

Article

Synthesis of Magnetic Nanoparticles Coated with Human Serum Albumin and Loaded by Doxorubicin

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Abstract: Magnetic iron oxide (II,III) nanoparticles (MNPs) are highly interested in biomedicine. However, their application is limited by oxidation, aggregation, rapid clearance from the body, and poor biodistribution. Coating by human serum albumin (HSA), the predominant blood plasma protein, can significantly influence properties, prolong circulation half-life, and enhance tumor capture efficiency. Here, we report the synthesis of oleic acid and Tween20-coated MNPs and their interaction with HSA. The influence of albumin coating on MNP size, zeta potential, aggregation ability, and toxicity was studied. The particles were characterized by dynamic light scattering, transmission electron microscopy, and Fourier transform infrared spectroscopy methods. The nanoparticles' relaxivities (r_1 and r_2) were assessed under a magnetic field of 1.88 T to evaluate their performance in MRI applications. The anticancer drug doxorubicin (DOX) loading capacity of up to 725 $\mu\text{g}/\text{mg}$ for albumin-coated MNPs was determined. DOX-loaded MNPs displayed pH-sensitive drug release during acidic conditions. The series of DOX-loaded nanocomposites indicated inhibition of A549 cell lines, and the IC_{50} values were evaluated. This research underscores the utility of HSA-coated MNPs in enhancing the efficacy and stability of drug delivery systems in biomedicine.

Keywords: magnetic nanoparticles; magnetic iron oxide; functionalization; oleic acid; coating; tween; serum albumin; magnetic resonance imaging; doxorubicin; drug delivery



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1. Introduction

Magnetic nanoparticles demonstrate excellent efficacy as thermoelectric materials, drug carriers, imaging agents, cell separation, catalysis, and biosensors [1–3]. Magnetic iron (II,III) oxide nanoparticles (MNPs) are valuable tools for biology, biochemistry, diagnostics, and medicine [4–7]. They are relatively easy to obtain, cheap, and, in most cases, biocompatible due to the involvement of iron ions in metabolism [8]. However, the use of MNPs is limited by their high surface energy, which causes them to aggregate and oxidize, causing a loss of magnetic properties, an increase in vivo toxicity, and the generation of reactive oxygen species [9–11]. Another significant issue is the rapid elimination from the bloodstream caused by the innate immune system [12–14]. Indeed, non-specific accumulation in various organs leads to less than 1% of injected MNPs reaching the tumor site [15]. Surface coatings are commonly used to enhance the colloidal stability and biocompatibility of MNPs [16]. Among various surfactants, biomolecules are promising but mostly unexamined ones within the framework of changing properties of MNPs [17–20].

Human serum albumin (HSA) is a major blood plasma transport protein with a long circulation half-life (19 days) [21]. It contains multiple sites that can bind fatty acids, steroids,

hormones, and drugs [22]. HSA provides drug and probe transport in tumor tissues through interaction with receptors (FcRn, gp60, and SPARC) [20,23–25] overexpressed in cancer cells. It makes it a versatile platform for therapeutic and diagnostic agent delivery [26–28]. As one of the abundant plasma proteins, albumin participates in drug metabolism and interacts with nanoparticle surfaces, changing their biodistribution [20,29–31]. HSA-coating on NPs provides prolonged circulation time [29], prevention of proteins' non-specific adsorption [32–34], reduced cytotoxicity and immune response [12,34–37], and cancer tissue uptake [29].

Doxorubicin (DOX) is an essential drug in cancer treatments [38–40]. Despite its high efficacy, it has significant cardiotoxicity, limiting clinical application [39]. Various DOX-loaded magnetic nanocomposites are being considered for targeted drug delivery [41–44]. However, the high drug capacity and pH-sensitive specific release in cancer cells are required for further biomedical applications [45].

In this work, we studied the suitability of albumin coating on MNPs and the features of the resulting nanoparticle properties. As model MNPs for this study, oleic acid (OA)-coated and double-layered (OA and Tween20 (TW20)) magnetite nanoparticles were synthesized. Magnetic nanocomposites coated with albumin (MNP_OA_HSA and MNP_OA_TW20_HSA) were analyzed using dynamic light scattering (DLS), transmission electron microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR) methods. The albumin-loading capacities up to 1.75 mg/mg and 1.43 mg/mg were obtained for MNP_OA_HSA and MNP_OA_TW20_HSA, respectively. The stability experiments of the initial and albumin-coated MNPs in various buffer solutions were provided to see the impact of HSA in different pH and ionic strengths. The nanoparticles' relaxivities (r_1 and r_2) at 1.88 T were determined to evaluate possible MRI applications. We studied the DOX loading capacity and pH-sensitive drug release. We deployed the synthesized DOX-MNPs in cytotoxicity experiments using A549 cell lines, and the IC₅₀ values were evaluated. We believe that albumin coating and drug loading lead to the smart, targeted theranostic construct for further intravenous cancer treatment.

2. Materials and Methods

2.1. Materials

Iron (III) chloride hexahydrate, iron (II) sulfate heptahydrate, concentrated hydrochloric acid, hydrogen peroxide 30%, aqueous ammonia 30%, sodium chloride, potassium hydroxide, boric acid, sodium acetate, and glacial acetic acid were purchased from Reachim (Moscow, Russia). Tris (hydroxymethyl)aminomethane (Tris) and Tris hydrochloride were obtained from Helicon (Moscow, Russia). The following reagents were purchased from manufacturers: oleic acid and methylene blue (Soyuzkhimprom, Novosibirsk, Russia), human serum albumin (PAN Biotech, Aidenbach, Germany), polysorbate Tween 20 (Merck, Darmstadt, Germany), doxorubicin (SOAO Ferein, Minsk, Belarus), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (AppliChem, Darmstadt, Germany). All solutions were prepared using deionized water (18 M Ω) obtained with Millipore Simplicity 185 (Millipore, St. Louis, MO, USA).

2.2. Characterization of NPs

DLS and ξ -potential measurements were carried out on Malvern Zetasizer Nano (Malvern Instruments, Worcestershire, UK) in deionized water at 25 °C, 173° scattering angle, and concentration of NPs ~300 μ g/mL and ~75 μ g/mL. Before measurement, every sample was sonicated (50 W, 35 kHz) for 3 min.

TEM data were obtained on a JEM-1400 instrument (Jeol, Tokyo, Japan) at an accelerating voltage of 80 kV. A micro-quantity solution of NPs was sonicated at 50 W for 3 min

and dropped on a copper plate with a carbon film grid for sample preparation. Samples were contrasted using uranyl acetate.

FTIR spectra were measured on a 640-IR FT-IR spectrometer (Varian, Peabody, MA, USA) from 4000 to 400 cm^{-1} at 25 °C, accompanied by a KBr pellet.

The colloidal stability of NPs was carried out by DLS using a 300 $\mu\text{g/mL}$ solution of NPs at 25 °C in deionized water, 0.15 M NaCl, PBS buffer, and 0.15 M acetate buffer (pH 4.0, 5.0, 6.0) at various time points.

2.3. Synthesis of Iron (II,III) Oxide Nanoparticles (MNP)

MNPs were produced according to the previously reported procedure [46]. Briefly, solutions of FeCl_3 (0.92 mL, 1 M), FeSO_4 (0.23 mL, 2 M), and hydrochloric acid (2.3 mL, 30%) were mixed. A solution of ammonia (11.54 mL, 30%) was added to the resulting mixture and incubated under stirring (600 rpm) for 30 min. The resulting black precipitate of MNPs was washed with deionized water to a neutral pH using magnetic decantation.

2.4. Synthesis of Oleic Acid-Stabilized MNPs (MNP_OA)

Oleic acid-stabilized MNPs (MNP_OA) synthesis was adapted from Kovrigina E. et al. [46]. Briefly, a 1.4 mL solution of FeCl_3 (0.346 M) and FeSO_4 (0.211 M) was heated at 90 °C. Then, 31 μL of oleic acid and 0.415 mL of 30% ammonia solution were added. The mixture was incubated at 90 °C under stirring (600 rpm) for 3 h. The resulting black precipitate was washed with deionized water via magnetic decantation until a neutral pH was reached.

2.5. Stabilisation of MNP_OA with Tween 20 (MNP_OA_TW20)

Tween 20 was used for additional stabilization of MNP_OA. The 1 mL 105 ± 30 mg/mL solution of MNP_OA was sonicated (50 W, 35 kHz) for 3 min. To the latter solution, 14 mL of deionized water and 30 μL of Tween 20 solution (25%) were added, mixed, and sonicated for 30 s.

2.6. Synthesis of Human Serum Albumin and Fluorescent Dye Cy5 Conjugate (HSA-Cy5)

Cys34 in HSA was reduced by dithiothreitol (DTT) [47]. Briefly, to 1.86 mL of HSA (0.3 mM) in PBS, a 1.4-fold excess of DTT (8.3 mM, 94.1 μL) in PBS was added. The mixture was incubated for 20 min at 37 °C under stirring at 250 rpm. The DTT excess was removed by centrifugation for 10 min at 10,000 rpm in Centricon filters (MWCO of 3-kDa, Amicon Centriprep YM30, Millipore, Bedford, MA, USA) using PBS as eluent. The procedure was repeated 10 times until there was no distinctive DTT odor in the sample and wash waters.

HSA-Cy5 synthesis was adapted from Chubarov et al. [48]. To 1.46 mL reduced HSA (HSA-SH) solution (0.25 mM) in PBS, 0.35 mL of a three fold excess of maleimide derivative of Cy5 dye in DMSO was added and incubated at 37 °C for 15 h under stirring 250 rpm. The unreacted Cy5 was removed by Centricon filters as described above. The yield of HSA-Cy5 was ~90%. UV-vis λ_{max} 652 nm $\epsilon = 2 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (~0.8 Cy5 per albumin).

2.7. HSA Adsorption on MNPs

MNP_OA and MNP_OA_TW20 were redispersed in buffer solution (0.15 M acetate pH 6.0, 0, 15 M Tris pH 7.4 PBS pH 7.4, or 0.15 M sodium-borate pH 8.5) to obtain 20 μL of 5 mg/mL solution. Then 5, 10, 20, 30, 40, or 50 μL of HSA-Cy5 solution (1 mM) in the same buffer was added. The volume of the mixture was adjusted to 0.1 mL with the same buffer, sonicated (50 W, 35 kHz) for 30 s, and incubated at 25 °C overnight under stirring at 300 rpm. NPs were separated by magnetic decantation, washed with 40 μL of deionized water, and stored in 0.1 mL of deionized water. The amount of adsorbed HSA-Cy5 was determined spectrophotometrically. The difference between the amount of albumin in the

solution before and after adsorption onto nanoparticles was calculated. The capacity of HSA-loaded MNPs was calculated as a mass of albumin divided by the mass of MNPs ($\text{capacity} = m_{\text{HSA}}/m_{\text{MNP}}$).

2.8. Doxorubicin Loading

Doxorubicin (DOX) adsorption was performed according to [46,49]. Briefly, solutions of NPs (0.1–3 mg/mL) and DOX (0.1 mg/mL) were mixed in 0.2 mL of 10 mM sodium-borate buffer solution (pH = 8.5). The solution was incubated at 25 °C for 12 h. Afterward, the NPs were magnetically decanted and washed twice with 1 mL of sodium borate buffer. The amount of remaining DOX was measured by fluorescence ($\lambda_{\text{ex}} = 480 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$) on a Clariostar fluorimeter (BMG Labtech, Ortenberg, Germany) using DOX solutions with known concentrations as a control.

2.9. DOX Release

DOX release was performed in acetate buffer solutions (100 mM, pH = 4.6 and 5.6) and PBS (10 mM, pH = 7.4) from the surface of NPs with the highest loading. The 1 mL of DOX-loaded NPs (20 $\mu\text{g/mL}$ calculated per MNP core) in buffers were incubated at 37 °C under stirring at 750 rpm. The 0.1 mL aliquots were taken at different times (20–120 min), and the concentration of DOX was measured by fluorescence, as presented in Section 2.8. The reaction mixtures were magnetically decanted to exclude NPs in the aliquot. After the measurement, the aliquot was returned to the reaction mixture to exclude the effects associated with the volume change of the solution. The kinetic curves were fitted into the mathematical models below to elucidate the mechanism of DOX release from the MNPs.

2.10. Cytotoxicity Assay

The cytotoxicity assay was performed using the colorimetric assay with the MTT compound [50]. Experiments were performed on A549 lung adenocarcinoma tumor cell lines. Cells were cultured at 37 °C and 5% CO_2 in a humidified atmosphere in IMDM media supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin (100 units/mL), and streptomycin (100 units/mL), respectively. The study was performed in a 96-well plate (4×10^3 cells per well). After overnight incubation, cells were treated with a medium containing MNPs and incubated for 48 h at 37 °C and in a 5% CO_2 atmosphere. The cells incubated with the medium were used as a control. The cell medium was removed, and an aliquot of MTT solution (10 μL , 25 mg/mL) in PBS was added to each well and incubated at 37 °C for 4 h. The medium was removed, and the formazan was dissolved in 0.1 mL DMSO. Formazan concentrations at 570 nm and background at 620 nm were determined on a Clariostar spectrophotometer (BMG Labtech, Ortenberg, Germany). Experiments were performed in at least three repetitions with a standard deviation calculation.

2.11. Relaxivities r_1 and r_2

The relaxation times T_1 and T_2 were measured for 0.5 mL samples of NPs at different concentrations using a Spinsolve 80 NMR spectrometer (Magritek, Aachen, Germany) at 80 MHz (1.88 T) at 25 °C. An inversion-recovery sequence with a fixed relaxation delay of at least $5 T_1$ was used to determine T_1 , and T_2 was evaluated using a Carr–Purcell–Meyboom–Gill sequence consisting of a 90° pulse followed by a series of 180° pulses covering the full signal decay. The relaxivities r_1 and r_2 were found by finding the tangent of the slope of the inverse of the longitudinal T_1 and transverse T_2 proton relaxation times for the NPs samples from iron concentrations ($1/T_i = 1/T_{i,d} + r_i \cdot C$, where T_i is the measured relaxation time, $T_{i,d}$ is the diamagnetic solvent contribution, and C is the iron concentration within nanoparticles). Relaxivity values were calculated from three independent experiment series with at least five NP concentrations.

3. Results and Discussion

3.1. Synthesis and Characterization of NPs

Iron (II,III) oxide nanoparticles stabilized with oleic acid (MNP_OA) were obtained according to the previously published co-precipitation method [46] (Figure 1). Stabilization with the amphiphilic compound TW20 was performed to slightly decrease the hydrophobicity of MNP_OA and make them dispersible in water solutions. Magnetite nanoparticles without surface stabilization were synthesized for comparison [46]. The size and ζ -potential of NPs were determined by DLS and TEM. The MNP and MNP_OA had a similar diameter of 131–134 nm in number size distribution by DLS (Table 1). However, a peak of ~ 770 nm was observed in intensity, indicating nanoparticle aggregation (Figure S1). According to the TEM, the mean size of these MNP_OA particles was 11 ± 2 nm, forming clusters of 179 ± 47 nm ($n = 20$) that were mainly close to spherical or ellipsoidal shape (Figure 2A). Clusters with sizes ~ 400 nm were also found in minor quantities. The size of MNP_OA_TW20 increased slightly to 167 nm (Table 1). Interestingly, only one peak was observed in Intensity and Volume size distribution by DLS, indicating higher colloidal stability and low ability to aggregate (Figure S1). Further analysis by TEM showed that small spherical particles of ~ 11 nm formed clusters covered with low-density shells (Figure 2B). The mean size of MNP_OA_TW20 is 165 ± 45 ($n = 20$) with a TW20 layer of ~ 40 nm.

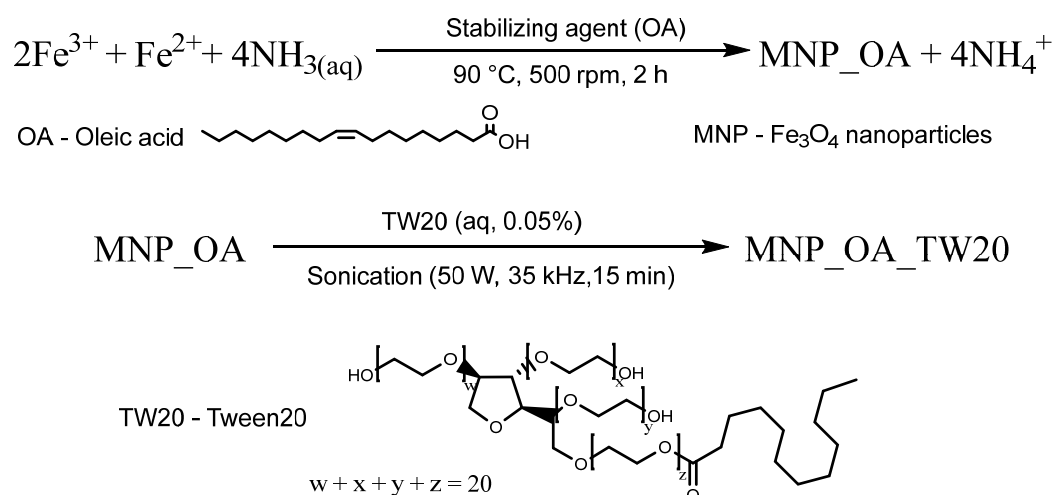


Figure 1. Synthesis of MNP_OA and MNP_OA_TW20.

Table 1. DLS data for MNP, MNP_OA, and MNP_OA_TW20.

NP	NP Size by Number, nm		PDI	ζ -Potential, mV
	Mean Size	Peak Width		
MNP	131 ± 27	67	0.40 ± 0.01	$+22.4 \pm 0.1$
MNP_OA	134 ± 16	41	0.36 ± 0.02	-19.5 ± 0.7
MNP_OA_TW20	167 ± 13	40	0.24 ± 0.02	-7.6 ± 0.4

MNP_OA has a ζ -potential of -19.5 ± 0.7 mV, corresponding to moderate physical colloidal stability. After TW20 coating, ζ -potential retained its sign but decreased to -7.6 ± 0.4 . Surprisingly, this result (lower 10 mV) usually indicates sufficient or low colloidal stability due to enhanced electrostatic repulsion among particles [51]. However, according to DLS data, no formation of aggregates was observed in aqueous conditions (Figure S1). Previously, we have found the same effect for nylon-coated MNP_OA [52]. We assumed that phenomena such as different layer charges, double-layer polarization,

and low-polar large outer layer as TW20 could reduce the ζ -potential value. Nevertheless, MNP_OA_TW20 showed high colloidal stability in aqueous conditions.

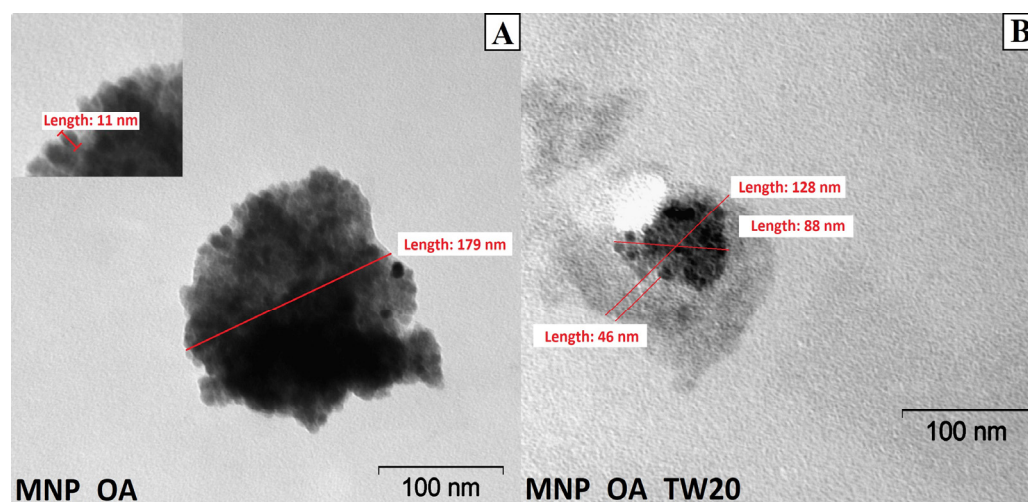


Figure 2. Representative TEM images of MNP_OA (A) and MNP_OA_TW20 (B).

MNP_OA and MNP_OA_TW20 were characterized by FTIR (Figure 3). The bands at 2935 cm^{-1} and 2868 cm^{-1} correspond to the antisymmetric and symmetric stretching of the CH_2 -group in OA and TW20 [53,54]. The bands at 1635 cm^{-1} and 1460 cm^{-1} represent asymmetric and symmetric stretching in a carboxylic group in OA [55,56]. The band at 1735 cm^{-1} corresponds to the $\text{C}=\text{O}$ stretching in ester TW20 in MNP_OA_TW20. The line at 1122 cm^{-1} corresponds to $\text{C}-\text{O}-\text{C}$ vibrations in the oxolane ring of TW20 [57]. The band at 590 cm^{-1} is a characteristic vibration of the $\text{Fe}-\text{O}$ bond related to the magnetite [58,59].

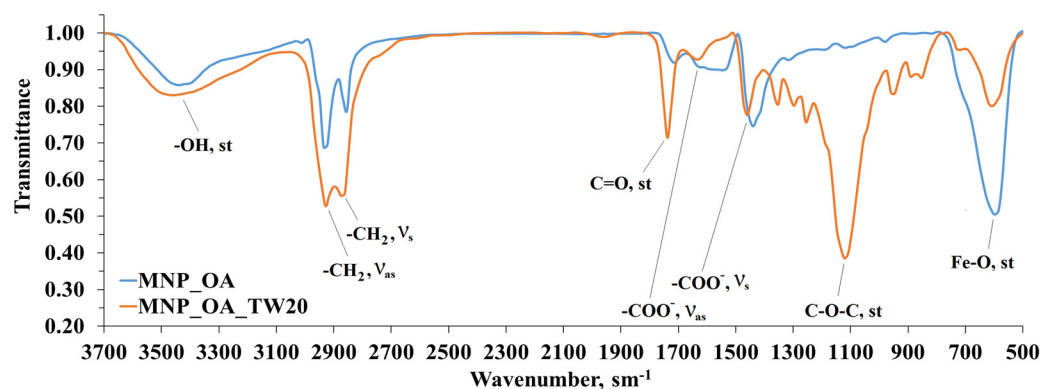


Figure 3. FTIR spectra of MNP_OA and MNP_OA_TW20.

3.2. Colloidal Stability of Nanoparticles in Different Solutions

Nanomaterials for therapeutic and diagnostic applications should be stable in salt solutions and various pH. For this reason, we investigated the stability of MNP_OA and MNP_OA_TW20 in deionized water, 0.15 M NaCl, 0.15 M acetic buffer solutions (pH 4.0, 5.0, 6.0), and PBS buffer (pH 7.4) by DLS (Figure 4).

Bare MNP particles showed a lack of stability and significant enlargement under almost all conditions investigated. Both their size and PDI dramatically increased over time, indicating high heterogeneity (Figure 4A,B). Fatty acids are frequently used in literature to stabilize MNPs without significant loss of magnetic properties [60]. However, due to high hydrophobicity, these particles are usually dispersed in organic solvents, which limits their biomedical applications [60,61]. In our case, OA coating increased the stability of MNP (Figure 4C,D). MNP_OA were stable in deionized water for 1 week of studies at $25\text{ }^{\circ}\text{C}$ and

not less than half a year in a fridge during the storage. However, a significant size and heterogeneity increase occurred in buffer and salt solutions. The high hydrophobicity of MNP_OA allows them to be stored only in deionized water, significantly limiting their application. This problem is usually solved with additional amphiphilic or hydrophilic stabilizing layers [62], such as TW20. The TW20 coating dramatically increases the stability of MNP_OA in all studied solutions (Figure 4E,F).

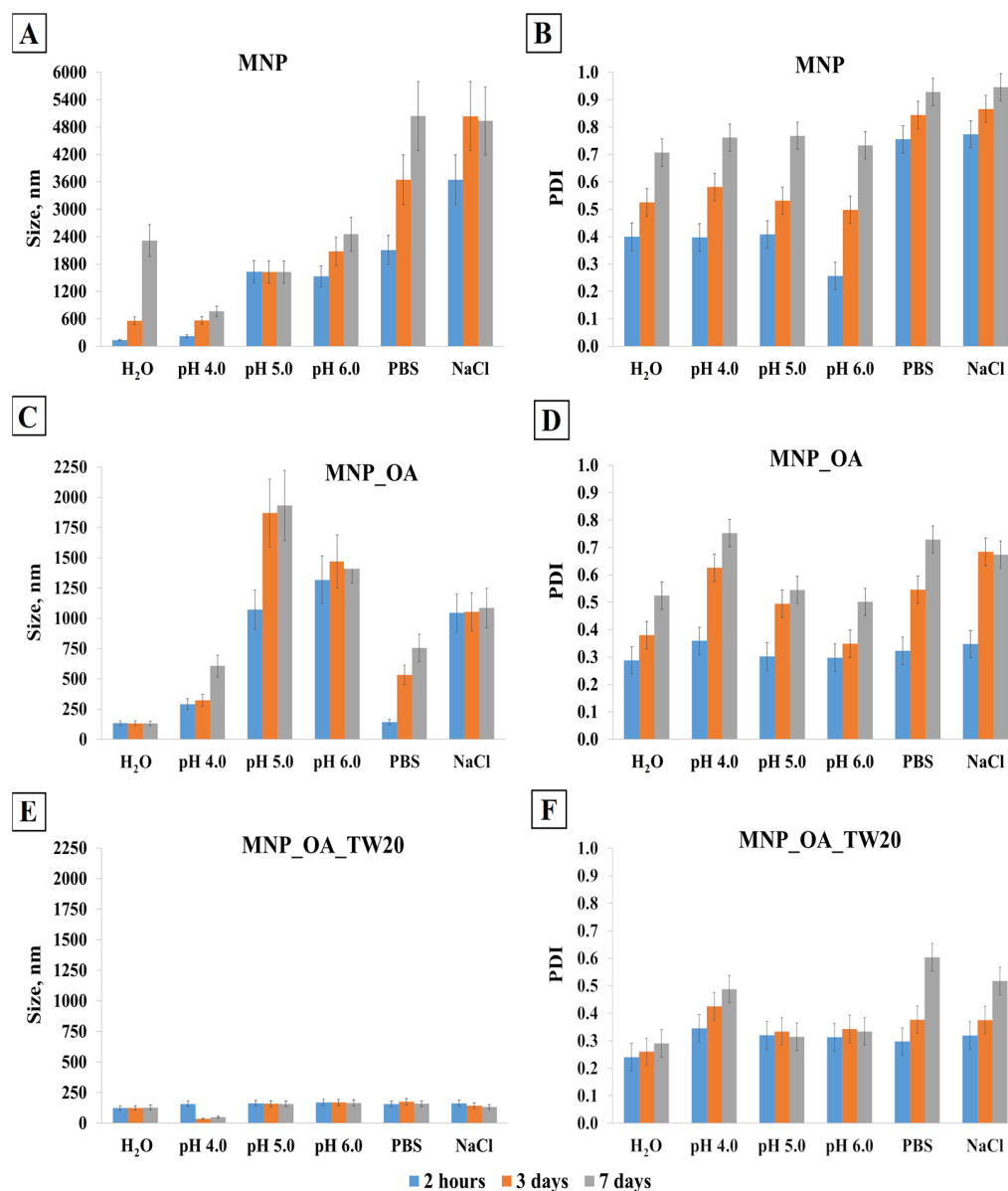


Figure 4. Stability of NPs (300 µg/mL) by DLS in number size distribution (A,C,E) and PDI (B,D,F) at various time points in deionized water, 0.15 M NaCl, 0.15 M acetic buffer solutions (pH 4.0, 5.0, 6.0), and PBS (pH 7.4).

3.3. Human Serum Albumin Coating of MNP_OA and MNP_OA_TW20

HSA can bind to fatty acids and other hydrophobic molecules [22]. This property of the protein can be used to enhance the solubility and increase the colloidal stability of NPs [20,63,64]. HSA adsorption on MNPs proceeded by several approaches. Among them, adsorption in solution [29,63,65], sonochemical adsorption [66,67], with linker molecules [64,68–70], photon immobilization [71], and free-radical binding [72,73] were evaluated. We chose adsorption in an aqueous solution to observe the formation of a protein corona under conditions close to physiological. The protein loading was assessed

at pH 6.0, 7.4, and 8.5 and at various albumin concentrations of 0.05–0.5 mM. However, before starting extensive protein absorption experiments, the stability of MNP_OA and MNP_OA_TW20 in these solutions was evaluated (Figure 5).

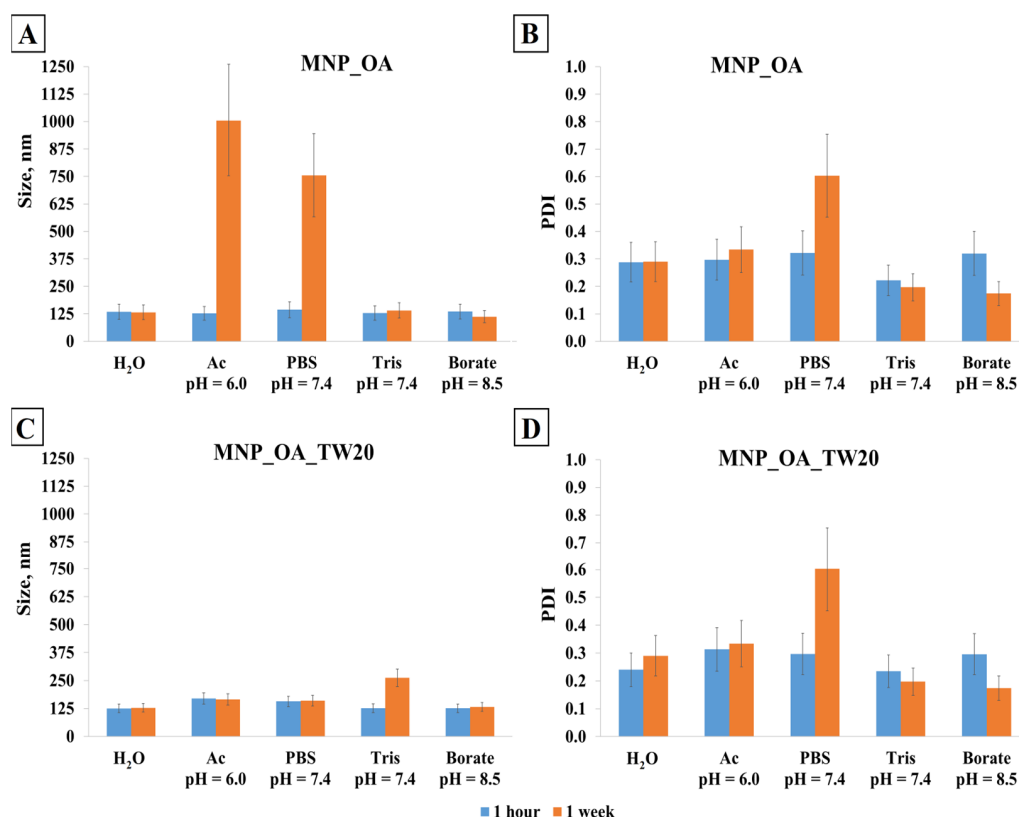


Figure 5. The size of NPs (A,C) and PDI (B,D) in deionized water and buffer solutions (0.15 M acetate buffer pH 6.0; PBS pH 7.4; 0.15 M Tris pH 7.4; and 0.15 M sodium-borate buffer pH 8.5) by DLS after 1 h and 1 week of the experiment.

The pH 7.4 mimics the pH of blood plasma. More acidified and alkalinized conditions (pH 6.0 and 8.5, respectively) were chosen to demonstrate the change in loading efficiency in different mediums. For pH 7.4, we used two buffer systems, PBS and Tris. There were no significant changes in the NP size and PDI for NPs after 1 h. However, after 7 days, MNP_OA was significantly enlarged in acetate buffer and PBS (Figure 5A). In the PBS buffer, the heterogeneity on NPs was increased after 7 days (Figure 5B,D, PDI data for both NPs). That is why further albumin loading experiments were done using Tris buffer (pH 7.4).

Cy5 dye was attached to albumin for better quantification of protein adsorption using a standard procedure (see Section 2.6). The HSA-Cy5 adsorption efficiency on NPs and the capacity of the obtained NPs are presented in Figure 6. Similar results were obtained for the non-labeled by dye albumin. The capacity of NPs increases with increasing protein concentration while the loading efficiency decreases (Figure 6). However, HSA loading efficiency in most cases did not exceed 5–7%. The higher albumin sorption on MNP_OA may be attributed to the higher hydrophobic behavior of these NPs. The highest capacity of 1.75 and 1.43 mg of protein per 1 mg of NP was obtained at pH = 7.4 in Tris/Tris-HCl buffer for MNP_OA_HSA and MNP_OA_TW20_HSA, respectively, using 0.5 mM albumin concentration. To demonstrate the efficiency of surface modification of NPs with HSA-Cy5, UV spectra for MNP_OA_TW20_HSA-Cy5 were recorded (Figure S2), providing an absorption peak at ~650 nm of Cy5 dye.

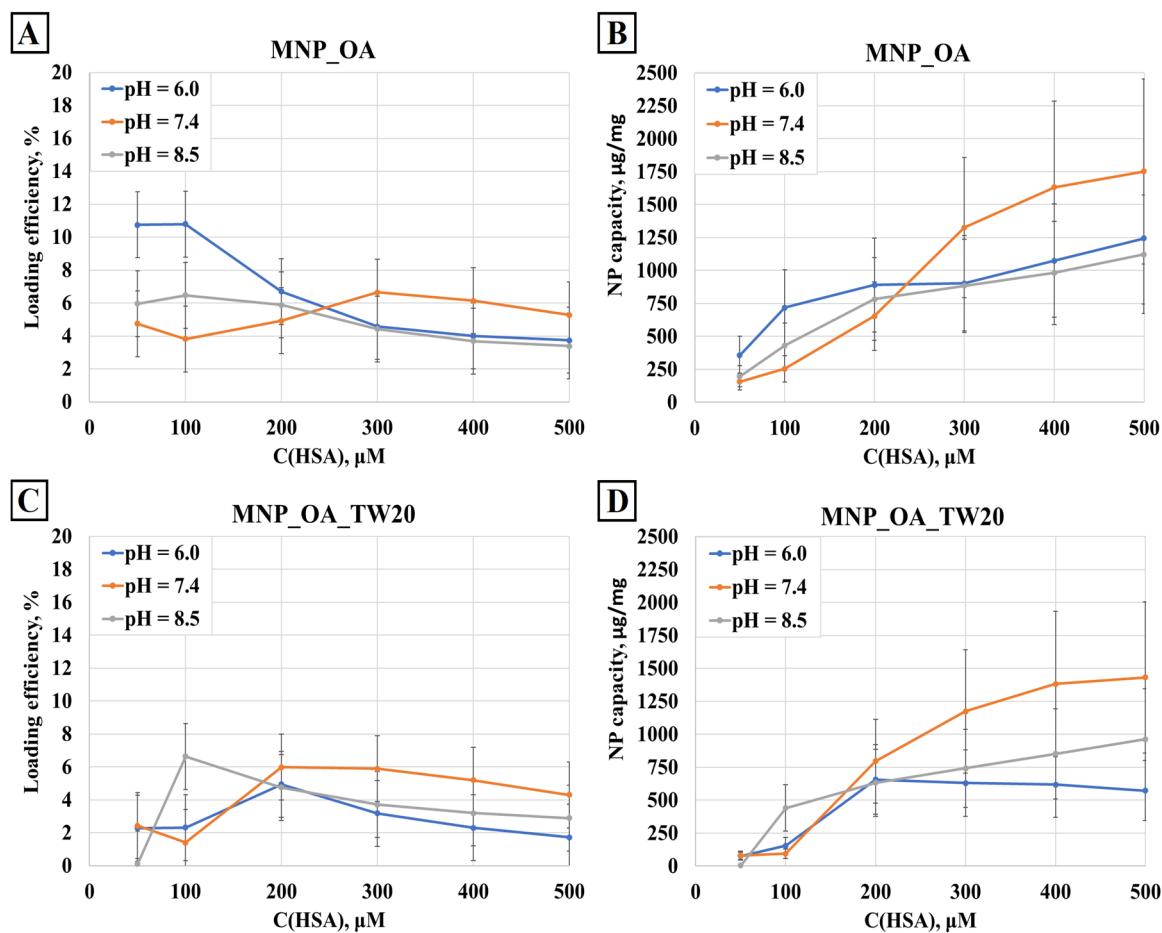


Figure 6. Adsorption efficiency (A,C) of HSA-Cy5 and NP capacity (B,D) at various pH and albumin concentrations in 0.15 M acetate buffer pH 6.0, 0.15 M Tris pH 7.4, and 0.15 M sodium-borate buffer pH 8.5. The capacity of HSA-loaded MNPs was calculated as a mass of albumin (μg) divided by the mass of MNPs (mg) (capacity = $m_{\text{HSA}}/m_{\text{MNP}}$).

The size of obtained MNP_OA_HSA and MNP_OA_TW20_HSA was measured by DLS after NP isolation and purification (Figure 7). The average diameter of the particles was 150–180 nm (Figure 7A,C, Figure S3). Similar results were obtained for the non-labeled by dye albumin. Mild agglomeration was observed at pH 6.0 for MNP_OA. MNP_OA_HSA-Cy5 and MNP_OA_TW20_HSA-Cy5, with the highest albumin capacity synthesized at pH 7.4, were used for further experiments. Their size was calculated as 176 ± 7 nm and 129 ± 6 nm, and peak widths 44 nm and 39 nm, respectively. HSA-coating slightly increased the mean size of MNP_OA and decreased it in the case of MNP_OA_TW20, which can be attributed to the sonification (30 s, 50 W, 35 kHz) of the reaction mixture and nanoparticles' structure reorganization in the presence of protein. The ζ -potential of HSA-coated NPs is presented in Figure 8. We observed the same ζ -potential for different albumin concentrations used for loading. The ζ -potentials are similar for MNP_OA_HSA and MNP_OA_TW20_HSA and mainly depend only on pH.

Obtained HSA-loaded NPs were characterized by TEM (Figure 9). It can be easily seen that the structure of MNPs has dramatically changed (cf. Figures 2 and 9). The structure of the nanoparticles can be described as “dandelion flowers”. They contain a core with high electron density surrounded by a friable layer with low electron density (Figure 9). The mean size of MNP_OA_HSA particles was 182 ± 104 nm ($n = 25$), with the mean core size of 94 ± 27 nm. The thickness of the outer layer was 82 ± 31 nm. The mean size of MNP_OA_TW20_HSA was 136 ± 80 nm ($n = 25$). Large particles with sizes exceeding

300 nm were rare in both MNP_OA_HSA and MNP_OA_TW20_HSA samples (Figure S4). This corresponds to DLS data in Intensity and Volume size distributions (Figure S3). The results of size measurements are summarized in Table 2. Significant changes in the structure of MNPs may contribute to changes in magnetic properties. However, we have not obtained notable differences during the magnetic decantation procedure (Figures S5 and S6).

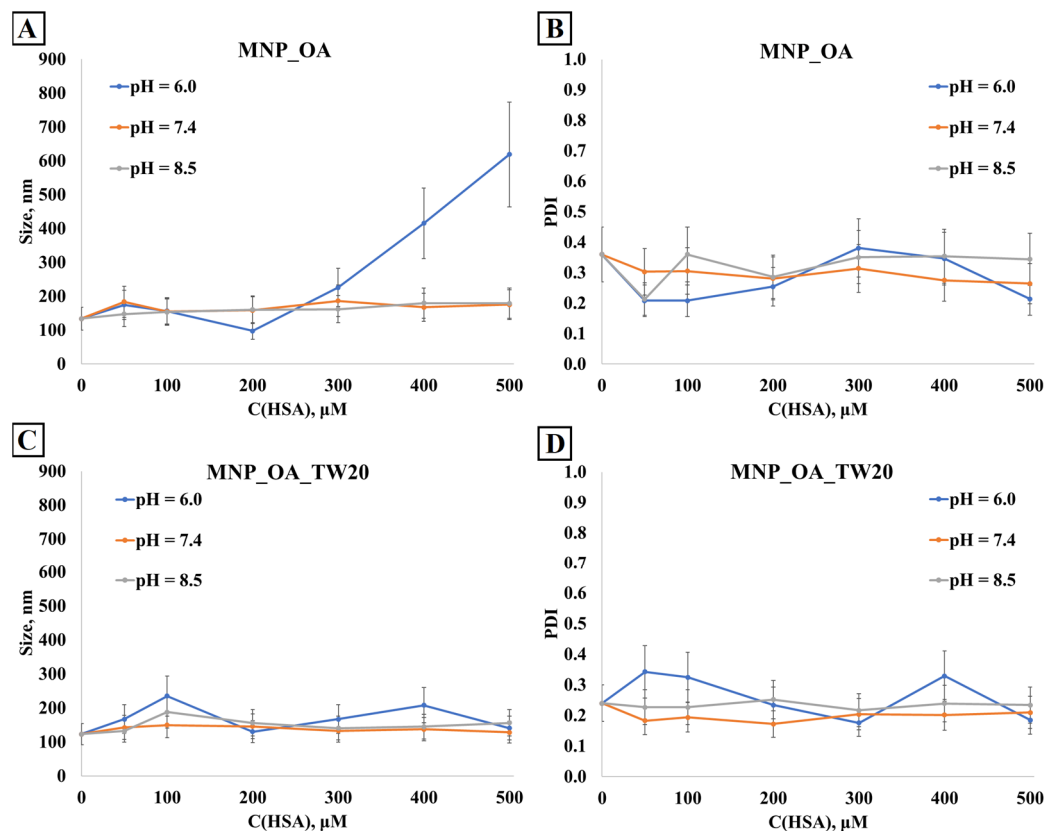


Figure 7. The size (A,C) and PDI (B,D) of HSA-coated NPs at different loading conditions were measured by DLS.

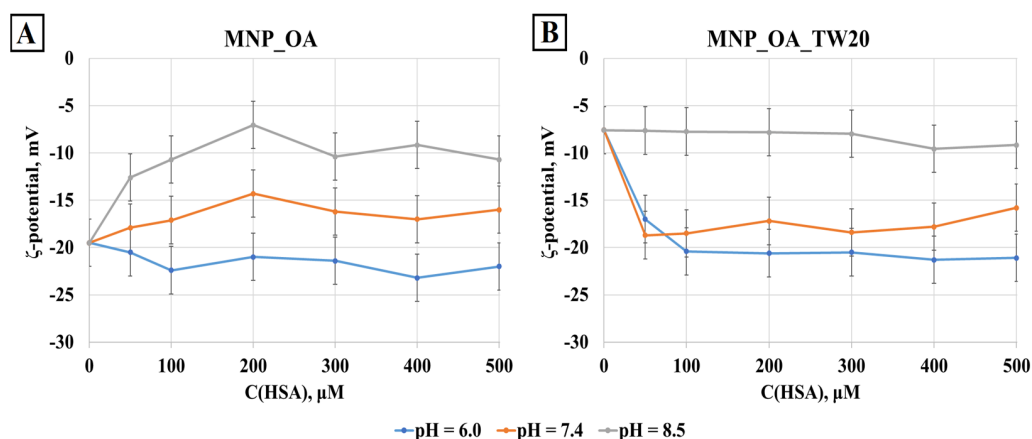


Figure 8. The ζ -potentials of the HSA-coated MNP_OA (A) and MNP_OA_TW20 (B).

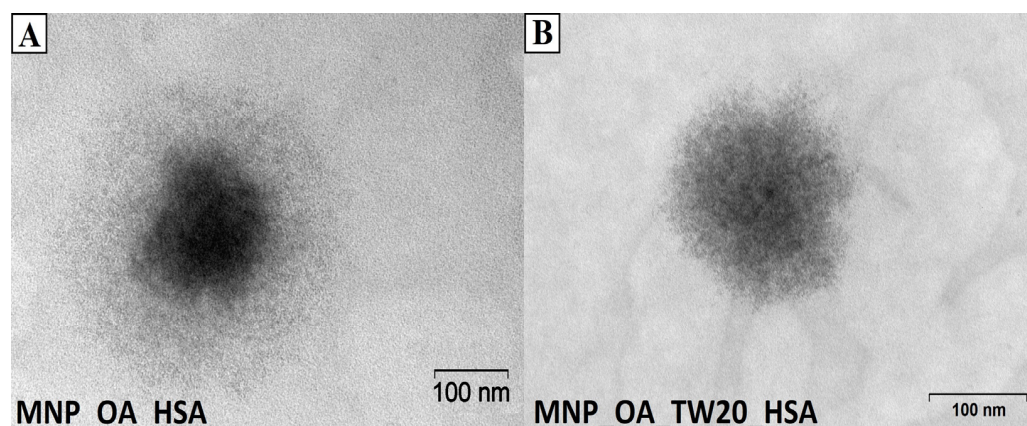


Figure 9. Representative TEM images of MNP_OA_HSA (A) and MNP_OA_TW20_HSA (B).

Table 2. DLS and TEM data for MNP_OA, MNP_OA_TW20 with and without HSA-coating.

NP	NP Size by DLS, nm			ζ -Potential, mV	TEM Size, nm
	Mean Size	Peak Width	PDI		
MNP_OA	134 $\pm$ 16	41	0.36 $\pm$ 0.02	−19.5 $\pm$ 0.7	179 $\pm$ 47
MNP_OA_TW20	167 $\pm$ 13	40	0.24 $\pm$ 0.02	−7.6 $\pm$ 0.4	165 $\pm$ 45
MNP_OA_HSA *	176 $\pm$ 7	44	0.26 $\pm$ 0.01	−16.0 $\pm$ 1.0	182 $\pm$ 104
MNP_OA_TW20_HSA *	129 $\pm$ 6	39	0.21 $\pm$ 0.01	−15.8 $\pm$ 0.1	136 $\pm$ 80

* Obtained at pH = 7.4 in Tris/Tris-HCl buffer, C (HSA) = 0.5 mM.

FT-IR spectra were recorded for HSA-stabilized NPs (Figure 10). The recorded spectra contain bands corresponding to magnetite and bands corresponding to proteins [74–76]. An intense peak at 3700–3100 cm^{-1} corresponds to the stretching of the OH group. The bands at 2935 cm^{-1} and 2868 cm^{-1} relate to the antisymmetric and symmetric stretching of the CH_2 -group. The bands at 1650 cm^{-1} and 1530 cm^{-1} are typical bands of amide bonds (Amide I and Amide II) in proteins. The band at 1460 cm^{-1} represents symmetric stretching in a carboxylic group. The band at 595 cm^{-1} is a characteristic vibration of the magnetite Fe–O bond.

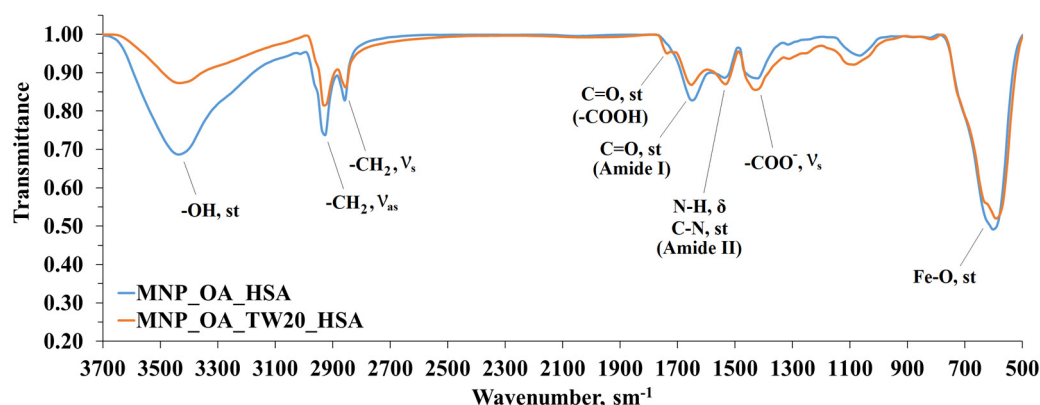


Figure 10. FT-IR spectra of MNP_OA_HSA and MNP_OA_TW20_HSA.

3.4. Stability of HSA-Coated Nanoparticles in Different Solutions

The stability experiments of albumin-coated nanocomposites were analyzed in acetate buffer (pH = 4.0, 5.0, 6.0), Tris/Tris-HCl buffer (pH = 7.4), PBS buffer (pH = 7.4), borate buffer (pH = 8.5), and 0.15 M NaCl solution. The NPs were stable at different pH values, confirming that HSA coating extensively improves the stability of MNPs (Figure 11) compared to MNP_OA and MNP_OA_TW20 (Figures 4 and 5). However, HSA-coated nanoparticles increase their sizes in acidic pH values of 5 and are more pronounced for

pH 4 (Figure 11A,C), which can be explained by structural changes that HSA undergoes in acidic environments [77–79]. Under acidic conditions, albumin loses its heart-shaped structure (N isoform) and unfolds, which can lead to the rearrangement of the surface protective layer of nanoparticles. Moreover, albumin has an isoelectric point pI of 4.7. The isoelectric point is the pH at which protein has no charge. Therefore, lower-than-isoelectric point albumin is positive and negative at a pH higher than 4.7, which will highly influence the tertiary structure of albumin and basically affects the MNP coating structure in the pH 4–5 range.

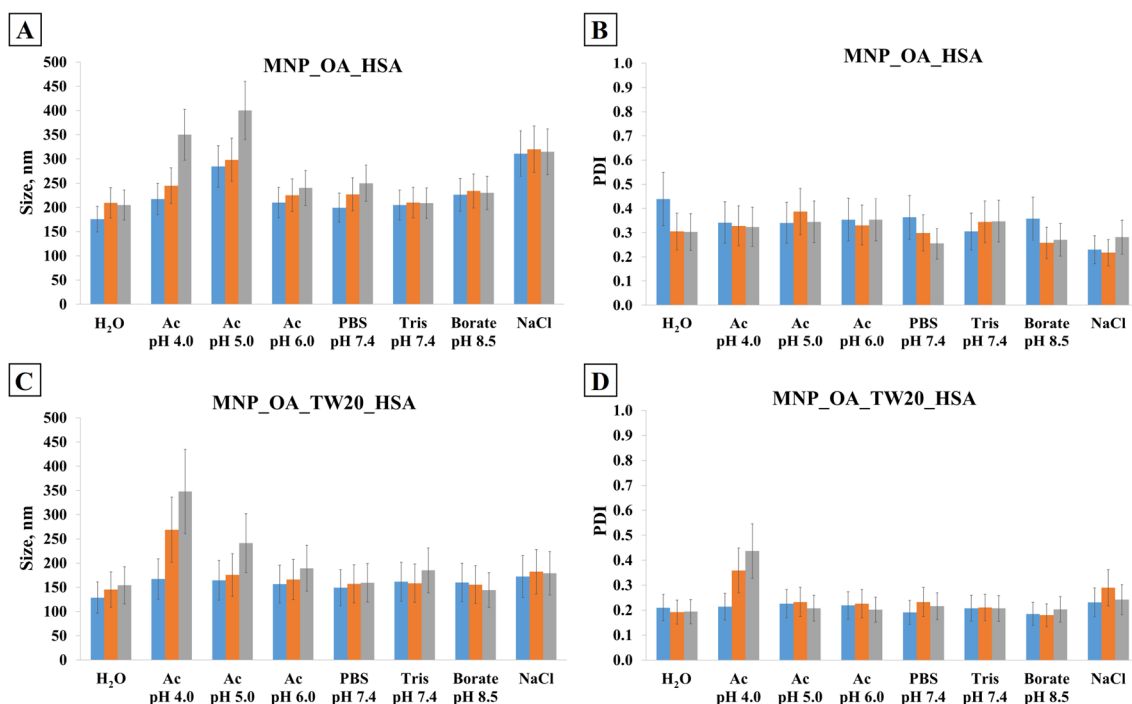


Figure 11. Stability of HSA-modified NPs in various solutions studied by DLS. Changes in size are on the left (A,C) and PDI are on the right (B,D).

3.5. Relaxivities r_1 and r_2 of MNPs

The relaxivities r_1 and r_2 strongly influence the sensitivity of T_1 - and T_2 -weighted MRI images on the NP concentration. A higher relaxivity value indicates possible higher MRI image contrast. To evaluate how obtained NPs could perform in the MRI method, the relaxivity parameters r_1 and r_2 were determined (Table 3, Figure S7). For comparison, the relaxivities of iron oxide nanoparticles coated with carboxydextran T_2 -contrast agent Ferucarbotran (Resovist®) at 1.5 T and T_1 -contrast agent Gd^{3+} -complex Gadovist are presented in Table 3 [80]. Magnetite (Fe_3O_4) is usually known as a T_2 -contrast agent as it has a much higher r_2 value than r_1 . The r_2/r_1 values for all studied MNPs are higher than for the T_1 -contrast agent Gadovist, which confirms their T_2 behavior (Table 3). HSA-coated NPs have higher r_1 values than MNP_OA and MNP_OA_TW20. The coating usually decreases r_1 but does not change r_2 values much due to the different relaxation mechanisms [20,63,81]. A possible explanation for r_1 enhancement might be nanoparticle reorganization (see TEM, Figures 2 and 9) and increasing water interaction with magnetite grains.

The r_2 value for MNP_OA_HSA was 35-fold higher than for MNP_OA. On the contrary, MNP_OA_TW20_HSA possesses two times less r_2 than for MNP_OA_TW20. The increase of r_2 was previously shown for 10 and 30-nm MNPs during albumin adsorption [63,81]. This discrepancy between the observed decrease of r_2 and expected values may be due to the thicker albumin coating in MNP_OA_TW20_HSA (Figure 9B), which would reduce the relaxivity. Nevertheless, values for r_2 for all studied MNPs are not high and magnitude

lower than for T₂-contrast agent Resovist (Table 3). However, the selective accumulation of stable, biocompatible HSA-coated nanoparticles in the tumor due to the properties of albumin can offset the lower r₂ value [20,29–31]. The twice-higher ratio compared to the Resovist r₂/r₁ ratio will increase the effectiveness of the contrast.

Table 3. Relaxivity r₁ и r₂ (mM^{−1} s^{−1}) per Fe concentration for MNPs measured at 1.88 T, 25 °C.

NP Type	r ₁ , mM ^{−1} s ^{−1}	r ₂ , mM ^{−1} s ^{−1}	r ₂ /r ₁
MNP_OA	0.05	0.16	3.2
MNP_OA_TW20	0.07	4.44	63.4
MNP_OA_HSA	0.36	5.58	15.5
MNP_OA_TW20_HSA	0.45	1.97	4.4
Resovist (Iron oxide, T ₂ -contrast, 1.5 T) [63]	8.7	61.0	7.0
Gadovist (Gd-complex, T ₁ -contrast) [80]	4.7	6.8	1.4

3.6. Anticancer Doxorubicin (DOX) Loading and pH-Sensitive Release

HSA-coated MNPs have great potential in such a field with magnetite core diagnostic applications enhanced by HSA coating that could act as a targeting and stabilizing agent, as well as a binder of various hydrophobic drugs [20,22]. Herein, we used a well-known anticancer drug, DOX, for a multifunctional system for therapy and diagnostics based on HSA-coated MNPs. DOX loading was carried out according to the previously optimized procedure in our laboratory [46,49]. In the present work, we mainly investigate how albumin coating influences the DOX loading process. The DOX loading efficiency and capacity of the obtained NPs as a function of NP concentration are shown in Figure 12.

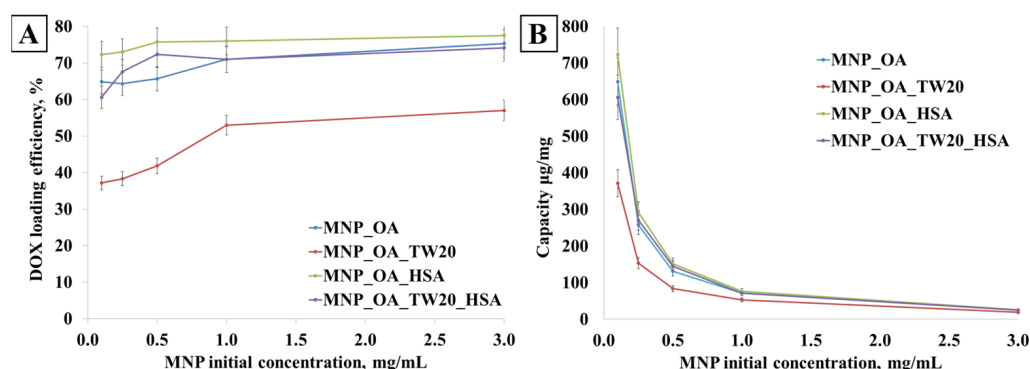


Figure 12. DOX loading efficiency (A) and capacity (B) of obtained NPs.

It can be easily seen that high NP concentrations lead to a decrease in the capacity (Figure 12B). It is necessary to use a low concentration of NPs to obtain a high capacity. However, when the concentration of NPs is lower than 0.1 mg/mL, it is difficult to isolate them by magnetic decantation. It takes a long time for the NP to concentrate near the magnet, which causes most of the product to be lost. The highest capacity of 725 µg/mg (DOX/MNP) was obtained for MNP_OA_HSA using 0.1 mg/mL concentration of NPs. A bit lower values were calculated for MNP_OA (650 µg/mg), MNP_OA_TW20_HSA (600 µg/mg), and almost twice lower for MNP_OA_TW20 (370 µg/mg). MNP_OA has a bit higher capacity than MNP_OA_TW20 due to the better interaction of amphiphilic drug with hydrophobic NP's surface [46]. HSA-loaded MNPs have higher capacity than corresponding to them uncoated NPs, which can be explained by DOX binding in albumin drug binding sites [82].

Composites with the highest loading capacities were selected to examine pH-sensitive drug release and investigated at 37 °C and pH 7.4, 5.5, and 4.5 (Figure 13). DOX-loaded NPs must be stable at a pH of 7.4 to avoid releasing the drug into the bloodstream. Effective drug release should occur in acidic conditions, mimicking lysosomes and cancer tissue. The

MNPs demonstrated a pH-dependent drug release profile (Figure 13) with higher efficiency at acidic pH. A more pronounced difference between DOX release at pH 7.4 and 4.5 was obtained for HSA-coated MNPs (Figure 13B,D). To study DOX-release kinetics, several different general kinetics models, such as zero- and first-order, Higuchi, and Korsmeyer–Peppas, are used [83–85]. In our case, the drug release profiles show two stages: a rapid release of DOX is obtained within 20 min, followed by a slower rate of process. Such a kinetic curve can only be evaluated by an exponential function. The first-order model is applicable to water-soluble drugs encapsulated in porous materials. The Higuchi model is suitable for drugs incorporated in solid matrices. Korsmeyer’s theory describes the medicine released from a polymeric system, which was supplemented by n value to characterize various release mechanisms [84]. The obtained rate constants for each model are presented in (Table S1). Common metrics for agreement of fit with each kinetics curve were then calculated as regression coefficient R^2 . The first-order model does not fit well with experimental data, as demonstrated by low R^2 (Table S1). Calculations by Higuchi provide better R^2 values than the first-order model. However, the highest R^2 of 0.999 was obtained with the Korsmeyer–Peppas model (Table S1). The release exponent (n) values are in the range of 0.09–0.18, which is less than 0.45, suggesting the DOX release mechanism associated with the Fickian diffusion process [83,84].

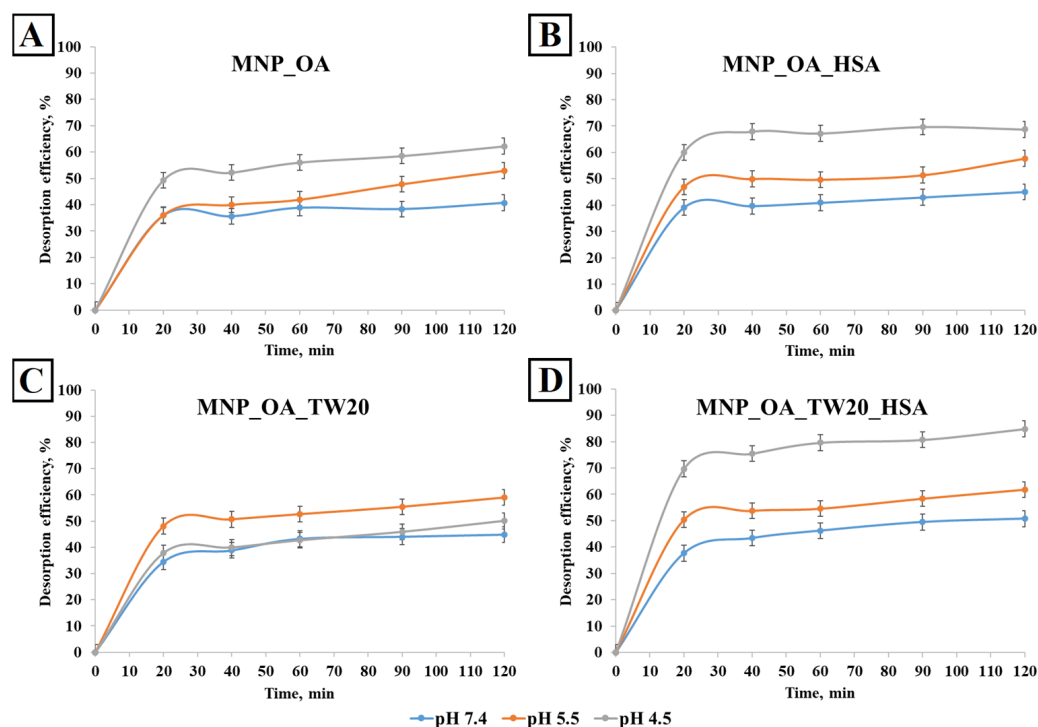


Figure 13. DOX release from NPs with capacities of 725 $\mu\text{g}/\text{mg}$ (MNP_OA_HSA, (B)), 650 $\mu\text{g}/\text{mg}$ (MNP_OA, (A)), 600 $\mu\text{g}/\text{mg}$ (MNP_OA_TW20_HSA, (D)), and 370 $\mu\text{g}/\text{mg}$ (MNP_OA_TW20, (C)) at various pH.

3.7. Cytotoxicity of Nanoparticles with and Without HSA Coating

Cytotoxicity experiments were performed on A549 lung adenocarcinoma tumor cell lines. Except for MNP_OA, the NPs did not exhibit significant cytotoxicity (Figure 14). Moreover, HSA-coated MNPs enhance cell viability with concentration increase, which is explained by cells utilizing the protein for their growth.

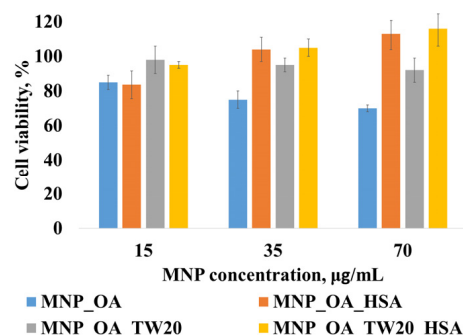


Figure 14. Cytotoxicity of albumin-coated and uncoated NPs on A549 cells for 48 h. Cells with buffer were used as a 100% survival control.

3.8. Cytotoxicity of DOX-Loaded Nanoparticles

MNPs with two different DOX capacities were used for cytotoxicity experiments on A549 cells. The loading capacity is mentioned in MNPs' name end as µg/mg (DOX/MNP) (Figure 14).

DOX-loaded NPs effectively inhibit cell growth in a concentration-dependent manner. More outstanding results were obtained for nanoparticles with higher DOX loading (Figure 15, top). Abnormal high cell viability was obtained for MNP_OA_TW20_HSA_DOX-145 (Figure 15, center), which might be attributed to protein utilization for cell metabolism. The IC_{50} values were calculated for the MNPs per MNP or DOX concentrations (Table 4). The best result was obtained for albumin-coated MNP with the highest DOX loading (MNP_OA_HSA_DOX-725, Figure 15 and Table 4).

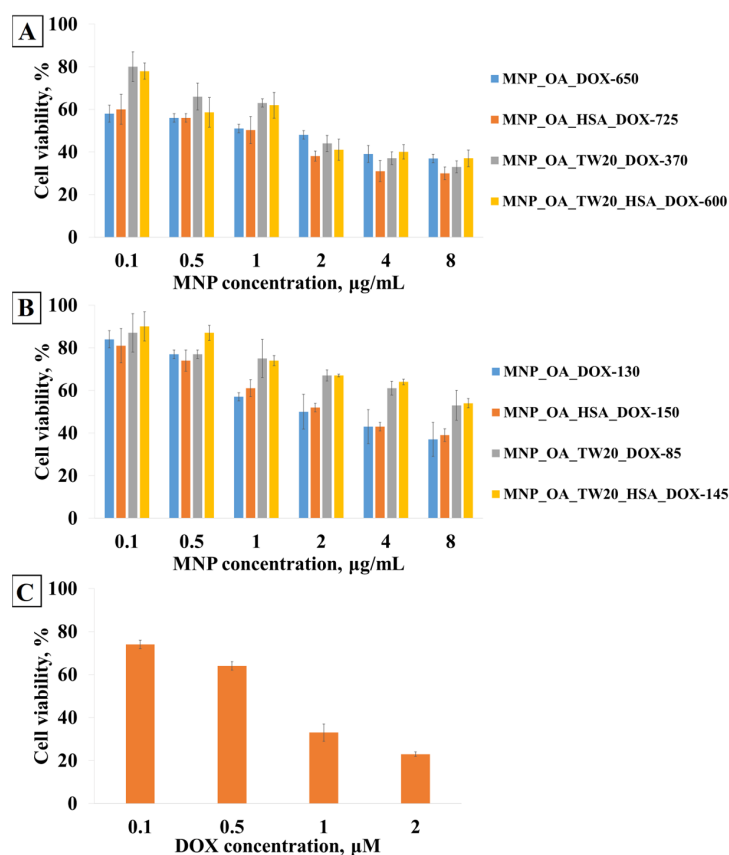


Figure 15. Cytotoxicity of MNPs (A,B) with different DOX capacities (numerically indicated in abbreviations in µg DOX per 1 mg NP) on A549 cells. Cells with buffer were used as a 100% survival control. Cell survival in the presence of DOX is given for comparison (C).

Table 4. IC₅₀ for the obtained MNPs.

NP-DOX	IC ₅₀ Per MNP, µg/mL	IC ₅₀ Per DOX, µg/mL
MNP_OA_DOX-650	1.2	0.8
MNP_OA_HSA_DOX-725	1.0	0.7
MNP_OA_TW20_DOX-370	2.0	1.4
MNP_OA_TW20_HSA_DOX-600	1.5	1.1
MNP_OA_DOX-130	2.1	0.3
MNP_OA_HSA_DOX-150	3.3	0.5
MNP_OA_TW20_DOX-85	13.4	1.1
MNP_OA_TW20_HSA_DOX-145	10.8	1.6
DOX	-	0.8

4. Conclusions

In this study, the adsorption of HSA on MNPs was studied to see how adsorption conditions affect the efficiency of coating formation. Two types of MNPs with different hydrophobicities were used as models to show the albumin-coating effect on structure and physical and biological properties. The efficiency of protein adsorption depends on the acidity and reaches a maximum at a pH of 7.4 among the values studied. At an albumin concentration of 0.5 mM, the highest HSA adsorption of 1.75 mg and 1.43 mg of HSA per 1 mg of MNP_OA and MNP_OA_TW20, respectively, was obtained. The synthesized MNPs and HSA-coated MNPs were characterized by TEM, DLS, and FT-IR methods. Not only does protein adsorption on the surface of nanoparticles occur, but also a partial or complete restructuring of the MNP structure, which was shown by TEM. Such findings can be helpful in further optimization of albumin-coating to improve the properties of MNPs and understand the effects of protein corona formation in the body for intravenous injection of smart constructions.

HSA coating provides high stability over a wide pH range and at high ionic strengths, except for a low pH of 4–5, which leads to a change in the protein structure, partial desorption, and MNP surface reorganization. The relaxivity r_1 increases after albumin adsorption. However, r_2 changes are dependent on the MNP type. Obtained r_2/r_1 values range from 4.4–15.5, indicating a preferable T₂-weighted image manner. The MNP_HSA exhibited low cytotoxicity even at high NP concentrations.

The efficiency of the interaction of MNPs with the antitumor drug DOX was investigated. The constructs effectively adsorbed DOX up to 725 µg/mg for MNP_OA_HSA. HSA improves DOX adsorption because of the drug binding in protein sites. DOX-loaded MNP showed pH-sensitive release with much higher efficiency at acidic pH. These constructions efficiently inhibit the growth of A549 cells with up to IC₅₀ values of 0.7 µg DOX/mL, which provide high therapeutic potential of MNP_HSA-DOX.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/magnetochemistry11020013/s1>. Figure S1: The hydrodynamic diameter of MNP, MNP_OA, and MNP_OA_TW20 by DLS in three distribution modes: Number, Volume, and Intensity; Figure S2: UV-vis spectra of MNP_OA_TW20_HSA-Cy5 in deionized water. The Cy5 peak is marked with an arrow. The NPs were obtained with 0.3- and 0.5-mM HSA-Cy5 during the protein-loading procedure at pH 7.4; Figure S3: The hydrodynamic diameter of MNP_OA_HSA and MNP_OA_TW20_HSA by DLS in three distribution modes: Number, Volume, and Intensity; Figure S4: Representative TEM images of MNP_OA_HSA and MNP_OA_TW20_HSA, high (a) and low (b) magnification; Figure S5: MNPs in mQ water on the magnetic rack at various time points. 1—MNP, 2—MNP_OA, 3—MNP_OA_TW20, 4—MNP_OA_HSA, 5—MNP_OA_TW20_HSA; Figure S6: MNP_OA (A) and MNP_OA_TW20 (B) without (left) and with HSA-coating (right) in mQ water on the magnetic rack at various time points; Figure S7: Relaxivities (r_1 and r_2) calculations

under a magnetic field of 1.88 T; Table S1: Correlation coefficients and release parameters based on drug-release data.

Author Contributions: Conceptualization, A.C.; Investigation, A.C., K.P. and E.R.; Resources, A.C. and E.D.; Data curation, A.C., K.P. and E.R.; Writing—original draft preparation, A.C. and K.P.; Writing—review and editing, E.D.; Supervision, A.C. and E.D.; Project administration, A.C.; Funding acquisition, A.C. and E.D. All authors have read and agreed to the published version of the manuscript.

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