protein sorption isotherms on NPs, data on visible spectroscopy of NPs colloids and dynamic light scattering (DLS). Magnetite based NPs were used owing to their pronounced magnetic properties, good sorption capacity and accessibility. Here we present our early results on the research of surface modification of NPS by albumin to study the patterns of PC formation.[†]

We used bovine serum albumin (BSA), one of the main components of blood plasma, as a model component of PC. Albumin is a transport protein, *i.e.*, it has specific binding points of components in its structure, therefore, it should be well sorbed on the surface of MNPs. Also, in the creation of theranostic agents, the presence of binding centers makes albumin a convenient compound for surface modification by biologically active compounds.[13–15,‡] The possibility of using albumin as a

[†] Magnetic NPs of the core–shell structure Fe_3O_4@SiO_2 (Shanghai Allrun Nano Science & Technology Co., Ltd). Iron content in particles 64%, silicon 6%, average particle size 140 nm (electron microscopy data, see Figure 1); ζ-potential in water –34 m*V*.

[‡] For constructing isotherms of BSA sorption on MNPs, 30–2500 μl of an aqueous BSA solution was added to 5.0 ml of MNP suspension (with a particle concentration of 0.3 mg ml^{-1}) and the mixture was incubated in a drying oven at 37–39 °C for 2 h with periodic shaking. NPs with the sorbed protein were separated using a permanent magnet. Filtrate containing unbound protein was analyzed for the sulfur content by inductively coupled plasma atomic emission spectrometry (IRIS Intrepid II Duo spectrometer, Thermo Electron Corp., USA). The precipitate of

compound that satisfactorily models whole blood serum for quantitative PC studies has been also shown.[16]

To analyze the sorption data on albumin, we applied a computational approach that was successfully used earlier on the example of sorption of a number of ionic surfactants on NPs. As a result, the sequential formation of ordered monomolecular surfactant layers was shown.[17,18] Previous studies[12,19,20] have shown that such ordered sorption is also characteristic for albumin adsorption on various NPs. Therefore, the calculation method outlined in detail below has been tested to describe albumin adsorption (as a model of plasma proteins) on NPs.

Sorption characterized by the formation of ordered sorbate layers can be quantitatively characterized by the expression:[17] $Q = \theta\, Q_0$, where Q is the amount of adsorbed modifier (mol m^{-2}), θ is the number of monolayers on the sorbent surface, Q_0 is the amount of modifier when the monolayer is fully filled. The value Q_0 is calculated by the formula $Q_0 = (s_0\, N_A)^{-1}$, where N_A is the Avogadro constant, s_0 is the surface area occupied by the BSA molecule in a continuous extremely compressed surface monolayer (the so-called 'molecular area'). In the case of the formation of an ordered layer, the value of s_0 (and, consequently, Q_0) is a constant determined only by the structural parameters of the sorbate molecule. Thus, the dimensionless value $\theta = Q/Q_0$ shows the degree of NP surface filling with modifier molecules.[§]

According to the modern concepts, PC consists of two layers: internal and external. The inner layer, so-called 'hard' PC, includes tightly bound protein molecules, and the outer layer ('soft' PC) is represented by proteins in dynamic equilibrium with the solution.[21] It is assumed that protein molecules of the 'hard' corona interact directly with the surface of the nanomaterial, while 'soft' corona proteins bind to 'hard' corona proteins using weaker protein-protein interactions. It is anticipated that the albumin sorption corresponding to $\theta = 1$ would be consistent with the complete formation of a 'hard' corona (ordered monolayer), at $\theta > 1$ the further albumin multilayer ('soft' corona) is formed.

Images of the initial NPs and their size distributions obtained by electron microscopy (EM) and DLS are shown in Figure 1. Comparing the size distributions resulting from EM data (allows for measuring the NPs size in dried form) and DLS method (determines the dimensions considering the hydrate shell and possible aggregation in the solution), it can be seen that in both cases the particles have comparable nanometer sizes, *i.e.*, there is no significant NPs aggregation in the aqueous medium.

The albumin sorption isotherms are shown in Figure 2. The graphical dependence of the sorption isotherm in water is the most informative, here, a curve break corresponding to $\theta = 1.0$–1.1 is observed, *i.e.*, a fully filled monolayer. Further, the sorption curve changes shape (the sorption mechanism also apparently changes): at $\theta < 1$ it is close to linear and at $\theta > 1$ it turns into sigmoidal shape, which is typical for polymolecular sorption, while sorption intensifies. Thus, the resulting isotherm of sorption from water makes it possible to clearly separate these two PC components: the formation of an ordered monolayer ('hard corona') and the build-up of subsequent layers ('soft corona'). If background compounds (sodium phosphate buffer and citrate buffer) are present, sorption is difficult because of their competition for the NPs surface with the protein. Apparently, the construction of the sorption isotherm in the absence of background components can give more correct information about the structure of the protein layer on the NPs surface.

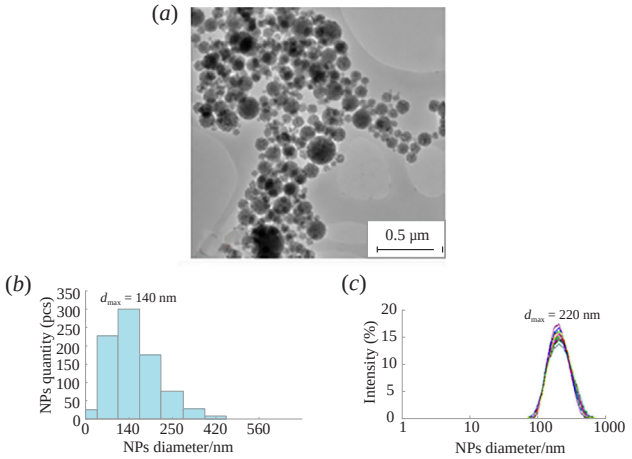

Figure 1 (*a*) TEM image of the initial NPs, (*b*) histogram of the NPs size distribution (EM data analysis) and (*c*) size distribution curve (obtained by DLS).

The formation of a precisely ordered surface BSA monolayer on the NPs has been revealed in a number of published works. To calculate sorption isotherms, we used the value of the surface area occupied by a BSA molecule in an extremely filled surface monolayer equal to 32 nm^2 which was calculated[12] based on studies of the kinetics of NPs aggregation. Inasmuch as the albumin molecule can be geometrically simplified by an equilateral triangular prism with sides of 8 nm and a height of 3 nm,[20] the calculation of the area of the prism base gives 28 nm^2, which is quite close to 32 nm^2. Also, based on the fluorescence correlation spectroscopy data, the thickness of the BSA monolayer was calculated as 3.3 nm, that is close to the theoretical height of the prism of 3 nm.[18] Thus, the analysis of the obtained and published data does show that an ordered monolayer structure is formed, where the sorbate molecules are arranged on the surface in a certain order. Given that the ordering of the sorption layer refers only to the 'hard' corona, the calculation of θ from the expression $Q = \theta\, Q_0$ will be correct only for it (*i.e.*, for $0 \leqslant \theta \leqslant 1$).

The NPs size and ζ-potential dependence on modification degree are shown in Figure 3. It can be seen that both indicators behave symmetrically if albumin sorption increases. Their slight decrease at $0 \leqslant \theta \leqslant 1$ can presumably be explained by structural

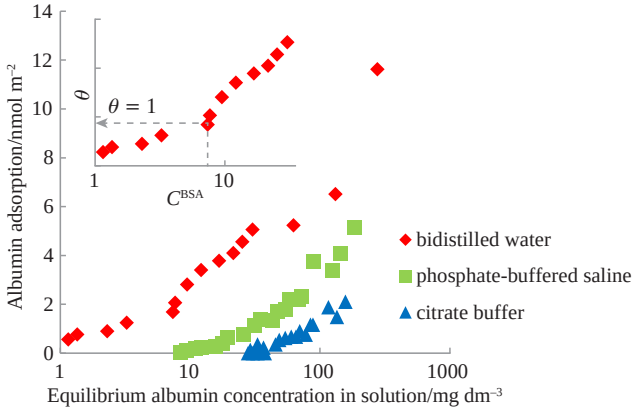

Figure 2 BSA sorption isotherms on Fe_3O_4@SiO_2 NPs in different background media in the coordinates of the equilibrium albumin concentration in solution C^{BSA} – albumin adsorption on MNPs. The inset depicts the initial part of the albumin sorption from water curve in the coordinates θ *vs.* C^{BSA}.

NPs was redispersed in 5 ml of bidistilled water; the particle size distribution and ζ-potential were determined in the resulting suspension (Zetasizer Nano ZS particle size analyzer, Malvern Panalytical Ltd., UK) and absorption spectra in visible region (UV-1800 spectrophotometer, Shimadzu, Japan). In experiments on the sorption of BSA, various background media were tested for simulating the internal environment of the body (sodium phosphate buffer and citrate buffer).

[§] BSA was used freeze-dried (Dia-m, Russia, at least 98% purity); the initial solution (0.1%) was prepared by dissolving the sample in bidistilled water.

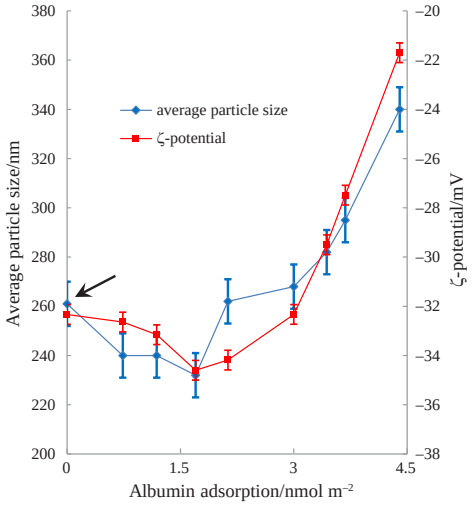

Figure 3 NPs average size and ζ-potential *vs.* degree of surface filling with the modifier (BSA). The arrow indicates a data point with no albumin addition (blank sample of pristine NPs).

rearrangements of the NPs hydrate shell during the formation of an ordered monolayer. At $\theta \geqslant 1$, there is a sharp transition to an increase in particle size, it can also indicate the formation of a protein surface multilayer ('soft PC'), apparently decreasing ζ-potential to some extent, which might be explained by the shielding of the NPs charge. The size of the unmodified NPs obtained here (260 nm) slightly exceeds the nominal value (220 nm), probably, because of the presence of a heat treatment stage (37–39 °C, 2 h).†

In order to further confirm the dynamics of changes in the size of NPs during albumin modification by an independent method, we applied the empirical Geller equation $D = K\lambda^{-n}$, which allowed us to estimate the particle size from the absorption spectra of dispersed systems.[22] In this equation, K is a constant (independent of the wavelength), D is the optical density, λ is the wavelength, n is a constant determined by the particle size. In theory, the dependence of n on the average particle size is inversely proportional, and n usually takes values from 2 to 4. Thus, in theory, n should decrease with increasing particle sizes, and *vice versa*. From the absorption spectra of colloids for different θ values (see Figure 4), the corresponding n values were calculated (shown in Table 1). The data qualitatively confirms the previously made conclusions about a decrease in $\bar{d}_{NP}$ (n increases from 1.92 to 2.87) at $0 \leqslant \theta \leqslant 1.1$ and a subsequent increase in $\bar{d}_{NP}$ at $\theta > 1.1$ (n decreases to 2.15).

Thus, according to the results of the comprehensive model study of proteins interaction with the NPs surface, the existence of an ordered maximally filled ($\theta \approx 1$) surface protein monolayer (the so-called 'hard PC') and then a multilayer ('soft PC') of a different structure has been confirmed. The degree of the

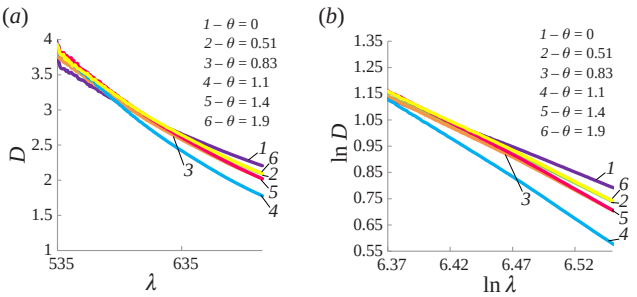

Figure 4 Spectra in the visible region for aqueous NPs colloids with varying degrees of surface modification: (*a*) D *vs.* λ and (*b*) ln D *vs.* ln λ.

Table 1 Calculated values of the constant n in the Geller equation depending on the average NPs diameter (according to the DSL data) and degree of the surface modification.

Q/nmol BSA m^{-2}	θ	$\bar{d}_{NP}$/nm	n
no BSA	0	261	1.92
0.77	0.51	257	2.15
1.3	0.83	253	2.20
1.6	1.1 (complete monolayer)	233	2.87
2.1	> 1 (multilayer)	241	2.32
2.8	> 1 (multilayer)	260	2.15

monolayer filling can be calculated *via* the proposed computational approach. The study was carried out by constructing isotherms of albumin adsorption on NPs and using a computational method, as well as using DLS method and studying the spectral NPs characteristics. The proposed integrated approach may be relevant both for the study of the formation and structure of BC and for the successful synthesis and application of theranostic multifunctional NPs.

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