Biological Compatibility of Polyol Synthesized Magnetite Nanoparticles

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The structural and magnetic properties of magnetite nanopowder obtained with polyol synthesis and its cytotoxicity at interaction with the cell culture are studied in a given work. As shown by x-ray diffraction method, the synthesized powder has spinel structure with the lattice parameter of 0.8393 nm. High dispersion of powder with the particle sizes of 10 nm and rather low aggregation are revealed in TEM studies. As found, there is 100% preservation of cell viability during 2 hours incubation for all the studied concentrations of magnetite. A reverse dependence of the cell viability rate on used concentration of nanoparticles with increasing the exposure time up to 24 hours is revealed; moreover, the decrease in cell viability by 50% is revealed at the concentration of the particles of 0.6 mg/ml Fe_3O.

В роботі досліджено структурні та магнетні властивості магнетитового нанопорошку, одержаного шляхом поліольної синтези, а також його цитотоксичности при взаємодії з клітинною культурою. Методою рентґеноструктурної аналізи показано, що синтезований порошок має структуру шпінелі з параметром ґратниці у 0,8393 нм. Дослідженням методою ПЕМ виявлено високу дисперсність порошку з розміром частинок у 10 нм та його доволі слабке аґреґування. Виявлено 100% життєздатність клітин при їх двогодинній витримці у всіх досліджених концентраціях магнетиту. Знайдено обернену залежність коефіцієнта життєздатности клітин від використовуваної концентрації наночастинок при зростанні часу витримки до 24 годин; крім того, виявлено зменшення життєздатности клітин на 50% при концентрації частинок Fe_3O_4 у 0,6 мг/мл.

В работе исследованы структурные и магнитные свойства магнетитового нанопорошка, полученного путём полиольного синтеза, а также его цито-

токсичность при взаимодействии с клеточной культурой. Методом рентгеноструктурного анализа показано, что синтезированный порошок имеет структуру шпинели с параметром решётки в 0,8393 нм. Исследованием методом ПЭМ установлена высокая дисперсность порошка с размером частиц в 10 нм и его довольно слабое агрегирование. Обнаружена 100% жизнеспособность клеток при их двухчасовом выдерживании во всех исследованных концентрациях магнетита. Найдена обратная зависимость коэффициента жизнеспособности клеток от используемой концентрации наночастиц при возрастании времени выдерживания до 24 часов; кроме того, обнаружено уменьшение жизнеспособности клеток на 50% при концентрации частиц Fe_3O_4 в 0,6 мг/мл.

1. INTRODUCTION

Currently, one of the top-priority tasks in the field of nanotechnology is the fabrication of biofunctional nanomaterials for target drug delivery [1, 2].

To solve such a problem, it is necessary that the powder particles should have possessed a complex of properties [1, 3].

First, nanoscale materials should have a high dispersivity for precise control of the targeting ligand density on their surfaces, and moreover, they should have the size of 5−20 nm providing slight penetration of nanoparticles into the cell to overcome biological barriers but not too rapid their elimination from tissues.

Second, the surface of nanoparticles should have certain coating to form a stable complex nanoparticle−drug, which will allow deliver of therapeutic agent to biotargets by means of the magnetic transport systems.

In addition, to improve targeted delivery of therapeutic agent by means of magnetic systems it is necessary that the nanoparticles should show necessary physical and chemical properties: sufficient specific magnetization, low coercivity, high dispersion, corrosion resistance, bactericidal action.

The magnetite particles show these properties and polyol method is used to synthesise nanopowder of magnetite.

However, in spite of the above-mentioned prospects of the polyol nanotechnology, the obtained relevant materials cause alarm regarding to their biocompatibility and possible adverse effects of the interaction with living organisms.

The structural and magnetic properties of nanopowder of magnetite obtained with polyol synthesis as well as their cytotoxicity at interaction with the cell culture are studied in a given work.

2. MATERIALS AND METHODS

Powder of magnetite Fe_3O_4 was obtained with polyol-synthesis as described in [9]. X-ray studies were carried out using x-ray diffractometer DRON-3.0 in CoK_{α} radiation. Magnetic characteristics of the synthesized powder were measured using a ballistic magnetometer.

Microstructure of powder was studied by means of transmission electron microscopy (TEM). On the grid-substrate coated with a thin formvar film, a drop of nanoparticles was applied. After drying, the samples with nanoparticles were investigated with the electron microscope ТЭМ-125К under an accelerating voltage of 75 kV, equipped with a system of image analysis SAI-01A ('SELMI', Sumy, Ukraine), including the CCD camera DX-2 and software package of 'KAPPA' company, Germany.

The object of this study was the alveolar macrophage (AM) culture obtained from the lungs of guinea pigs [8]. All manipulations with animals were carried out in accordance with the regulations of the 'European Convention for the Protection of Vertebrate Animals Used in Experimental and Other Scientific Purpose' (Strasbourg, 1985) and were approved by the Committee on Bioethics IPCiC NAN of the Ukraine [6]. The cells concentration in culture was 10^6 for 1 ml. Macrophages were separated from other alveolar cells by sedimentation on the plastic surface of the dish when placed in an incubator (37°C) in the 5% CO_2.

To determine the content of nanoparticles in cells of the culture, method of cytochemical staining of iron into blue colour using specific dye Prussian blue was used [6, 8]. Nanoparticles were added to cell culture in a concentration range from 0.06 to 1.8 mg/ml. The incubation time of AM culture with nanoparticles was 2 and 24 hours. After exposure, cells were washed from nanoparticles by medium 199 and fixed with methanol, for staining they were placed into equal amounts of 2% solution of Prussian blue and 2% solution of 0.1 n·HCl at the temperature of 50−560°C.

To quantify the number of interaction of cells with nanoparticles, the principle of Astaldi based on identifying variations of specific staining degrees intensity was used [8]. The investigated cells were divided into three groups: non-coloured, partially coloured and fully coloured. To quantify the results, 100 cells in 5 different fields were counted, differentiating them by the above principle. The experimental results are presented as a percentage (%) in relation to samples in which all cells were completely stained.

Cytotoxicity of a nanomaterial was determined using MTT-test [12] based on the ability of living cells mitochondrial dehydrogenases to convert soluble 3-(4.5-dimetiltiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT) to formazan, which crystallizes within viable cells. Af-

ter incubation of AM with nanoparticles, supernatant was removed, to the cells in each Petri dish, MTT (0.5 mg / ml) was added and incubated them for 4 h at 37°C in presence of 5% CO_2; the resulting crystals of formazan were dissolved by dimethyl sulfoxide (DMSO). Optical density was measured at a wavelength of 540 nm using photocolorimeter CPK-2-UHL-4.2. The experimental results are presented as a percentage (%) to control. Cell viability (Cv) was calculated using the formula: $C_v = (OPn/OPk) \cdot 100\%$, where OPn is optical density of cells incubated with nanoparticles; OPk—average optical density of control cells incubated without nanoparticles. The colour intensity is proportional to the number of viable cells in suspension.

Investigation of lysosomal function was monitored by neutral red dye, which can be accumulated in the lysosomes of viable cells. After exposure of the cells with nanoparticles, to the AM culture, 2 ml of medium 199 containing 100 µg/ml neural red was added and incubated for 3 h at 37°C with presence of 5% CO_2. Then, the cells were washed with physiological solution and placed into 1 ml of the solution (1% acetic acid in 50% ethanol) for 10 minutes to complete cell lysis. Concentration of neutral red in cells was photocolorimetrically determined at a wavelength of 540 nm and presented as a percentage (%) relative to cells incubated without nanoparticles. The colour intensity is proportional to the number of viable cells in suspension.

Lactate dehydrogenase (LDH) activity determination was carried out in the extracellular medium by the kinetic method using standard test set LDH 'CPF' Pyruvate [9]. The principle of the method based on the ability of this enzyme to recover lactate in a pyruvate, transforming the NAD^+ into $HADH/H^+$. LDH activity was measured by spectrophotometric method on an CF-4 at a wavelength of 343 nm and presented as a percentage (%) relative to the LDH activity at 100% cells lysis treated with 0.1% Triton X-100.

Statistical analysis was performed using Student's t test (T test).

3. RESULTS AND DISCUSSION

As shown by x-ray diffraction method, synthesized powder has spinel structure with the lattice parameter of 0.8393 nm, which is consistent with the tabular data for magnetite. The average particle size was determined by the broadening of the diffraction lines using the formula of Selyakov−Scherrer approach [10], and in our case, this size was $\cong 6$ nm. This was in agreement with the analysis of microstructure (Fig. 1, b). A high degree of dispersion and low enough aggregation ability was revealed analysing the powder microstructure. The following magnetic characteristics of the synthesized powder were obtained: the coercivity $H_c = 7$ Oe, the residual magnetization $B_r = 46$ G, and the saturation magnetization $\sigma_s = 43$ G·cm^3/g.
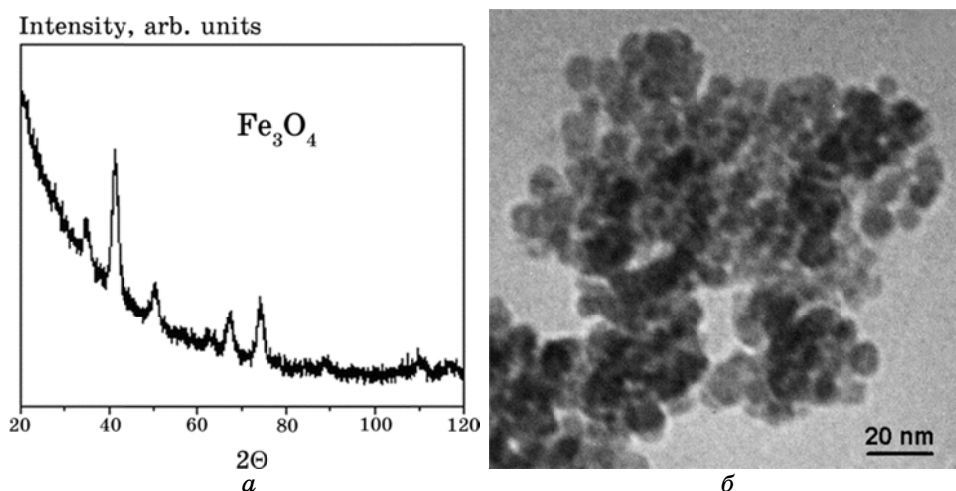
Fig. 1. Diffraction pattern (*a*) and microstructure (*b*) of polyol synthesised nanoparticles Fe_3O_4.
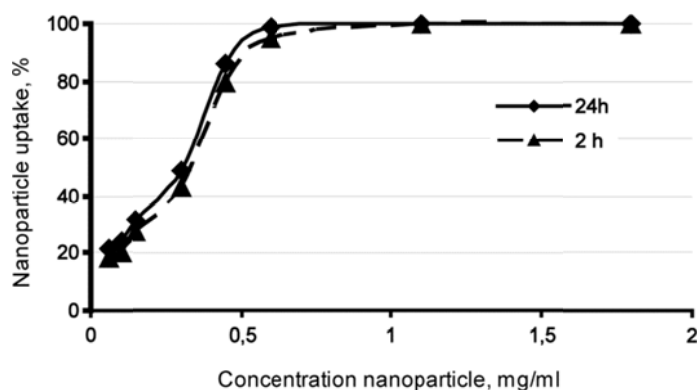
Fig. 2. Effect of incubation duration of 2 or 24 hours and concentration on uptake of nanoparticles into cells.

Pointed above finding of the nanoparticle dimension, the relatively low coercivity force, high values of the saturation magnetization and residual induction and resistance to aggregation are evidences for the nanopowder to be used in biology and medicine for magnetically controlled transport of drugs [1, 2, 3].

Allegedly, one of the possible 'transporters' of medical nanosystems are immunocompetent blood cells (leukocytes, monocytes, dendritic cells) and tissues (Kupffer in the liver, macrophages in the lungs, etc.) that can interact with nanoparticles and migrate to different sites of inflammation [11]. Analysis of the nanoparticles interactions (Fig. 2)
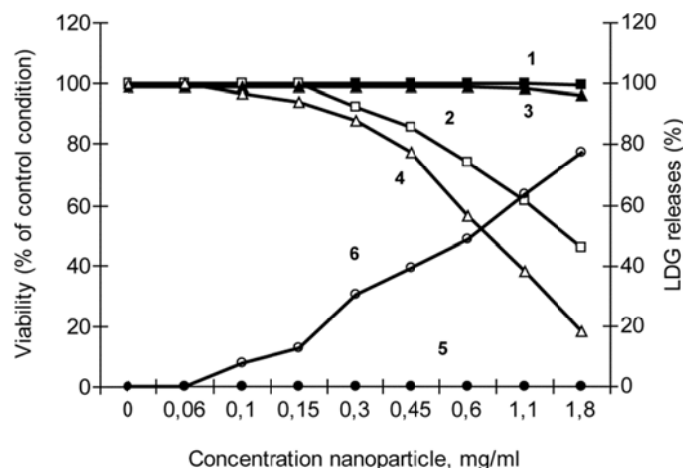
Fig. 3. Effect of incubation duration of 2, 24 hours and concentration of nanoparticles on cell endocytosis by the dye neutral red (curves *1*, *2*), MTT test (curves *3*, *4*) and LDH leakage into the culture medium (curves *5*, *6*).

with different concentrations (0.06–1.8 mg/ml in the incubation medium) with the culture AM have found that the 'full saturation' of cells with nanoparticles had detected from the concentration of 0.6 mg/ml Fe_3O_4 and practically had shown low dependence on incubation time.

According to the literature [13, 14], under the interaction with biological objects, nanoparticles can cause toxic effects such as oxidative stress inducing and free radical formation, oxidative DNA damage, mutagenesis and activation of inflammatory processes.

In our experiments, the cytotoxic effect of the nanopowder was assessed by changes in the following cellular functions: the total activity of mitochondrial enzymes (MTT test), reduction of endocytosis of the dye neutral red, and lactate dehydrogenase (LDH) leakage into the culture medium, indicating damage to the plasma membrane of cells (see Fig. 3).

As follows from Figure 3, there is complete preservation of the analysed parameters of cell viability (curves *1*, *3*, *5*) in all range of concentrations of magnetite Fe_3O_4 at 2-hour incubation. Whereas with an increase of the exposure time to 24 hours, an inverse relationship of cell viability level of the value used by the concentration of nanoparticles is registered (Fig. 3, curves *2*, *4*, *6*). In this case, suppression of 50% of studied cellular functions (IC_{50}—suppression of activity of the studied enzyme to 50%) is observed at the concentration of 0.6 mg/ml of Fe_3O_4. Comparing these data with the data presented in Fig. 2, it should be noted that the output on the plateau of 'saturation' of cells by nanoparticles occurs at a concentration of 0.6 mg/ml Fe_3O_4 (Fig. 2). These experimental data clearly indicate that about 50% of cells were

damaged at the full 'saturation' AM with magnetite nanoparticles Fe_3O_4 after 24 hours incubation, whereas at lower concentrations of studied Fe_3O_4 (from 0.06 to 0.15 mg/ml) higher viability of AM (Fig. 3) was observed.

It is obviously related with the fact that lower nanoparticles' concentration correlates with their lower cells uptake, which is confirmed by cytochemical analysis [14].

As shown above, the suppression of cellular functions by 50% is observed only in those cells, in which uptake of magnetite Fe_3O_4 was observed (Fig 2, 3).

Our experiments demonstrated (Fig. 3) that mitochondrions are the most sensitive structural component of cells to the damaging effect of magnetite Fe_3O_4 nanoparticles, since the tendency for decline in mitochondrial dehydrogenases activity with increasing of nanoparticles concentration is stronger than the decrease in lysosomal function and increased permeability membrane of the cell. This is probably due to the fact that the inhibition of mitochondrial respiration is an early stage of the toxic effect of high nanoparticles concentrations leading to cell death [9]. It should be noted that the complete preservation of all investigated cellular functions of AM after two hours exposure with nanoparticles in the studied concentration range, on one hand, and a more expressed suppression of mitochondrial function after 24-hours incubation, on another, is indirect evidence of uptake of nanoparticles into the cells and their interactions with intracellular components.

Thus, the experimental data presented above allows concluding that usage of high-dispersed nanoparticles obtained by polyol-synthesis demonstrates the efficiency of their interaction with cells as well as the determined range of the concentrations and the time parameters can be used in the further biomedical researches.

4. CONCLUSIONS

1. Polyol synthesized nanoparticles of magnetite Fe_3O_4 have a high degree of dispersion, small size of about 10 nm and show the relevant level of magnetic properties (specific saturation magnetization and coercivity) suitable for use in biology and medicine for target drug delivery.
2. Uptake of Fe_3O_4 nanoparticles by AM cells depended on their concentration and did not depend on incubation duration 2 and 24 hours. Complete 'saturation' of the cells by nanoparticles occurred at the concentration of 0.6 mg/ml Fe_3O_4.
3. Cytotoxicity of Fe_3O_4 nanoparticles was observed after 24-hours exposure within the magnetite concentration range from 1.1 mg/ml to 1.8 mg/ml. As shown, the decrease in 50% of cells viability was registered at the concentration of 0.6 mg/ml in the incubation medium.

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