Magnetic nanoparticles for medical application with a coating deposited with various methods

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Abstract. The work is devoted to the evaluation of the effectiveness of the methods of modifying magnetic nanoparticles for the development of targeted drug delivery systems. Both chemisorption by aminated particles and adsorption by unaminated particles are considered. Various methods for the determination of albumin and the study of the kinetics of dissolution of the albumin coat are given.

1. Introduction
Nanoparticles of magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃) are most often used in biomedical research. These particles should be non-toxic and non-immunogenic, and their size should allow penetration through the capillaries into tissues and organs without the formation of thromboses for successfully in vivo application. In practice, most often used magnetic nanoparticles coated with organic compounds – fatty acids, dextran, starch, polyvinyl alcohol, polyethylene glycol, various proteins [1]. These coatings should provide not only electrostatic stabilization of nanoparticles, the safety of their use, but also the possibility of conjugation of medications and compounds that increase the targeting of delivery to the lesion sites. The promising material for the creation of such coatings is albumin. Albumin does not have the toxicity of many polymeric carriers, largely binds highly lipophilic compounds and provides transport of the sorbed drug substance to the cell, which in particular allows the use of the anti-cancer drug paclitaxel without the highly toxic solvent Cremophor EL [2]. Moreover, albumin carriers with a 10 nm size bind to the cell non-specifically, providing intracellular delivery of the preparation. However, the main advantage of albumin is the ability for active transport of drugs inside the endothelial cell. This is due to the fact that a specific substrate for albumin binding, the gp60 receptor, is expressed on the cell surface. Interaction with this receptor stimulates the phagocytosis of the albumin-gp60 complex by the cell. Subsequent exocytosis through the basolateral part of the cell causes the active delivery of an albumin-bound drug into the tumor interstitium [3].

To date, various methods have been developed for creating protein coatings on the surface of iron oxide nanoparticles. In [4], in order to obtain a protein coating on Fe₃O₄-γ Fe₂O₃ nanoparticles at the stage of the formation of magnetic particles, trypsin was used in an aqueous solution close to neutral pH at a temperature of 4° C. The authors of patent JP635019 used to create a binding layer containing
organosilicon compounds on the surface of particles, the functional groups of which were used for carrying out the polymerization reaction and immobilization of biological components, on magnetic particles with polymer coatings containing immobilized biological components, including proteins. A method for the immobilization of trypsin on the surface of magnetite is known [5] including the treatment of the surface of magnetite with 3-aminopropyltriethoxysilane, the modification of the resulting derivative with glutaraldehyde, and subsequent binding of trypsin to the carrier. In work [6], to fix the adsorption layer of albumin macromolecules on magnetite nanoparticles, carbodiimide was used as the cross-linking agent. In the patent [7], a method for creating a stable protein coating based on the property of proteins to undergo cross-linking by the action of free radicals, which are generated on the surface of nanoparticles under the action of an initiator – hydrogen peroxide, was proposed.

It is obvious that the evaluation of the efficiency of using the above methods of modifying magnetic nanoparticles (MNPs) for the development of targeted drug delivery systems and the development of new methods is an urgent task, and this work is aimed at solving it.

2. Materials and methods

2.1. Synthesis of MNPs

The synthesis of the MNPs was carried out as follows [8]. To a solution containing a mixture of ferric sulfate and ferrous sulfate in a molar ratio of 2:1 and a volume of 700 ml, with constant stirring a mixture of 25% ammonium hydroxide solution and 1% ammonium acetate solution was added at a rate of 4 ml/min. Thus, the ratio of iron and ammonium acetate was 2:1:0.1. The synthesis was carried out until a saturated black color was fixed and a pH value of 8–9 was established. The next day, the resulting colloidal product was separated with centrifugation and washed four times with distilled water. To prepare the dry sample, the obtained MNPs were filtered and subjected to freeze-drying at a temperature of –50 °C and a pressure of 3 Pa for 48 hours.

2.2. Amination of MNPs

The modification of the MNPs surface was carried out according to the following procedure. In a 50 ml round-bottomed flask, 2 grams of dry MNPs and 25 ml of a 5% solution of 3-aminopropyltriethoxysilane were placed in pre-dried benzene. The reaction mixture was refluxed for two hours at 80° C using a thermostated cell connected to a LT-105a liquid thermostat (LOIP, Russia). The excess reagent was removed with repeated washing with dry chloroform using magnetic separation. At the final stage of washing, ethyl alcohol was used. During the modification and washing, the reaction mixture was vigorously stirred by means of a magnetic stirrer.

The total content of amino groups in the samples was determined as follows. 1 ml of 0.1N hydrochloric acid was added to the aminated MNPs and set to neutralize the amino groups for 15 minutes, periodically shaking. The suspension was then centrifuged for 5 minutes at 3000 min⁻¹. The supernatant was titrated with alkali (NaOH) with an acid-base indicator of methylorange 0.1. The final total content of amino groups was calculated from the amount of alkali.

The total content of amino groups does not give an idea of how much of the active substance can be chemisorbed on the modified surface. Therefore, the number of available amino groups by capacitance over the fluorescent dye indocyanine green (ICG) was determined. To this, 1 ml of a fluorescent dye solution of 1 mg/ml and 1 ml of distilled water were added to a suspension containing 50 mg of pre-aminated nanoparticles in 2 ml of water. Sorption was carried out in 15-ml polypropylene tubes on a LS-220 shaker (LOIP, Russia) at a stirring rate of 300 min⁻¹ for 2 hours. The solution was then centrifuged for 5 minutes at 3000 min⁻¹ and washed 5 times with distilled water and centrifuged.

Upon termination of the sorption of the ICG, its amount was determined. To this, 10 ml of a 0.1 N sodium hydroxide solution was added to the washed precipitate and stripped for 15 minutes. The solution was then centrifuged and 1 ml of supernatant was taken. The resulting solution was analyzed for the fluorescent dye content with the spectrophotometric method using the method [9] with a Unico 2802S spectrophotometer (Unico Sys, USA) at a wavelength of 700 nm.
The total amount of amino groups was 0.81 mmol/g, the amount of available amino groups was 0.048 mmol/g. The amount of available amino groups is about an order of magnitude lower than their total content in the sample. This can be explained by the fact that access of relatively large molecules to most amino groups in the shell is difficult. This fact was already considered by the authors in [10].

2.3. Formation of albumin coating on the surface of MNPs
The formation of the albumin coating on the MNPs surface was carried out with several methods:
- adsorption immobilization of albumin on the MNPs surface;
- chemisorption of albumin on the surface of aminated MNPs;
- cross-linking of the coating with glutaraldehyde.

Adsorption immobilization and chemisorption of albumin were carried out with a mixing device LS-220 at a frequency of 300 min\(^{-1}\) for 2 hours. To do this, 50 mg of MNPs was placed in a 15 ml polypropylene tube and 2 ml of a 20% albumin solution was added. Human albumin was used (Waxter AG, Vienna, Austria). After sorption, the resulting preparation was washed five times with magnetic separation.

Crosslinking of the coating with glutaraldehyde was carried out as follows. A sample of 50 mg of aminated MNPs was treated with a solution that was prepared by diluting 25 μl of 50% glutaraldehyde (Sigma Aldrich, Germany) solution with 10 ml of water followed by the addition of 1.3 ml of albumin (20% solution) for 1 hour with stirring, then repeatedly washed from the excess of reagents with water with magnetic separation.

2.4. Determination of the amount of albumin in aqueous solution
The method of determination of albumin with the aid of bromocresol green dye [11] was used in the work. It was believed that in the sample there were no substances interfering with the analysis [12]. Serum albumin, being a weak acid, binds in citrate buffer with bromocresol green to form a complex of gray-blue color. The photometric measurement is based on the presence of an absorption peak with a maximum at a wavelength of 585 nm by the molecules of the colored complex. All other components of the solution have weak absorption at this wavelength. Thus, the increase in the optical density of the reaction mixture is directly proportional to the content of albumin in the analyzed sample [13].

The preparation for determining the amount of albumin was prepared by adding to a citrate buffer of 500 ml volume and pH = 4.2 500 μl of Tween 80 and 90 mg of bromocresol green dye. To 5 ml of the preparation different amounts of albumin were added and spectrophotometric studies were performed at the 10th minute at a wavelength of 585 nm against a blank sample.

Similarly, a calibration was performed to determine the albumin in a Krebs-Henseleit solution [14]. For this, 2 ml of the preparation and 2 ml of the Krebs-Henseleit solution were taken.

2.5. Determination of the amount of albumin in the coating with thermogravimetry
The study of the amount of albumin in the coating was carried out with thermogravimetry by heating to 900 °C in the air at a rate of 10 °C/min on the SetaramSetsys Evolution (Setaram Instrumentation, France). It was assumed that the entire organic shell consists of albumin and burns in the air.

2.6. Investigation of the physical properties of samples
Microphotographs of the MNPs were obtained with a transmission electron microscope (TEM) with JEM-1400 STEM (JEOL, Japan). The static magnetic properties of the MNPs were studied using a vibrating sample magnetometer Lake Shore 7410 (Lake Shore Cryotronics Inc., USA) in air at standard temperature.

3. Results and Discussion
As can be seen from the obtained TEM images (Fig. 1), samples with albumin sorbed on the MNPs and chemisorbed on the aminated MNPs are individual coated particles. In this case, the coating is thicker in samples with albumin sorbed on unaminated MNPs. The sample with albumin cross-linked with glutaraldehyde is a cluster of which several MNPs are combined by a common coating. The average size of such clusters is 150 nm, while the size of individual nanoparticles is within 10–20 nm.
3.1. Determination of the amount of albumin adsorbed and chemisorbed on MNPs

An analysis of the amount of albumin adsorbed on the MNPs surface and chemically adsorbed on the surface of aminated MNPs was performed on the residual protein content. After immobilization, the initial solution was analyzed for the presence of albumin, which for adsorption was 7.7 mg, and for chemisorption was 7.3 mg for 50 mg of MNPs.

![Figure 1(a,b,c). TEM images of MNPs coated with albumin with various methods: (a) sorption by MNPs; (b) chemisorption by aminated MNPs; (c) – crosslinking with glutaraldehyde.](image)

3.2. Determination of the amount of albumin in the cross-linked shell by thermogravimetry

The thermogram of heating the MNPs sample with albumin crosslinked with the glutaraldehyde method is shown in Figure 2. Denaturation of the protein crosslinked on the nanoparticle surface usually occurs at temperatures below 100°C [15]. This stage coincides with the stage of water loss by the sample. Both processes are endothermic and therefore not separable. With a further increase in temperature, a two-stage burnout of albumin denaturing products is observed. In the approximation that the sample is dry, that all organic compounds burn out, it is possible to estimate the amount of albumin cross-linked on the MNPs surface, which is 16.7%. This figure correlates with the amount of adsorbed and chemisorbed protein (about 15%).

![Figure 2. Thermogram of MNPs sample with albumin, cross-linked glutaraldehyde method.](image)

3.3. Investigation of kinetics of dissolution of albumin coating in Krebs-Henseleit buffer

Samples were washed from the excess of the active substance and lyophilized. A sample of 50 mg was placed in a conical tube and poured 2 ml with a Krebs-Henseleit buffer solution, which was similar to the salt composition of the blood. Dissolution of the coating was carried out on a LOIP LS-110 agitator at a speed of 300 min⁻¹. At certain time intervals (0.5, 1, 2, 3, 5, 5 hours), the solutions...
were centrifuged and analyzed for albumin content as described above. However, according to the data obtained, it was not possible to establish the release of albumin. This suggests that albumin, in any method of immobilization, forms a strong coating that is insoluble in Krebs-Henseleit buffer solution.

Thus, obtained nanoparticles can be used in the development of magnetically controlled delivery products.

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