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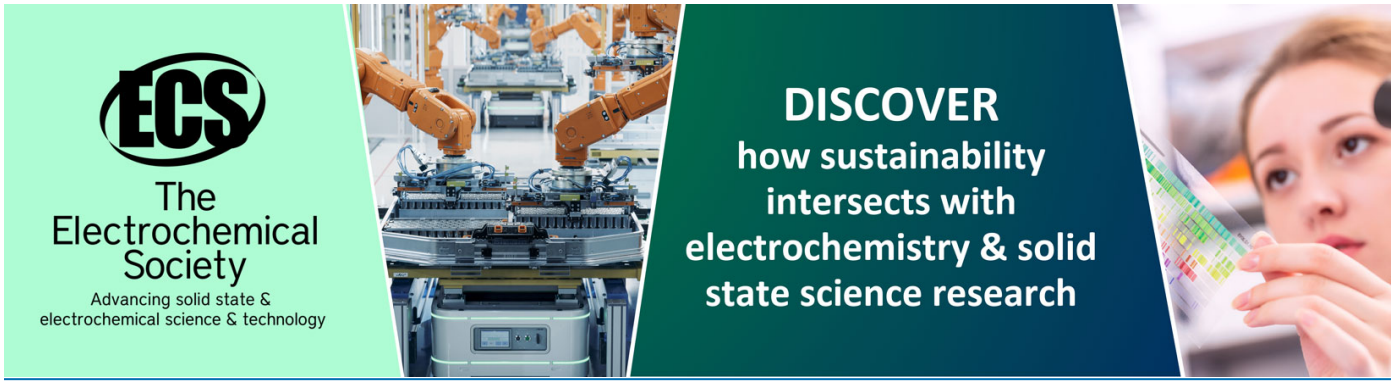

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Comparative study of cytotoxicity of ferromagnetic nanoparticles and magnetitecontaining polyelectrolyte microcapsules

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Abstract. The cytotoxicity of magnetite nanoparticles (MNP) stabilized with citrate acidand polyelectrolyte multilayer microcapsules containing these particles in the shell is analyzed. Microcapsules were prepared by co-precipitation of iron (II) and (III) chlorides. Polyelectrolyte microcapsules synthesized by the layer-by-layer method from biodegradable polymers polyarginine and dextran sulfate. Cytotoxicity of the synthesized objects was studied on the L929 cells culture and human leucocytes. It was also investigated the phagocytic activity of leukocytes for the MNP and magnetite containing polyelectrolyte microcapsules (MCPM). A set of tests (MTT assay, neutral red uptake assay, lactate dehydrogenase release assay) was used to study the cytotoxicity in vitro. All the tests have shown that the magnetic nanoparticles have a greater cytotoxicity in comparison with microcapsules containing an equivalent amount of magnetite. In contrast to the mouse fibroblast culture, human leukocytes were more resistant to the toxic effects of magnetite. At the concentrations used in our studies no significant reduction in the viability of leukocytes has been registered. Both MNP and MCPM undergo phagocytosis, however, the phagocytic activity of leukocytes for these particles was lower than for the standard objects (latex microparticles).

Introduction

Polyelectrolyte microcapsules (PM) are a promising system for packaging and magneticaly driven delivery of verious substances. Microcapsules are commonly synthesized by layer-by-layer (LBL) technology when oppositely charged polyelectrolytes are applied in layers on a core of inorganic material which can contain different substances[1]. Then the core is dissolved by an acid and the substance remains in the PM. Inclusion of different functional groups into the core or shell of capsules allows to control the delivery and release of the drug.A method for the synthesis of PM from biocompatible and biodegradable polymers (polyarginine and dextran-sulphate) with nanoparticles of magnetite incorporated into the shell was proposed in paper [2]. It has been shown in in-vitro

experimentsthat these capsules are more actively phagocytosed by cells [2].andcan act as intracellular anchors that allow manipulating cells by an external magnetic field [3].

However, there are several problems that limit the use of this technology. One of the main problems is the toxicity of magnetic nanoparticles. Iron is not a foreign substance to the body being a part of its major systems (hemoglobin, cytochrome p450, etc.). There are several systems involved in assimilation, transportation and excretion of iron in the human body. However, magnetically driven drug delivery requires parenteral administration of rather large amount of iron which can lead to some side effects [4]. In particular, the ionized iron can activate lipid peroxidation and be toxic for cell membranes [5]. However, the isolation of iron from contact with the blood cells and endothelium can decrease the cytotoxicity [6].

In the present paper, the cytotoxicity of magnetite nanoparticles (MNP), and magnetite containing polyelectrolyte microcapsules (MCPM) with these nanoparticles included in the shell was studied.

1. Materials and methods:

1.1. Reagents

Iron chloride (III) (FeCl3 • 6H2O) (99.99%, Sigma-Aldrich),
Iron chloride (II) (FeCl2 • 4H2O) (99.99%, Sigma-Aldrich),
Ammonium hydroxide 25% (99.8% Vekton, Russia)
Citric acid (99.99%, Sigma-Aldrich),
Sodium ethylenediaminetetraacetate (99.9% Vekton)
Sodium carbonate (99.9% Vekton)
Calcium chloride (99.9% Vekton)
Sodium dextran sulfate (DS) (M ~ 100 kDa, Sigma-Aldrich),
Polyarginine hydrochloride (PA) (M ~ 15-70 kDa, Sigma-Aldrich),
Sodium chloride (99.9% Vekton)
Hydrochloric acid 38% (99.8% Vekton).
Dulbecco's Modified Eagles Media (DMEM),Biolot (Russia).
Fetal Bovine Serum (FBS), Biolot (Russia).
Isopropanol (99.9% Vekton)
Neutral red, Sigma-Aldrich.
MTT (3- [4,5- dimethylthiazol-2-yl-2,5-difeniltetrazolium bromide), Sigma-Aldrich.
LDH Cytotoxicity kit, (The Thermo Scientific, USA).
The latex particles (PanEco, USA).
Trypan blue, Sigma Aldrich.
All reagents were used without further purification.

1.2. Synthesis of ferromagnetic nanoparticles

Magnetite nanoparticles were prepared by coprecipitation of iron (II) and (III) chlorides in a molar ratio of 1:2 [Volodkin] using citric acid as a stabilizer. Iron chlorides (2.35g of $FeCl_3 \cdot 6H_2O$ and 0.86 g of $FeCl_2 \cdot 4H_2O$) were dissolved in 40 mL of deionized water. The obtained solution was poured into three-necked flask and heated to 80°C under nitrogen. Then ammonium hydroxide (5 ml of 25% solution) were slowly added to a heated solution with vigorous stirring and kept for 15 minutes. Then citric acid (4 ml of25% solution) was added to the particles colloidand kept stirring for 1 hour at 90°C. The magnetite particles were purified by dialysis(cellulose membrane Q1210-55 F3 ("Orange scientific", Belgium) with 12-14 kDa pore, the eluent 0.15 MNaCl, the volume ratio suspension/eluent equal 1/1500, the treatment time 24 hours). Then the purified colloid was centrifuged at 16 000 min^{-1} for 5 minutes to remove large particles.

1.3. Synthesis of magnetite containing polyelectrolyte microcapsules

Polyelectrolyte microcapsules were prepared by the «layer-by-layer» method with the calcium carbonate core using the technique described by Volodkin et al [1]. Synthesis of core was performed by rapid mixing of calcium chloride and sodium carbonate solutions in equal volume proportions at room temperature. The cores were precipitated by centrifugation (2000 min^{-1} for 30 seconds) and washed three times with deionized water. Calcium carbonate core were added in a 1 mg/mlPA solution.The suspension was shaked for 15 minutes washed three times with deionized water and then transfered into DS solution. The same manipulation was repeated for subsequent adsorption layers. Magentic nanoparticles having a negative surface charge were deposited on the layer of PA. After deposition ofthe magnetite NP the layer was supplementedwithDSfor compensation of remaining cationic charges. The next layer of magnetite was deposited on the 3-th layer of PA in the same way. As a result, we have obtained the microcapsule shells with following composition:PA/DS/PA/Fe_3O_4NP+DS/PA/Fe_3O_4NP+DS/PA/DS.

In the last stage of synthesis, cores were dissolved with a solution of 0.2M EDTA (pH 6,5). After dissolving of the cores, the microcapsules were washed three times with deionized water.

Polyelectrolite microcapsules without magnetite (PA-DS-M) were synthesed by the similar procedurefor the control group in cytotoxity studies.

1.4. Characterization of the objects

1.4.1. Particle size. Determination of dimensional characteristics of Fe_3O_4 nanoparticles was performed by dynamic light scattering (Nano-Flex analyzer, Microtrac Inc., Germany).

1.4.2. Zeta potential. Measurement of zeta potential was carried out by electrophoretic mobility measurements (Stabino analyzer, Microtrac Inc., Germany).

1.4.3. Magnetite concentration. Quantitative determination of the magnetite in the samples (MNPand MCPM) was carried out by photocolorimetry. This method is based on the measurement of the optical density of colored iron (III) complexes with sulfosalicylic acid in an alkaline medium. Colloid of MNP (30 μl) or microcapsule suspension (30 μl containing capsules 260 mln / ml) was dissolved in 100 μl of 36% hydrochloric acid. A solution of hydrogen peroxide (200 μl of 6% solution) was added for the oxidation of iron to Fe^{3+}. The mixture was heated to 100°C and held at this temperature for 25 minutes. Then 1 ml of 17% sulfosalicylic acid and 1 ml of 10% ammonia solution were added. Then the mixture was resolved with deionized water to 50 ml and held for 10 minutes. The optical density of the solution was measured at λ = 430 nm (spectrophotometer Shimadzu UV2600). Iron concentration was determined with the calibration curve. The concentration of magnetite was obtained from the iron concentration by the formula:

C (Fe_3O_4) = 1.38 C(Fe).

Calculation of the content of magnetite in a capsule was carried by the formula:S = C (Fe3O4)/n, where C is the concentration of magnetite, and n is concentration of capsules in the suspension.

1.4.4. Visialization. Scanning Electron Microscopy (SEM). For SEM analysis, samples were prepared by applying a drop of the particle suspension to a glass slide and then drying overnight. Then samples were sputtered with gold and measurements were conducted using aGemini Leo 1550 instrument at an operation voltage of 3 keV (AFM, NT-MDT Ntegra system, Spectrum Instrument).

1.4.5. Magnetic properties. The magnetization of samples was measured by the induction method with a vibration magnetometer (EZ11, Microsense).

1.5. Cytotoxicity

Cytotoxicity of the synthesized nanoparticles and magnetite containing polyelectrolitemicrocapsules was investigated in accordance with the international standard operation procedure "Tests for in vitro cytotoxicity" (ISO 10993-5). [7].To investigate cells viability a series of tests were performed: MTT-test, neutral red uptake (NRU) test, lactate dehydrogenase (LDH) release test.The study was carried outaccording tothe Integrated Testing Strategies (ITS), because the median effective dose (ED_{50}) determined by asingle in-vitro toxicity test (such as MTT or NRU) have a low correlation (50-70%) with its real value (false negative results are frequently detected) [8].

1.5.1. Cell cultures and treatments. We used the culture of mouse fibroblast L929 obtained from tissue culture collections of D.I. IvanovskyInstitute of Virology. Cells were cultivated in flasks of DMEMsupplemented with 10% fetal bovine serum under the conditions: 5%atmosphere of CO_2, t = 37 ° C, humidity equal to 5% (incubator Sanyo, Japan). The cells in exponential growth phase were reseeded in a 96-well plate ($2 \cdot 10^4$ cells/well) containing 100 μl per well of DMEM supplemented with 10% FBS. Cells were incubated for 24 hours and then the mediumwas replaced with a fresh one with addition of MNP andMCPM in test series. The medium for the first control series (Control 1)contained 50 μl of phosphate buffer per well. The biodegradable capsules without iron oxide was added in the wells for second control series (Control 2).The concentration of objects were determined by the containing of magnetite. In every test series we used 6 levels of magnetite concentrations: 0.003; 0.032; 0.322; 0.644; 1.610;3.220 mg / ml. We used three values of exposure time: 24, 48 and 72 hours. This stage of the sample preparation was idetical for all tests.

1.5.2. MTT-test. MTT (3- [4,5- dimethylthiazol-2-yl-2,5-difeniltetrazolium bromide) is a water soluble salt of tetrazolium.It is transformed into an insoluble formazan which is accumulated in cytoplasm. The amount of formazan depends on activity of mitochondrial succinate dehydrogenase and is proportional to the number of living cells. The concentration of formazan can be determined by a colorimetric method. MTT test was performed as follows. After the sample preparation described above, we aspirated the medium and added 50 μl of 5 mg / ml MTT solution. Cells were incubated for 2 hours. After that the medium was removed. Then 100 μl of isopropanol was added in the well, and the plate was vortexed for 20 minutes until the formazan was dissolved completely. Optical density was measured by a microplate reader (EFOS 9305, Russia) at a wavelength of 492 nm with a reference wavelength of 620 nm. The cell viability was determined as the ratio of the optical density (OD) of the sample to the OD of the control solution, and expressed as a percentage.

1.5.3. Neutral Red Uptake test. The method is based on the original protocol proposed by Borenfreund and Puerner (1984) [9] and detects the accumulation of neutral red dye in the lysosomes of intact viable cells. It was realised in the following way. After incubation, medium was removed and 90 μl of fresh DMEM medium with 10 μl of 0.33% neutral red solution was added to each well. The cells were held in the incubator for 2 h. Then, the medium was carefully removed and the cells were fixed quickly with 0.5% formalin solution supplemented with $CaCl_2$. The fixing solution was removed by washing with a phosphate buffer. After washing,the cell monolayerwas dissolved by 1% acetic acid solution in ethanol. For solubilization of dye, plates were shaked for 10 min at room temperature, then supernatant was carefully aspirated and transferred to a clean plate for further measurements. Optical density was measured on a microplate ELISA reader (EFOS 9305, Russia) at a wavelength of 450 nm against a reference of 620 nm. The viability calculations were carried out similarly to the MTT test.

1.5.4 LDH release test. The method is based on determining of lactate dehydrogenase enzyme which is normally located in the cytoplasm and is released into the cultural medium whencells are irreversible damaged [10]. LDH content was determined using a colorimetric method with commercial kit (Pierce LDH Cytotoxicity Assay, The Thermo Scientific, USA). It was realised as follows. After the sample preparation, the medium was carefully aspirated and transferred to a clean plate.According

to the procedureprotocol, reagents were added. Finally the stop-reagent was added and the absorbance was evaluated at 490 nm wavelengthagainst 680 nm reference.

1.6.Phagocytosis

Phagocytosis of particles was studied in the in vitro reaction with human whole blood [11]. The particles were added to the blood of healthy donors stabilized with sodium citrate. The latex particles (PanEco, USA) were added in the control series. Samples were incubated for 30 minutes at 37° C. After incubation, the samples were separated to two parts. In the first one, neutrophil viability was evaluated by the intravital staining with trypan blue. From the second part, smears were made and stained byRomanovsky-Giemsa.The activity of phagocytosis was evaluated by light microscopy. We calculateoverall phagocytic capacity (Percent phagocytosis (%P)) as the percentage of phagocytizing cells related to the total number of phagocytes. The phagocytic index (PI)was defined as the average amount oflatex particles internalized by one phagocyte.

1.7. Statistics

Statistical processing was made using t-test by MS Excel.

2. Results and discussion

2.1. Characterization ofMNP and MCPM

The size distribution for magnetite nanoparticles is shown in Figure 1.

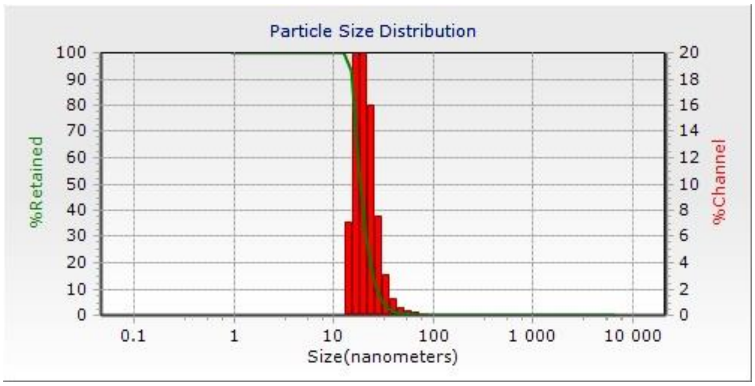

Figure 1. Size distribution of MNP.

The average size is 18±4 nm,zeta potential is equal to –39±2 mV at 7.0±0.1 pH. Suspension of MNP did not contain large aggregates and it was stable for 4 weeks. The SEM-images of MCPM are shown in Figure 2.

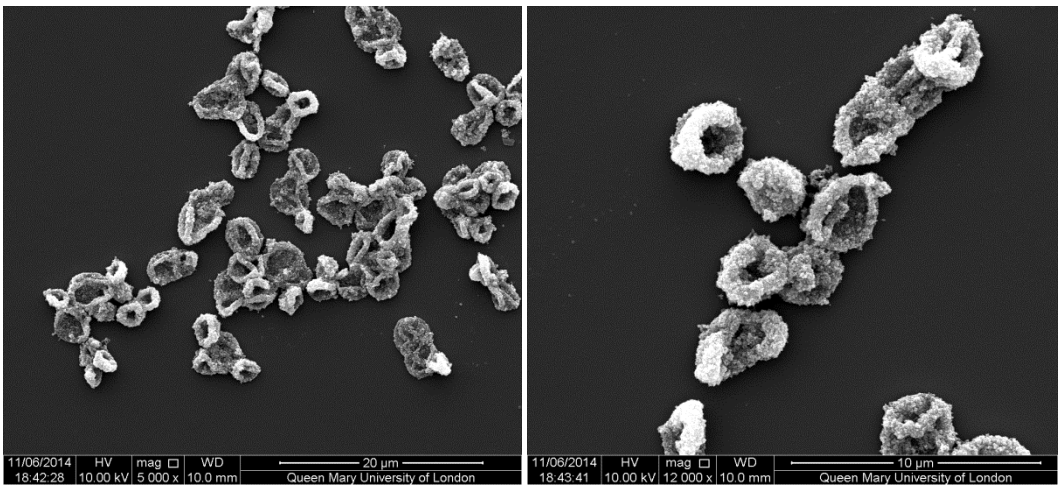

Figure 2. The SEM-images of MCPM.

One can see that the microcapsules have spherical or biconcave shape and average size of 2,9±0,7µm. The content of the magnetite in the colloid was3,24±0,05µg/ml or ≈0,012 ng per 1 capsule. These data is in agreement with the results obtained previously by other authors using the same synthesis method [12].

2.2. Magnetic properties

The hysteresis curves for MNP and MCPM samples are shown in Figure 3. The dependence of magnetization *M* on magnetic field *H* represented in the figure is typical for small magnetic particles in a liquid [13]. The saturation magnetization M_s (renormalised to the magnetite mass) was 55 emu/g and 70 emu/g for MNP and MCPM respectively. The saturation magnetization values for both samples are of the same order of magnitude as the value of M_s for bulk magnetite (about 90 emu/g). This result allows to conclude that magnetic properties of magnetite are basically preserved both in MNP and MCPM. Consequently, both the objects can be used in the development of new tools for magnetically driven target drug delivery.

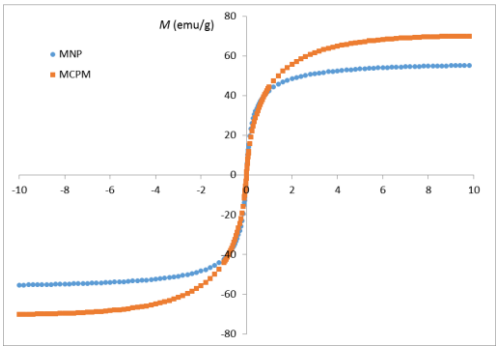

Figure 3. Magnetization hysteresis curves for MNP and MCPM samples. Magnetization is renormalized to the mass of magnetite in the sample.

For further studies of cytotoxicity, we have prepared solutions of colloid MNP and MCPM with identical concentrations of magnetite.

2.3 Cytotoxicity

Results of Cytotoxicity analysis of MNP and MCPM are shown in Figure4.

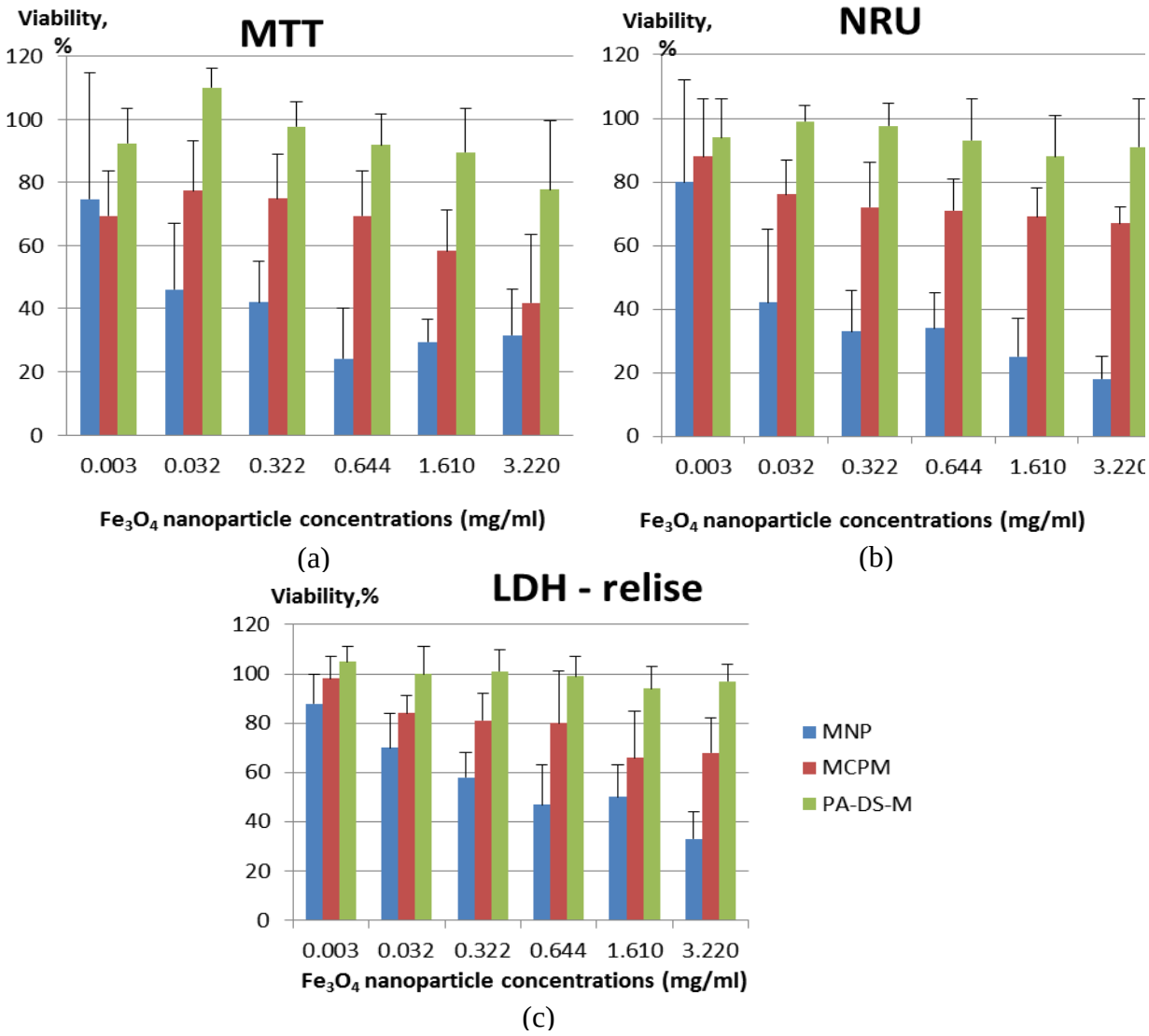

Figure 4. The viability of L929 cells after 24 h incubation with MNP and MCPM particles and PA-DS-M capsules according to the MTT test (a), NRU test (b) and LDH release test (c).

Analysis of MTT assay data (Figure 4, a) shows that a minimal concentration of MNP necessary for viability inhibition of L929 is 0.322 mg / ml. In the concentration range from 0.322 to 3.22 mg / ml, fraction of viable cells was 42-23%. Unlike MNP, polyelectrolyte capsules containing the equivalent amount of magnetite provided significantly less impact on the MTT assay results. In this case, the minimum concentration of magnetite causing a significant reduction in viability of cells was equal to 3.22 mg / ml. Polyelectrolitemicrocapsules without magnetite do not have a negative impact on the culture of L929.Similar results were obtained by NRU test (Figure 4, b). In the LDH release test,it wasalso found that thecytotoxicity of microcapsules is lower compared to the free magnetite nanoparticles. However, this method had significantly lesssensitivitythan the others. The minimal concentration of MNPcausing a cytotoxic effect determined by this testwas equal to 0.644 mg/ml. There was no significant cytotoxic effect observed by this method in the MCPM series as well as in the control series with the empty polyelectrolyte capsules.

Low sensitivity of LDH release method is explained by the fact that damage of cell membranes (deteced by this method)occureat the final stage of the cell death process. At the same time, MTT and NRU tests detect earlier and "finer" violations ofcell functions (thedamage of oxidative enzymes) [14].

Cytotoxicity studies have shown that the most sensitive test for estimation of the cells viability was MTT assay. According to this test, minimal doses providing significant effect on the cell viability was 0.322 mg/ml and 3.22 mg/ml of iron for MNP and MCPM respectively. To investigate the time-dependence of cytotoxicity the viability of L929 cells for these concentrations was estimated with MTT assay in 24, 48 and 72 hours after addition of MNP and MCPM to the incubation medium. The results are shown in Figure 5. It can be seen that at both concentrations cytotoxic effect reaches a maximum in 48hours and remains at approximately the same level for 72 hours.

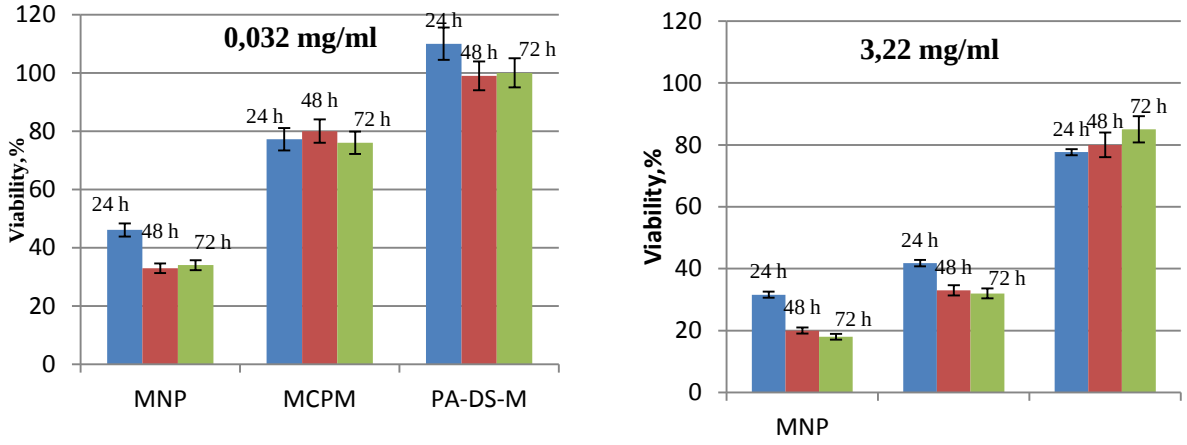

Figure 5. Cell viability studies at different time of exposureof MNP and MCPM particles and PA-DS-M capsules according to the MTT test.

The results of study of interaction between white blood cells and the magnetite containing objects are shown in Table 1. It is found that there is not significant reduction of phagocytes viability after incubation with MNP and MCPM.

Table 1. Impact of the magnetite containing objects on white blood cells viability and phagocytosis.

Parameter	Latex particles (control)	MNP	MCPM
Whitebloodcellsviability,%	93,1±3,3	95,1±1,7	92,9±0,7
Percent phagocytosis(%P), %	95±3,7	39±3,7 (p_{1-2}=0,002)[a]	46,7±3,1 (p_{1-3}=0,002)[b] (p_{2-3}>0,2)[c]
Phagocytic index (PI), n	8,7±0,9	-	3,5±1,5 (p_{1-3}=0,03)[b]

a - :p_{1-2} – statistically significant differences MNP vs Control (latex),
b - p_{1-3} –statistically significant differences MCPM vs Control (latex),
c - p_{2-3} - statistically significant differences MNP vs MCPM.

It was found by the light optical microscopy of leukocytes incubated with MNP and MCPM that both of these objects undergo phagocytosis. Neutrophils with inclusion of brown magnetite granules as well as cells with internalized microcapsule were observed in the blood smears. Phagocytosis index did not differ significantly in the groups with MNP and MCPM but it was two times lower compared to the control. The intensity of MNP phagocytosis was not determined because the particle size did not allow them to be identified using light microscopy. The magnetite containing microcapsules inside phagocytes were visualized clearly enough. The phagocytosis number for MCPM was 2 times lower in comparison with the one for latex.

The toxicity of iron oxide was discussed in detail in a number of studies. It was found that high concentrations of iron oxide nanoparticles induce cell necrosis [15,16]. Most authors proposed that the mechanism of necrosis inducing is associated with the generation of reactive oxygen species (ROS) [17]. Although antioxidant mechanisms present in the living systems, excess of ROS can cause lipid peroxidation and damage of cell membranes, proteins and DNA.

Autophagy can be another possible mechanism of toxicity. The majority of nanoparticles penetrating into the cell by endocytosis merges with lysosomes and form autophagosomes.[18, 19]. Internalization allows nanoparticles to reach subcellular structures and interact with various organelles (mitochondria, endoplasmic reticulum, nuclear membrane, chromosomes). The integration of these structures with autophagosomes blocks the cell functions. Critical increase in the autophagosome number leads to cell necrosis[20, 21].

The polymer shell of microcapsules blocks the toxic effects of ferromagnetic nanoparticles. The most likely mechanism for reducing of cytotoxicity is shielding of iron oxide nanocrystals from the intracellular environment. Thus incapsulation reduces their catalytic effect on the lipid peroxidation processes.

Tests with native blood did not show toxic effects of the magnetite containing objects in contrast to the tests with cell cultures. Both MNP and MCPM did not reduce the viability of neutrophils. This may be due to the presence of the protein in the environment. It is known that interaction of nanomaterials with many plasma proteins form so-called “protein crown” around the particles [22,23]. In this case, the particles interact substantially less intensive with cellular membranes and consequently produce less damage on them. This data illustrates the differences of in vitro and in vivo toxicity and suggests that the toxic effect for MNP and MCPM in the living organism is to be lower compared to cell cultures.

Concerning to the interaction of nanomaterials with immune cells, many questions still remain unresolved [24].It is known that nanoparticles having no antigenic determinants are not recognized by immune cells. In our study, we have shown possibility of phagocytosis of ferromagnetic nanoparticles and polyelectrolyte capsule in human blood but its activity was significantly lower than that of the latex which is the standard object for the study of phagocytosis. Most likely, this is due to low antigenicity and non-specific adsorption of plasma proteins. Even registered phagocytosis level can make a significant contribution to the biokinetics of nanoparticles and microcapsules. Furthermore, phagocytized magnetic microcapsules can be used for intracellular delivery of various compounds, as well as for remote control of phagocytes by external fields.

3. Conclusions

The interaction of magnetite nanoparticles and polyelectrolyte microcapsules containing these particles in the shell with L929 cells and human white blood cells was investigated. The dose-dependent effect of the substances on the viability of the L929 cells was observed. It is shown that iron oxide nanoparticles have higher cytotoxicity than microcapsules containing an equivalent amount of magnetite. The maximum cytotoxic effect was registered in 48 hours after administration of the substance in the incubation medium and persisted for 72 hours. It is found that nanoparticles and microcapsules do not affect significantly on the viability of neutrophils. It is shown that both the microcapsules and nanoparticles undergo phagocytosis, but the phagocytosis activity was lower for them than that for the latex. The results indicate the possibility for in vivo administration of magnetite

containing microcapsules. Moreover, the capsules are the promising tool for the development of targeted delivery systems based on autologous or homologous blood cells.

Acknowledgements
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