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Iron Oxide Nanorods as a Tool for the Formation of Tissue Structures by Magnetic Field and Laser Irradiation †

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Abstract. This paper presents the results on the development of a method for the formation of tissue structures using nanorods under of magnetic fields and laser irradiation in the near-infrared spectrum. Polyacrylic acid (PAA)-coated and bovine serum albumin (BSA)-coated nanorods were synthetized. The cytotoxicity of the iron oxide nanorods was determined by MTT test on L929 cells. It was shown low cytotoxicity of iron oxide nanorods at concentrations ranging from 5–60 μg (Fe)/mL. After laser irradiation in the near-infrared spectrum (808 nm) during 10 min cell death was 93%. The double staining of fluorescence dyes (acridine orange and ethidium bromide) showed that the main mechanism of the cell death after laser irradiation was necrosis. The possibility of forming cell structures by exposing cells with nanorods to a magnetic field has been shown. Our synthesized nanorods have high potential for application in optical hyperthermia and magnetic formation of tissue structures.

Keywords: magnetic nanoparticles; nanorods; cytotoxicity; magnetic formation; laser irradiation

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1. Introduction

Reconstructing the integrity of tissue anatomy and its functionality is the most important task of modern tissue bioengineering. The different ways of solving are aimed at replacing damaged and lost tissue structures [1]. The goal of tissue bioengineering is to design and grow living, functional tissues or organs outside the human body for subsequent transplantation into the patient to replace or induce regeneration of damaged organs or tissue [2]. The main strategy of tissue engineering is the selection of a biocompatible cell carrier, so that cells can interact with each other.

Iron oxide nanoparticles have shown great advantages in many applications and have been extensively studied and explored in various application fields because of their low cost, nontoxicity, and biocompatibility [3,4]. Nanomaterials have shown superior performance in various application fields such as tool for magnetic hyperthermia, MRI agent etc. [5]

The analysis of selected studies showed that the using magnetic nanoparticles has a good cost-benefit approach, which allows for rapid and easy 3D spheroids formation driven mostly by the presence of the magnetic field, with no nanoparticles-induced damage in cell populations. Although the use of magnetic nanoparticles and the magnetic fields did not influence the viability of the cells, their use could influence cell morphology and the final shape of the m3D structure archived. The use of 3D systems has been increasing in the last years, most using levitation and bioprinting methods. The magnetic ring structures seem to be an emerging tool for generating bigger 3D aggregates [6].

Modern and comprehensive approaches in biomedical applications enable the construction of tissue structures using novel strategies that include magnetic field and/or laser irradiation [7,8]. In order to form, by magnetic field, certain tissue structures, nanoparticles with high magnetization value and sufficient biocompatibility are required. Magnetic iron oxide nanoparticle have adsorption by cells and reduction of cytotoxicity. The selection of a coating to maintain stability during storage, sufficient particle adsorption by cells and reduction of cytotoxicity is important. In this regard, the main goal of this work is the study of main proprieties and cytotoxicity of iron oxide nanorods with or without laser irradiation in near-infrared spectrum.

2. Materials and Methods

For the synthesis nanorods were used: iron (III) chloride hexahydrate (FeCl3·6H2O) (99.99%, Sigma-Aldrich); sodium chloride (NaCl) (99.9% Vecton); sulfosalicylic acid (99.9% Vecton); hydrochloric acid (HCl) (37% Vecton); sodium acetate (99.9%, Vecton); ethylene glycol (99% Vecton); polyethylene glycol-2000 (Mw = 2000 Da, Sigma-Aldrich); sodium polyacrylate (PAA) (Mw~5100 Da, Sigma-Aldrich); 1,2-Hexadecanediol (90%, Sigma-Aldrich); bovine serum albumin (BSA) (Mw~66,000 Da, Sigma-Aldrich).

The characteristics of nanoparticles. The morphological characteristics of the synthesized nanorods were evaluated by transmission electron microscopy (TEM). Z-potential of iron oxide nanorods was measured by particle charge analyzer Stabino (Microtrac Inc., Germany). Quantitative determination of iron in suspension of iron oxide nanoparticles was carried out using photocolorimetric method based on measuring of optical density of yellow colored complexes of iron (III) ions with sulfosalicylic acid in alkaline medium [9]. Magnetic properties. Magnetization of the particle suspension was measured by induction method using an EZ11 vibrating magnetometer (Microsense Inc., Lowell, MA, USA) at room temperature (≈25 °C).

The cytotoxicity of the iron oxide nanorods was determined by MTT test on L929 mouse fibroblast cell culture. The L929 cell line was obtained from The National Research Center for Epidemiology and Microbiology named after Honorary Academician N.F. Gamaleya. The cytotoxicity of the nanomaterials was investigated in accordance with the procedures of International SOP (ISO) 10993-5 "Tests for in vitro cytotoxicity". L929 cells were pre-cultured in a 96-well culture plate and incubated for 24 h under standard conditions (37 °C and 5% CO_2) in DMEM (Paneco, Russia) with 10% fetal bovine serum (Corning, France). Prepared dilutions of polyacrylic acid (PAA)-coated and bovine serum albumin (BSA)-coated nanorods at concentrations of 3.9–500 μg/mL were added to cells. After 24 h of an incubation, cells with nanorods were treated with laser 808 nm during 10 min. After 24 h, MTT test was carried out to determine cell viability. For this, MTT 5 mg/mL dissolved in a fresh medium was added in the each well and cells were incubated for 3.5 h under standard conditions. Optical density was measured on Varioscan Lux reader (Thermosciencific, USA) at 570 nm and 650 nm reference wavelength. Cell viability was assessed as the ratio of the optical density of the sample to control (untreated cells). Cells were evaluated morphologically using an inverted microscope Micromed (Russia). The mechanism of cell death was assessed by double staining with fluorescent dyes: acridine orange and ethidium bromide. Acridine orange stains the nuclei of living cells with green color; ethidium bromide only penetrates in dead cells, staining the nuclei with red-orange color. Fluorescence microscopy was performed on inverted fluorescence microscope BM35FXT (ICOE, China).

Statistical processing of the data was performed with a one-factor analysis of variance (ANOVA test) in GraphPad Prism 8.0 software. Differences were considered significant at $p < 0.05$. Results are presented as Mean ± SD.

3. Results and Discussion

The synthesis of nanorods consists of two stages. At the first stage 3 g $FeCl_3$-$6H_2O$ was dissolved in 20 mL of deionized water, the salt solution was placed in an autoclave and incubated in an oven at 120 °C for 4 h. Yellow β-FeOOH particles were collected by centrifugation and purified several times with water and ethanol. The purified precipitation was dried at 65 °C for 10 h. The dry powder of β-FeOOH particles was dispersed with ultrasound (100 W, 15 min) in 15 mL ethylene glycol. The resulting particle suspension was hydrothermally treated in an autoclave at 200 °C for 24 h. The suspension changed color from yellow to black that indicate the formation of Fe_3O_4 particles.

PAA and BSA with a concentration of 12 mg/mL were added to the purified particles (to give greater biocompatibility) and the suspension was incubated for 24 h under constant stirring. The Fe_3O_4@PAA and Fe_3O_4@BSA nanorods were washed with deionized water 3 and dispersed in 0.9% NaCl solution. Figure 1 shows TEM images of the magnetic nanorods.

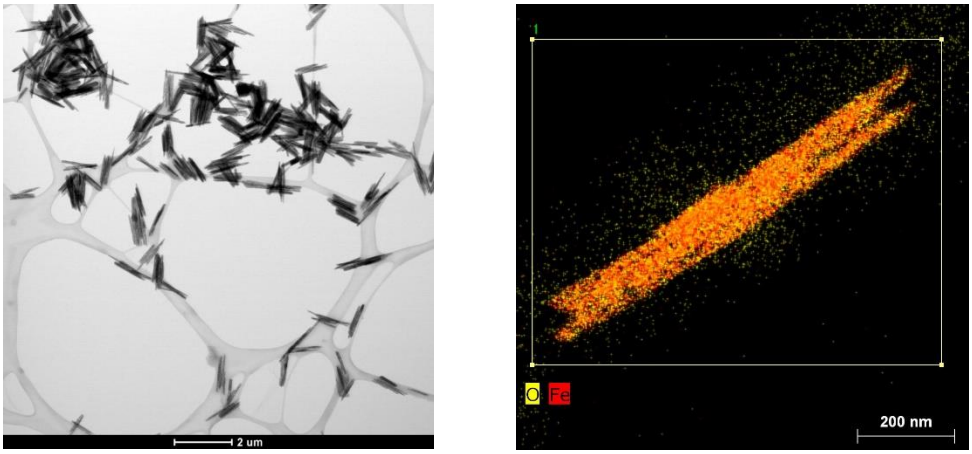

Figure 1. TEM images of Fe_3O_4 nanorods.

The particles disperse well in aqueous medium. The ζ-potential was −72 ± 6 and −79 ± 5 mV at pH = 7.1 for Fe_3O_4@BSA and Fe_3O_4@PAA, respectively. According to the results of quantitative analysis, the Fe concentration was 18 and 16 mg/mL for Fe_3O_4@BSA and Fe_3O_4@PAA suspensions, respectively. The saturation magnetization of the Fe_3O_4 nanorods was 80 emu/g.

MTT test shows that Fe_3O_4@PAA and Fe_3O_4@BSA at concentrations range of 125–500 (Fe)μg/mL were toxic to L929 cells. The high toxicity at these doses is most likely due to the biodegradation of nanorods in cell lysosomes, which produces large amounts of iron ions, which in turn can potentially lead to abnormal mitochondrial function, altered membrane potential, formation of reactive oxygen species and the uncoupling of oxidation and phosphorylation. These processes underlie are the main mechanisms of cytotoxicity [10]. Previous studies have noted that the increased production of reactive oxygen species and lipid peroxidation is most often associated with cytotoxicity of magnetic nanomaterials [11]. However, at concentrations of 3.9–62.5 μg/mL, nanorods had little effect on cell viability. Cell survival was more than 70% for Fe_3O_4@BSA and Fe_3O_4@PAA. In recent study, it was shown no affect on L929 cell viability by nanorods with PEG in an overall concentration range of 15.6–500 μg/mL after an incubation period of 24 h [12]. The cytotoxicity tests on L929 has shown that modified nanorods had less toxicity that non-modified [13]. That is why we used PAA and BSA for covering of nanorods. In our study, we showed that Fe_3O_4@PAA and Fe_3O_4@BSA nanorods in concentration of 62.5 μg/mL and less are safety for further studies on cell cultures and animals.

Laser irradiation of nanorods showed almost total cell death. After laser irradiation (808 nm) during 10 min cell death was 93% (Figure 2A). As revealed by the study of

photothermal effect of nanorods, less than 10% of 4T1 cells remained alive at the nanorods concentration of 50 μg(Fe)/mL after irradiation with a NIR laser [14]. Zhou et al. have prepared PEGylated iron/iron oxide core/shell nanoparticles (Fe@ION) [15]. In the in vitro study, HeLa cells were incubated with Fe@ION for 1 h in the absence or presence of a magnet beside the cells. Then, cells were irradiated by an 808 nm laser (0.31 $W \cdot cm^{-2}$) for 10 min. Nearly all of the cells that were incubated with the nanoparticles and laser irradiated underwent the ablation, while for the control group, only a few dead cells were observed. Contrary to the affirmation that a certain extent of aggregation of iron oxide nanoparticles is necessary to achieve a photothermal effect at safe irradiation doses, Chen et al. found that highly crystallized iron oxide nanoparticles with a delicate polymer coating could be used for efficient photothermal ablation [16]. In another study, Chu et al. investigated the photothermal effect of iron oxide nanoparticles of several shapes (spherical, hexagonal, and wire-like) coated with carboxyl-terminated PEG-phospholipid [17]. When the iron oxide nanoparticles (0.5 mg/mL) were taken up by Eca-109 cells (human esophagus carcinoma cells), the viability and cell structure was not affected. Upon irradiation with a laser of 808 nm however, the cell viability was suppressed (<60% in the three types of iron oxide nanoparticles). Our Fe_3O_4@PAA nanorods demonstrated good photothermal effect with near-infrared laser irradiation. This ability of nanorods we could use to modify a tissue structure from cells incubated with iron oxide nanoparticles.

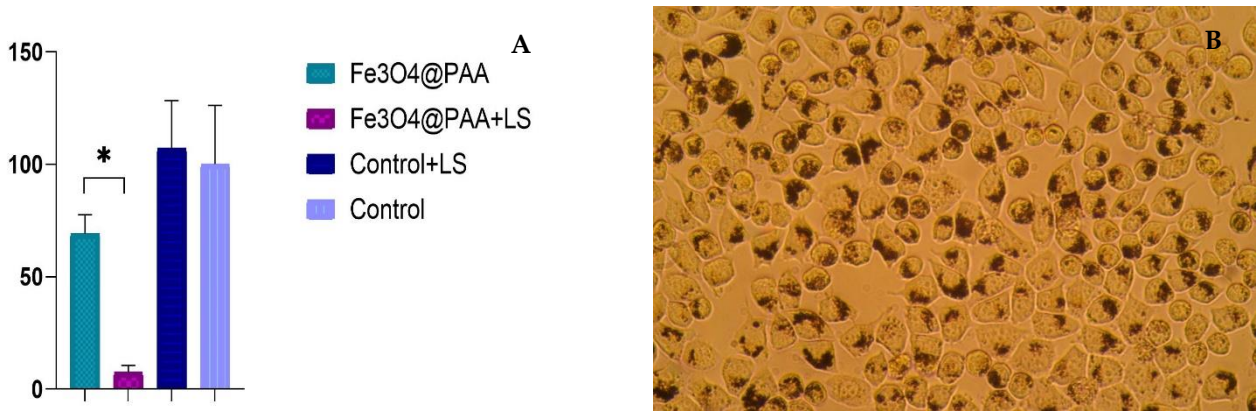

Figure 2. (**A**)—Cell viability of Fe_3O_4@PAA at concentration 31.5 μg/mL before and after laser irradiation 10 min 808 nm (* $p < 0.05$), LS—laser irradiation 10 min, control—cells without treatment. (**B**)—The morphology of L929 cells incubated with Fe_3O_4@PAA after 48 h (400× magnification).

The double staining with acridine orange and ethidium bromide confirmed the data of MTT test and showed that necrosis was the main cause of cell death after laser irradiation (Figure 3). Cells with green nuclear are alive (with Fe_3O_4@PAA without laser). Red color of nuclear tells that cells died (cells with Fe_3O_4@PAA after laser irradiation for 10 min).

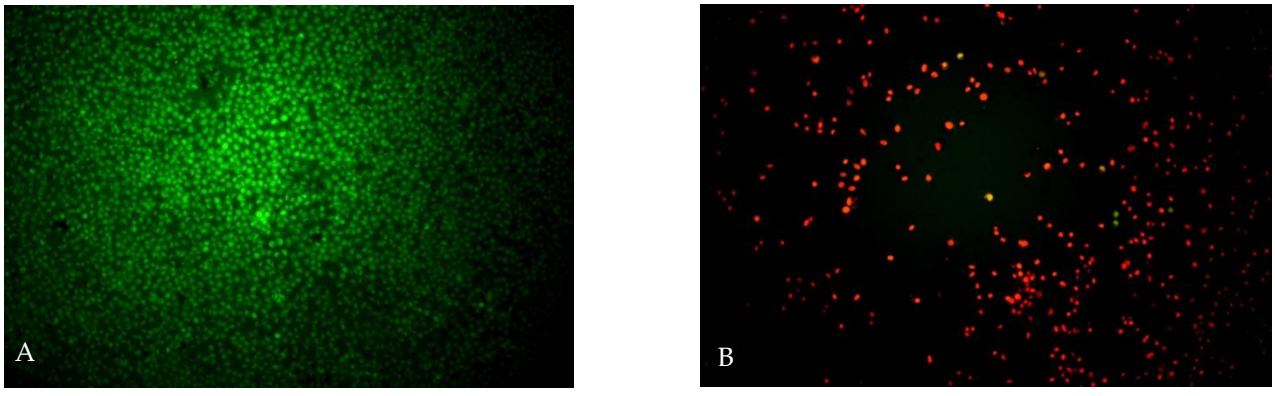

Figure 3. Cell viability of L929 cells treated with Fe_3O_4@PAA (31.5 μg/mL) with or without laser irradiation. Live-dead staining images of L929 cells with different treatments: (**A**) without irradiation; (**B**) NIR irradiation (808 nm, 10 min). Live and dead cells were stained by acridine orange and ethidium bromide and presented in green (live cells) and red (dead cells) colors in those images (100× magnification).

The possibility of forming cell structures with nanorods by magnetic field has been shown (Figure 4). On the Figure 4, we can see that cells with nanorods are concentrated on the shape of a magnet after 24 h incubation with permanent magnetic field. Cells are still viability. The most of cells have green color after double staining with acridine orange and ethidium bromide. Further research is planned on the possible formation of cell structures by magnetic field and laser irradiation.

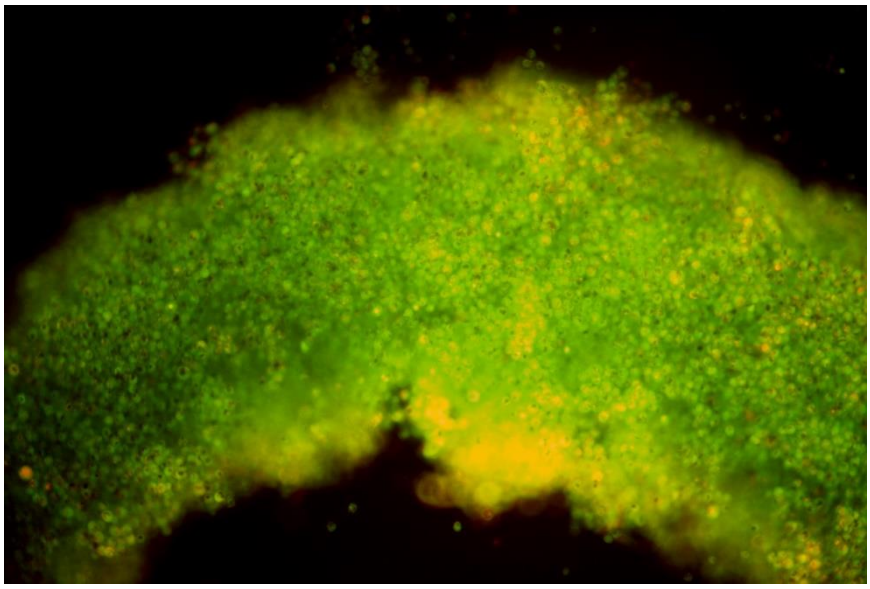

Figure 4. L929 cells with nanorods after 24 h incubation with permanent magnetic field. Live and dead cells were stained by acridine orange and ethidium bromide (100× magnification).

4. Conclusions

Thus, concentrated, highly dispersible Fe_3O_4@BSA and Fe_3O_4@PAA nanorods were prepared using a modified synthesis method. A cytotoxicity study shows that the nanorods were not toxic to fibroblasts at concentrations of 62.5 μg/mL and less. IC50 was 135.7 μg/mL for Fe_3O_4@PAA. The results showed that nanorods stabilized with PAA have the best absorption capacity on L929 cells. The laser irradiation in the near-infrared spectrum almost completely kills cells incubated with Fe_3O_4@PAA. This allows cells structures obtained to be corrected using laser irradiation. Fe_3O_4@PAA nanorods present a number of advantages, such as excellent magnetic properties, good compatibility, and absence of toxicity. The magnetic properties of iron oxide nanorods allow them to be magnetically targeted and used as tool for the formation of tissue structures.

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