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Protein-Coated Magnetic Nanoparticles: Creation and Investigation

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A novel universal approach to cross-linking of protein macromolecules on the surface of magnetite nanoparticles has been developed. The approach is based on protein liability to free radical modification, leading to the formation of intermolecular covalent cross links. Free radicals are locally generated on the surface of nanoparticles. Using a set of physicochemical methods, it has been proven that stable coatings composed of protein macromolecules are formed around individual nanoparticles. The proteins fixed on nanoparticles do not lose their activity as a result of adsorption and free radical modification. Fluorescent probe approach for evaluation of the native functional properties of serum albumin as a part of coating is suggested.

Keywords: Stable Coating, Magnetic Nanoparticles, Magnetite, Adsorption, Free Radicals, Electron Spin Resonance, Spin Labels, Ferromagnetic Resonance, Fluorescent Probe.

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1. INTRODUCTION

Magnetic nanoparticles (MNPs) have many applications in different areas of biology and medicine such as hyperthermia, magnetic resonance imaging, immunoassay, cell and molecular separation, a smart delivery of drugs to target cells [1–5]. Nanosystems for biomedicine consist of one or more magnetic cores and biological or synthetic molecules which serve as a basis for polyfunctional coatings on MNP surface. Proteins are promising materials for creation of coatings on MNPs due to their biocompatibility, an ability to protect magnetic cores from influence of biological liquids and prevent agglomeration of MNSs in dispersion, their possible functional activity as therapeutic products and biovectors [6]. The model which is often used when nanosystems are developed is presented in Fig. 1.

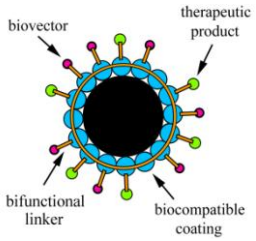

Fig. 1 – The classical scheme of magnetically targeted nanosystem for a smart delivery of therapeutic products

The creation of stable protein coatings with retention of native properties of molecules still is an important biomedical problem because of disadvantages of the commonly used methods such as formation of a polydisperse ensemble of particles, nonselective linking of proteins leading to cross-linking of macromolecules in solution, and desorption of coatings [7–8].

It is known that proteins can be chemically modified in the presence of free radicals with formation of cross-links [9]. The goals of the work were to create stable protein coatings on the surface of individual MNPs using a fundamentally novel approach based on the ability of proteins to form interchain covalent bonds under the action of free radicals and estimate activity of proteins in the coating; to use approaches based on magnetic resonance spectroscopy and develop a spectral and fluorescent method for studying adsorption processes of proteins and their functional properties in MNP coatings.

2. MATERIALS AND METHODS

2.1 Magnetic Sorbent Synthesis

Nanoparticles of magnetite Fe_3O_4 were synthesized by co-precipitation of ferrous and ferric salts in water solution at 4°C and in the alkaline medium according to the procedure [10]:

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4\downarrow + 4H_2O$$

MNPs were stabilized by double electric layer with the use of US-disperser ("MELFIZ", Russia). To create the double electric layer 0.1 M phosphate-citric buffer solution (0.05 M NaCl) with pH value of 4 was introduced. MNP concentration in hydrosol was equal to 15–40 mg/ml.

2.2 Protein Coating Formation

Bovine serum albumin (BSA), human serum albumin (HSA) and thrombin (TR) with activity of 92 units per 1 mg ("Sigma-Aldrich", USA) were used for protein coating formation. We used 2.8 ml of protein solution with a concentration of 1−3 mg/ml in 0.05 M phosphate buffer pH 6.5 (BSA, HSA) or pH 7.3 (TR), 50 μl of 3% hydrogen peroxide solution, and 0.35 ml of MNP hydrosol (the sample obtained were labeled as A-NP-1 and T-NP-1). We also used the samples prepared without H_2O_2 and labeled as A-NP-0 and T-NP-0 and the samples of supernatant solutions taken from A-NP-1 and A-NP-0 in the process of magnetic separation and labeled as s-A-NP-1 and s-A-NP-0 respectively. HSA

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and MNP solutions with concentrations of 2.6 and 1.6 mg/ml, respectively, were used as the references (labeled as R-A and R-NP, respectively).

Hydrogen peroxide interacts with ferrous ion on MNP surface with formation of hydroxyl-radicals by Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

A-NP-1-acid was a reaction mixture, containing 10 μl of ascorbic acid with concentration of 150 mg/ml. Ascorbic acid is known to form free radicals in reaction with H_2O_2 and generate free radicals in solution but not only on MNP surface.

2.3 Study of Protein Adsorption on MNP

The sizes of MNPs, proteins and MNPs in adsorption layer were analyzed using dynamic light scattering (Zetasizer Nano S "Malvern", England) with detection angle of 173° at temperature 25°C.

The study of protein adsorption on MNPs was performed using ESR spectroscopy of spin labels. The spectra of the radicals and MNPs were recorded at room temperature using Bruker EMX 8/2.7 X-band spectrometer at a microwave power of 5 mW, modulation frequency 100 kHz and amplitude 1 G. The first derivative of the resonance absorption curve was detected. The samples were placed into the cavity of the spectrometer in a quartz flat cell. Magnesium oxide powder containing Mn^{2+} ions was used as an external standard in ESR experiments.

Spin label technique allows studying adsorption of macromolecules on nano-sized magnetic particles in dispersion without complicated separation processes of solution components [11]. The principle of quantitative evaluation of adsorption is the following. Influence of local fields of MNPs on spectra of radicals in solution depends on the distance between MNPs and radicals [12–13]. If this distance is lower than 40 nm for magnetite nanoparticles with the average size of 17 nm [14] ESR spectra lines of the radicals broaden strongly and their amplitude decreases to zero. The decreasing of the spectrum amplitude is proportional to the part of radicals which are located inside the layer of 40 nm in thickness around MNP. The same happens with spin labels covalently bound to protein macromolecules. An intensity of spin label spectra decreases as a result of adsorption of macromolecules on MNPs (Fig. 2). We have shown that spin label technique can be used for the study of adsorption value, adsorption kinetics, calculation of average number of molecules in adsorption layer and adsorption layer thickness, concurrent adsorption of macromolecules [10, 15–16].

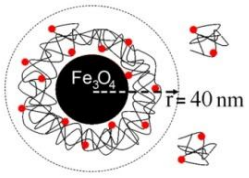

Fig. 2 – Magnetic nanoparticle and spin-labeled macromolecules in dispersion

The reaction between the radical 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid N-hydroxysuccinimide ester and protein macromolecules was conducted at room temperature. Average amount of spin labels on protein macromolecules reached 1 per 4–5 albumin macromolecules and 1 per 2–3 thrombin macromolecules. The portion of adsorbed protein was calculated from amplitude of the low-field line of nitroxide radical triplet I_{+1}.

The method of ferromagnetic resonance (FMR) was also used to study adsorption layer formation. Resonance conditions for MNPs in magnetic field of spectrometer include a parameter of the shift of FMR spectrum proportional to H_1 which is a local field created by MNPs in linear aggregates in spectrometer field. The local field is proportional to MNPs magnetic moment (μ) and inversely proportional to a cubic number of MNPs in aggregate (n) and D^3 which is a cubic distance between MNPs in linear aggregates [17]. Coating formation and the thickness of adsorption layer influence on the distance between MNPs and decrease dipole interactions. As a result the centre of FMR spectrum moves to higher fields.

To study the systems containing MNPs and HSA, the probe dye 3,3'-di-(γ-sulfopropyl)-4,5,4',5'-dibenzo-9-ethylthiacarbocyanine betaine (TCB) was used. In aqueous solution, TCB occurs mainly as the cis-dimer (λ_{abs} = 535 nm, practically nonfluorescent) and can form stable J-aggregates (characterized by narrow peaks with λ_{abs} = 645 nm and λ_{fl} = 660 nm). In the presence of HSA, a noncovalent complex of the trans-monomer (λ_{abs} = 612 nm) is formed. The great spectral shift, the high binding constant (>10^6 l mol^{-1}), and the high fluorescence quantum yield of the dye bound to the protein (0.57) make TCB efficient spectral and fluorescent probe for HSA [18]. Upon fluorimetric titration, equal volumes of solutions of the samples were added to a buffer solution (pH 6.5) with constant concentration of the dye (2−5 10^{-6} mol l^{-1}). The estimation of HSA interaction with TCB in the samples was performed in terms of the effective protein concentration (c_{eff}) equal to the ratio of the fluorescence intensities of the complex TCB-HSA in the samples and in the R-A solution (dimensionless value).

The absorption spectra of the dye were measured on a SF-2000 (OKB "Spectr", Russia) spectrophotometer and fluorescent measurements were made on a Flyuorat-02-Panorama (Russia) spectrofluorimeter in standard (1 cm) quartz cells at room temperature. The fluorescence spectra were corrected for the spectral characteristics of the excitation channel of the instrument and for the transmission signal of the sample.

2.4 Coating Stability Analysis and Analysis of Selectivity of Free Radical Process

The property of fibrinogen (FG) to replace BSA previously adsorbed on MNP surface was proved by complex study of systems containing MNPs, spin-labeled BSA and FG with spin label technique and FMR [10, 15]. It was used in this work for estimating BSA coating stability. A 0.25 ml of FG ("Sigma-Aldrich", USA) solution with concentration of 4 mg/ml in 0.05 M phosphate buffer with pH 6.5 was added to 1 ml

of the samples A-NP-0, A-NP-1. The cluster formation was observed by dynamic light scattering.

The samples A-NP-0, A-NP-1, T-NP-0, T-NP-1 were centrifuged at 120,000 g during 1 h on "Beckman Coulter" (Austria). On these conditions MNPs precipitate, but macromolecules physically adsorbed on MNPs remain in supernatant liquid. The precipitates containing MNPs and protein fixed on MNP surface were dissolved in buffer solution with subsequent evaluation of the amount of protein by Bradford colorimetric method [19].

Free radical modification of proteins in supernatant liquids of A-NP-0, A-NP-1 and the additional sample A-NP-1-acid were analyzed by IR spectroscopy using FTIR spectrometer Tenzor 27 ("Bruker", Germany) with DTGS-detector with 2 cm^{-1} resolution in order to reveal the selectivity of free radical process in A-NP-1.

2.5 Enzyme Activity Estimation

Estimation of enzyme activity of protein fixed on MNP surface was performed on the example of thrombin. Thrombin may lose its activity as a result of free radical modification and the rate of the process of conversion of fibrinogen to fibrin may decrease. So estimation of enzyme activity of thrombin cross-linked on MNP surface during free radical modification was performed by comparison of the rates of conversion of fibrinogen to fibrin under the influence of thrombin contained in reaction mixtures. 0.15 ml of the samples T-NP-0, T-NP-1 and thrombin solution was added to 1.4 ml of FG solution with concentration of 4 mg/ml. Kinetics of fibrin formation was studied by Rayleigh light scattering on spectrometer 4400 ("Malvern", England) with multibit 64-channel correlator.

3. RESULTS AND DISCUSSION

The signal intensity of spin-labeled macromolecules decreased after introduction of MNPs into the solution that testifies to the protein adsorption on MNPs (Fig. 3). Spectra of the samples A-NP-0 and T-NP-0 consist of nitroxide radical triplet, the third line of sextet of Mn^{2+} (the external standard) and FMR spectrum of MNPs. Rotational correlation time of spin labels does not change after MNPs addition. The dependences of spectra lines intensity for spin-labeled BSA and thrombin in the presence of MNPs on incubation time are shown in Table 1. Signal intensity of spin-labeled BSA changes insignificantly. These changes correspond to adsorption of approximately 12% of BSA after the sample incubation for 100 min. The study of adsorption kinetics allows establishing that adsorption equilibrium in T-NP-0 takes place when the incubation time equals to 80 min and ~41% of thrombin is adsorbed. The value of adsorption A may be estimated using the data on the portion of macromolecules adsorbed and specific surface area calculated from magnetite density (5200 mg/cm^3), concentration and size.

The FMR spectra of the samples A-NP-0, T-NP-0 and MNPs are characterized by different position in magnetic field (Fig. 4). This phenomenon of FMR spectrum centre shift we observed in the system A-NP-0 after FG addition [10]. The similar centre positions of FMR spectra of MNPs without coating (3254±3 G) and MNPs in BSA coating (3253±3 G) point to a very thin coating and low adsorption of BSA. According to FMR centre position (3449±3 G) the thrombin coating on MNPs is thicker than BSA coating (the spectrum of MNPs with thick coating becomes similar to FMR spectra of isolated MNPs). This result is consistent with the data obtained by ESR spectroscopy.

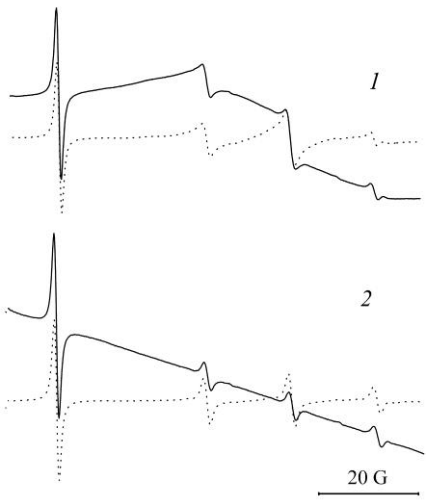

Fig. 3 – ESR spectra of spin labels on BSA (*1*) and thrombin (*2*) macromolecules before (dotted line) and 75 min after (solid line) addition of MNPs to protein solution at 25°C. Protein concentration equals to 1 mg/ml. External standard is Mn^{2+}

Table 1 – The dependence of relative intensity of low-field line of triplet I_{+1} of nitroxide radical covalently bound to BSA and thrombin macromolecules, and the portion N of the protein adsorbed on incubation time t of the samples A-NP-0 and T-NP-0

	BSA		Thrombin	
t, min.	I_{+1}, rel. un.	N, %	I_{+1}, rel. un.	N, %
0	0.230 ± 0.012	0 ± 5	0.25 ± 0.01	0 ± 4
15	-	-	0.17 ± 0.01	32 ± 4
35	0.205 ± 0.012	9 ± 5	0.16 ± 0.01	36 ± 4
75	0.207 ± 0.012	10 ± 5	0.15 ± 0.01	40 ± 4
95	-	-	0.15 ± 0.01	40 ± 4
120	0.200 ± 0.012	13 ± 5	0.14 ± 0.01	44 ± 4

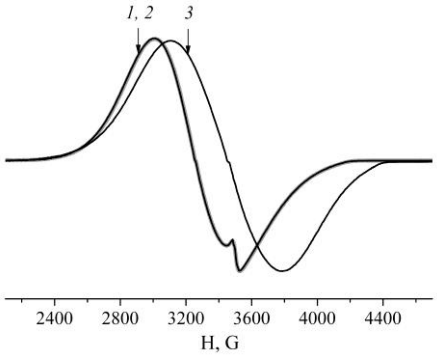

Fig. 4 – FMR spectra of MNPs (*1*), MNPs in the mixture with BSA (the sample A-NP-0) after incubation time of 120 min (*2*), and MNPs in the mixture with thrombin (the sample T-NP-0) after incubation time of 120 min (*3*). Protein concentration equals to 1 mg/ml

FG ability to replace BSA in adsorption layer on MNP surface is demonstrated in Fig. 5. Initially there is bimodal volume distribution of particles over sizes in the sample A-NP-0 that can be explained by existence of free (unadsorbed) BSA and MNPs in BSA coating in dispersion. After FG addition the distribution changes. Micron-sized clusters form in the sample that proves FG adsorption on MNPs [15]. In the case of A-NP-1 volume distribution is also bimodal. The peak of MNPs in BSA coating is characterized by particle size of maximal contribution to the distribution of ~23 nm. This size is identical to MNPs in BSA coating in the sample A-NP-0. It proves that H_2O_2 addition does not lead to uncontrollable linking of protein macromolecules in solution or cluster formation. Since MNPs size is 17 nm, the thickness of adsorption layer on MNPs is approximately 3 nm. After FG addition to A-NP-1 micron-sized clusters do not form. So adsorption BSA layer formed in the presence of H_2O_2 keeps stability. This stability can be explained by formation of covalent bonds between protein macromolecules [9] in adsorption layer as a result of free radical generation on MNP surface. Stability of BSA coating on MNPs was demonstrated for the samples A-NP-1 and A-NP-1 incubated for more than 100 min before FG addition. Clusters are shown to appear if the incubation time is insufficient.

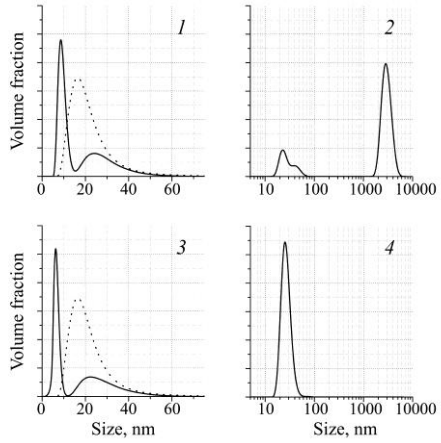

Fig. 5 – Volume distributions of particles in sizes in systems without (*1*, *2*) and with (*3*, *4*) H_2O_2 (A-NP-0, A-NP-1) incubated for 2 h before (*1*, *3*) and 20 min after (*2*, *4*) FG addition. Dotted line is the volume distribution of nanoparticles in sizes in dispersion

According to Bradford colorimetric method the precipitates of systems with H_2O_2 (A-NP-1 and T-NP-1) were shown to contain more protein than the same systems without H_2O_2 (A-NP-0 and T-NP-0). Therefore in the samples containing H_2O_2 the significant part of protein molecules does not leave MNP surface when centrifuged while in the samples A-NP-0 and T-NP-0 the most of protein molecules leaves the surface. This indicates the stability of adsorption layer formed in the presence of free radical generation initiator and proves cross-link formation.

Analysis of content of supernatant liquids obtained after ultracentrifugation of reaction systems containing MNPs and BSA that differed by H_2O_2 and ascorbic acid presence (A-NP-0, A-NP-1 and A-NP-1-acid) allows evaluating the scale of free radical processes in the presence of H_2O_2. As it was mentioned above in the presence of ascorbic acid free radicals generate not only on MNP surface but also in solution. So both molecules on the surface and free molecules in solution can undergo free radical modification in this case. The IR-spectrum of A-NP-1-acid differs from the spectra of A-NP-0 and A-NP-1 in the region of 1200–800 cm^{-1} while the spectra of A-NP-0 and A-NP-1 almost have no differences. The changes in this area are explained by free radical oxidation of amino acid residues of methionine, tryptophane, histidine, cysteine, and phenylalanine. These residues are sulfur-containing and cyclic ones which are the most sensitive to free radical oxidation [9, 20]. The absence of differences in A-NP-0 and A-NP-1 proves that cross-linking of protein molecules in the presence of H_2O_2 takes place only on MNP surfaces.

The results of the fluorimetric titration are shown in Fig. 6. Introduction of solutions of A-NP-0, A-NP-1, s-A-NP-0, and s-NP-1 into a cell with TCB leads to a growth of the fluorescence intensity (*1-4*, complex of the trans-monomer). In case of R-NP, fluorescence did not grow. The values of c_{eff} for the samples A-NP-0 and A-NP-1 were found to be similar (69±3% and 68±3%, respectively). The decrease of c_{eff} with respect to that for R-A can be explained by the effect of steric shielding of binding sites of HSA by MNPs as a result of protein adsorption on their surface. As a result of adsorption, the hydrophobic region of albumin molecules (which binds TCB) apparently becomes inaccessible to the solvent. Hence, binding the fluorescent probe to such protein is impossible. For the sample s-A-NP-0, c_{eff} = 93±5%, which can be due to practically complete release of noncovalently bound albumin macromolecules from the surface of MNPs in the process of magnetic separation and retention of the ability of these molecules to bind the probe. This apparently points to recovery of the initial native conformation of HSA molecules upon desorption. For s-A-NP-1, c_{eff} of the protein was found to be lower (42±3%) than that for A-NP-0 and A-NP-1. This result is probably explained by the fact that the coating fixed on MNPs by the free radical mechanism contains protein molecules capable of binding TCB, which turned out in the sediment as a result of magnetic separation. This conclusion is confirmed by coincidence of the c_{eff} values for the samples with their different size (~25 and ~20 nm for A-NP-1 and A-NP-0, respectively).

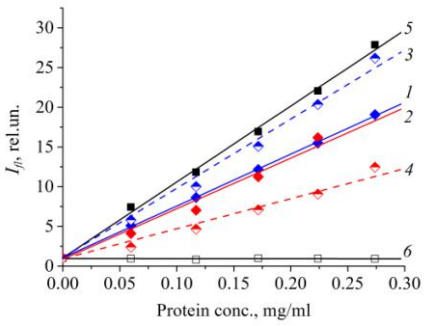

Fig. 6 – Dependences of the TCB fluorescence intensity (c_{TCB} = 5 10^{-6} mol l^{-1}) on the total HSA content in the samples (*1*) A-NP-0, (*2*) A-NP-1, (*3*) s-A-NP-0, (*4*) s-A-NP-1, (*5*) R-A, and (*6*) R-NP

Estimation of enzyme activity of thrombin cross-linked on MNP surface was performed by comparison of the rates of conversion of fibrinogen to fibrin under the influence of thrombin contained in reaction mixtures T-NP-0, T-NP-1 and thrombin solution. The curves for the samples containing thrombin and MNPs that differ by the presence of H_2O_2 illustrated preservation of enzyme activity of thrombin during free radical cross-linking on MNP surface. Fibrin gel was formed during ~15 min in both cases in the presence of MNPs. Rayleigh light scattering intensity was low when thrombin solution was used and small fibrin particles were formed in this case. Enzyme activity of thrombin is known to decrease spontaneously in solution [21]. The method of free radical cross-linking of proteins seems promising for enzyme immobilization because the proteins keep their activity longer when adsorbed on MNPs.

4. CONCLUSION

The novel method of fixation of proteins on MNPs proposed in the work was successfully realized on the example of serum albumin and thrombin and magnetite nanoparticles. The method is based on the ability of proteins to form interchain covalent bonds under the action of free radicals. Hydrogen peroxide induced into reaction mixture served as initiator. By the set of physical (ESR spectroscopy, ferromagnetic resonance, dynamic and Rayleigh light scattering, IR spectroscopy, fluorescence) and biochemical methods it was proved that the coatings obtained are stable and formed on individual nanoparticles because free radical processes are localized strictly in the adsorption layer. The free radical linking of thrombin on the surface of nanoparticles has been shown to almost completely keep native properties of the protein molecules. The use of the fluorescent dye TCB provide information on accessibility of its hydrophobic pocket to the solvent. As a first approximation, the results support that the native functional properties of serum albumin as a part of coating are retained. Our study reveals principally novel technologies of one-step creation of biocompatible magnetically targeted nanosystems with multiprotein polyfunctional coatings which meet all the requirements and contain both biovectors and therapeutic products (Fig. 7).

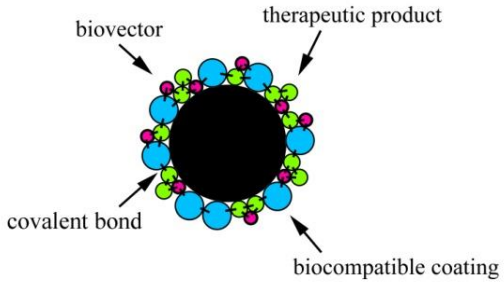

Fig. 7 – The scheme of magnetically targeted nanosystem for a smart delivery of therapeutic products based on the free radical protein cross-linking

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