REVIEW

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In vivo evaluation of Fe_3O_4 nanoparticles

ROXANA CRISTINA POPESCU, ECATERINA ANDRONESCU, ALEXANDRU MIHAI GRUMEZESCU

Department of Science and Engineering of Oxide Materials and Nanomaterials, Faculty of Applied Chemistry and Materials Science, Politehnica University of Bucharest, Romania

Abstract

Our review summarizes the latest approaches regarding *in vivo* biocompatibility evaluation of magnetite nanoparticle-based systems. The paper follows the applications of Fe_3O_4 nanoparticles in cancer diagnosis and treatment, by means of nanoparticle-mediated magnetic hyperthermia, respectively by targeted delivery of chemotherapeutics. The long-term biodistribution in relevant organisms is also discussed, due to the need of knowing the exact course of magnetite nanoparticles after the fulfillment of their function. Several commercial Fe_3O_4 systems used as contrast agents for medical imaging and cancer treatment by hyperthermia are briefly presented in the last section.

Keywords: magnetite nanoparticles, *in vivo* evaluation, biocompatibility, biodistribution.

Introduction

The biocompatibility evaluation is an important step in proving the functionality when obtaining nanosystems with medical applicability, such as those based on Fe_3O_4 nanoparticles. Its magnetic properties and its heating capability due to an external alternate magnetic field (AMF), make possible the usage of those nanoparticles as contrast agents for medical imaging [1], systems for magnetic hyperthermia cancer treatment [2], nanocarriers for controlled delivery of drugs [3], etc.

The phases of a biocompatibility study consist in a logic succession of biological tests, starting from *in vitro* evaluation, using cell cultures, going to *in vivo*, preclinical tests using animals, ultimately being applied in human organisms, as different clinical tests (Figure 1).

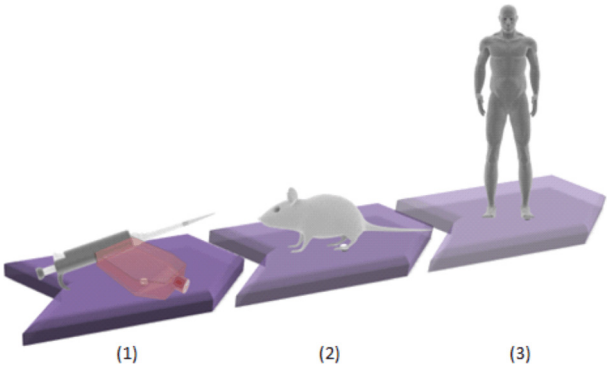

Figure 1 – ***The required phases of biocompatibility evaluation for nanosystems based on Fe_3O_4 magnetic nanoparticles (MNPs) used in medical applications: (1) in vitro evaluation, using cultured cells; (2) pre-clinical in vivo evaluation, using animal models; (3) clinical in vivo evaluation, using human subjects.***

In vitro tests

In vitro tests consist of relevant experiments realized on a controlled media that prove the effect of the evaluated material upon a particular or different cell cultures. The controlled conditions resemble the properties of the human organism media, yet the physiological complexity could not be reproduced by these means. Among the advantages of those tests, we can list the following aspects: (1) the cellular models are easier to understand, due to their reduced complexity, compared to the *in vivo* tests; (2) the biocompatibility evaluation using *in vitro* methods allows to obtain rapid information, with reduced costs; (3) the development of such methods determines a reduction in the number of sacrificed animals, preclinical tests being realized only after obtaining favorable results for the *in vitro* tests. Generally, to evaluate the biological effects of different materials and substances, monolayer adherent cells are preferable, only hematopoietic cells being cultured in suspension. Also, the most available and used are immortalized cells (commercial lines), because of the reproducibility and the easy supervision of the resulting outcomes. For more eloquent results, primary cell cultures offer properties closer to those exhibited in the *in vivo* media [4].

Preclinical *in vivo* tests

Preclinical *in vivo* tests use animals to evaluate the biocompatibility of materials, by means of analyzing the direct interaction with the structures of the living organism. This is an important step, preceding the clinical tests and offering better results than *in vitro* tests. It implies the selection of a suitable animal model, the most commonly used in MNPs research being: (1) mice: BALB/c [5], C57BL/6 [6], Kunming [7], C3H [8]; (2) rats: Swiss albino [9], Wistar [10]; (3) swines [11]; (3) zebrafish [12] (as vertebrate models); (4) *Daphnia magna* [12] (as invertebrate models); (5) chick embryos [13] (as embryo models). The results of these test are obtained after long periods of time (compared to *in vitro* tests), having a more difficult interpretation and higher costs.

Clinical *in vivo* tests

Clinical *in vivo* tests are carried on human subjects. These are performed only after obtaining favorable results in preclinical investigations, raising issues regarding the

ethics. Usually are made on volunteers, a control group being necessary [14].

In vivo evaluation of Fe_3O_4 MNPs-based nanosystems for cancer diagnostic

The majority of *in vivo* evaluation studies of Fe_3O_4 MNPs have been developed in order to verify its functioning potential as contrast agents for medical imaging techniques, as magnetic resonance imaging (MRI), offering the possibility for an improved diagnostic. MRI imaging uses the difference between the magnetic relaxation of water protons in biological solutions and surrounding tissues [15]. The principle of Fe_3O_4-based systems consists in the lowering of relaxation times T1 (longitudinal relaxation – spin-lattice, which determines the bright regions in the image) and T2 (transversal relaxation – spin-spin, assuring the dark regions of the image), characteristic for two independent relaxation processes of the protons [15]. The relaxivity ($mM^{-1}s^{-1}$) parameter measures the efficiency of the contrast agent, its growth determining an improved image contrast [15].

Kumagai *et al.* developed a system for MRI, consisting in intraperitoneal (i.p.) administration of PEG-PAsp (poly ethylene glycol–poly aspartic acid)-Fe_3O_4, which functions after 24 hours from the i.p. administration of TGF-β inhibitor (transforming growth factor β inhibitor). It was highlightened the accumulation of MNPs, as revealed by MRI images acquired two hours after the treatment with the designed MNPs, in comparison with Resovist (commercial dextran-Fe_3O_4 MNPs). From the histopathological exam, it was proved an accumulation of the obtained MNPs in fibrotic regions [5].

Tsuchiya *et al.* evaluated, by comparison, the bio-dynamics of superparamagnetic iron oxide nanoparticles (SPIONs) and ultrasuperparamagnetic iron oxide nanoparticles (USPIONs), with dextran shell, used in MRI. The study used 36 C57BL/6 mice, i.p. injected with MNPs, from which were determined the Fe-positive areas (Prussian blue staining) in different harvested organs. Such larger areas where observed mainly in the liver, being three times bigger for USPIONs injected mice, compared to SPIONs injected mice; the MNPs accumulation took place especially in the parenchyma. For mediastinal lymph nodes, the USPIONs, respectively the SPIONs accumulated in almost equal proportions. It is recommended the application of USPIONs in MRI imaging of the liver, while for the imaging of lymphatic nodules one can use either USPIONs or SPIONs, for close results [6].

Roohi *et al.* studied the influence of dimensions and coverage upon the blood half time of MNPs, but also the MRI performance of CDX (carboxydextran)-SPIONs, PAA (poly acrylic acid)-SPIONs and PEG-SPIONs. For this purpose, Wistar rats were injected i.v. in the tail vein with 100 μmol Fe kg^{-1} MNPs. For all of the specimens were harvested 0.4 mL of blood samples, before and after the treatment (from one to 360 minutes past the administration time), the plasma was isolated and it was determined the Fe relaxation rate (r_1 and r_2 relaxivity). Also, MRI images were recorded for two hours and 31 minutes from the administration time (at every 2.82 minutes). The following aspects resulted: (1) an increased half blood time with the administrated dose; (2) a lower half blood time for MNPs with greater dimensions; (3) for the same hydrodynamic size (50 nm), the ascending order for the half blood time was determined for PAA-SPIONs, CDX-SPIONs, PEG-SPIONs [16] (Table 1).

Table 1 – ***Examples of in vivo evaluation studies for Fe_3O_4 MNPs-based nanosystems for cancer diagnostic***

MNPs description	Diagnostic	Targeting	*In vivo* efficiency evaluation
PEG-PAsp-Fe_3O_4	Solid pancreatic tumors	Active (TGF-β inhibitor)	BALB/c nude mice, contrast enhancement [5].
Dextran-Fe_3O_4	Liver, mediastinal lymph nodes	Passive	C57BL/6 mice, rapid accumulation for USPIONs in the liver, compared to SPIONs; equal accumulation in mediastinal lymph nodes [6].
Citrate-Fe_3O_4	Head and neck	Passive	Swine (35–70 kg), contrast enhancement (a brighter image) after MNPs use [11].
Fe_3O_4 SPIONs	–	–	Swiss albino rats subcutaneously injected with Ehrlich carcinoma cells – SPIONs accumulated in the central tumor area providing efficient magnetic resonance hyperthermia treatment and imaging [9].
Mannan-SPIONs	Metastatic lymph nodes	Active	Balb/c mice mannan-SPIONs accumulated in metastatic lymph nodes [17].
MWCNTs (multi-walled carbon nanotubes)-Fe_3O_4	Liver and spleen	–	Kunming mice; a darkening of the spleen and liver was observed for the treated mice, in comparison with untreated mice (control) [7].
Anti-VEGF (vascular endothelial growth factor)-dextran-Fe_3O_4	Colon cancer	Active	Balb/c mice; efficient accumulation in the tumor site after systemic administration of MNPs [18].
scAb (prostate cancer stem cells antibody)-PLGA-SPIO/Docetaxel	Prostate cancer	Active	BALB/c nude mice MRI exam proved an enhancement of the negative contrast, comparing to the images acquired before the treatment [19].
Fe_3O_4-NH_2-AF (folic acid)	Hepatoma	Active	Wistar rats *in vivo* hepatoma tumor targeting efficiency proved; the toxicity studies demonstrated negligible effects for low concentrations (3 mg/kg) [10].
Anti-EGFR mAbs (monoclonal antibody anti-EGFR)-PEG-Fe_3O_4	EGFR-positive breast cancer adenocarcinoma	Active	Balb/c mice, proving the MNPs accumulated at the tumor site and also a modification of the intensity of magnetic relative signal was observed [20].
Fe_3O_4-CMD (carboxymethyl dextran)-AF	Oral epidermoid tumor tissue	Active	Mice, improved darkening capacity of MRI images and tumor specificity [1].

MNPs description	Diagnostic	Targeting	*In vivo* efficiency evaluation
Fe_3O_4-PEI (poly ethylene imine)-HA (hyaluronic acid)-FI (fluorescein isothiocyanate)	Cervical cancer, glioblastoma	Active	Balb/c mice, the highest contrast is obtained at four hours after the administration, respectively at 24 hours, U87MG model proving a better effect; similar results were obtained for MNPs HA_{31K} system [21].

In vivo evaluation of Fe_3O_4 MNPs-based nanosystems for magnetic hyperthermia cancer treatment

Nanoparticle-mediated magnetic hyperthermia is a therapeutic technique based on phenomenon like Néel relaxation, Brownian relaxation and hysteresis loss [22], in order to raise the local temperature of a tissue to about 41–43^{0}C by applying an alternated magnetic field (AMF) for a defined period of time [23]. The supplementary energy from the external AMF accumulated in the nanoparticles is converted in thermal energy to assure the relaxation state [15]. The specific absorption rate (SAR) parameter (Wg^{-1}) measures the continue ability of MNPs to produce thermal energy [15]. The nanoparticles accumulation in tumor tissues is made by passive targeting, due to the permeability effect and the enhanced retention, but also by active targeting, by magnetic directioning or due to the specific functionalizing with different agents, like folic acid [1], Gly-Arg-Gly-Asp-Ser [2], etc. The increased sensitivity of tumor tissues at 41–43^{0}C temperatures, compared to healthy tissues, which resist up to 46–47^{0}C, makes possible the differential treatment by magnetic hyperthermia. Generally, to insure these parameters, but also to obtain reduced adverse effects, the applied AMF must have values under 5×10^9 $Am^{-1}s^{-1}$ and frequencies under 1 MHz [15] (Table 2).

Table 2 – ***Examples of in vivo evaluation studies for Fe_3O_4 MNPs-based nanosystems for magnetic hyperthermia cancer treatment***

MNPs description	Treatment	Targeting	*In vivo* efficiency evaluation
NPrCAP (N-propionyl-cysteaminyl phenol)-Fe_3O_4	Melanoma	Active	C57BL mice; the tumor suppression increasing effect by magnetic hyperthermia for NPrCAP-Fe_3O_4 (compared to Fe_3O_4) [24].
DNR (Danorubicin)-Fe_3O_4	Leukemia cancer tissue	Active	Nude mice (; tumor growth suppression by bimodal treatment (hyperthermia and chemotherapy) [3].
Fe_3O_4 SPIONs	Ehrlich carcinoma	–	Swiss albino rats; the decrease in tumor growth rate by MRI mediated hyperthermia [9].
GRGDS (Gly-Arg-Gly-Asp-Ser)-poly (N-isopropyl acrylamide-acrylamide-allylamine)-PLGA-Fe_3O_4	Melanoma	Active	C57BL/6 mice MNPs accumulation in tumor tissue [2].

In vivo evaluation of Fe_3O_4 MNPs-based nanosystems for chemotherapy

The most common method used in cancer therapy is chemotherapy. However, the systemic administration of a high quantity of anti-tumor substances determines the exposure of the whole body, chemotherapeutic substances having numerous adverse effects such as cardiotoxicity, hepatotoxicity, myelosuppression, nephrotoxicity, etc. [25].

Mejías *et al.* evaluated *in vivo* the effect of DMA-MNPs used as controlled delivery agents for anti-tumorigen cytokine IFN-γ. The MNPs targeting consisted in magnetic guidance by an external AMF. The biodistribution of the designed MNPs was evaluated before and after the functionalizing with the active agent. Also, the team evaluated the accumulation efficiency at tumor site, the IFN-γ delivery efficiency at the targeted tissue and the effects regarding the tumor evolution. An increased accumulation of IFN-γ-DMSA-MNPs was observed at the tumor site, which determined an infiltration of T-cells and macrophages, promoting an anti-angiogenic effect. The combination of phenomena determined a reduction of tumor dimensions. The experiment used 16 C57BL/6 female mice (12-week-old), divided in control group (injected with phosphate buffered saline – PBS) and other groups that received injections with DMSA-MNPs (300 μg Fe/injection) and respectively IFN-γ-DMSA-MNPs (10 000 U IFN-γ + 300 μg Fe/injection). Some of the groups were exposed to an AMF of 0.4 T for one hour, after the MNPs administration (the treatment was administered two times/week, for two weeks). After one hour from the last injection, the mice were euthanized in order to collect samples from spleen, liver, heart, brain, lungs, kidneys and blood. Five groups of 30 mice each were subcutaneous injected with murine pancreatic cancer cells Pan02 (2.5×10^6 in 100 μL PBS). After one week, when the tumors began to grow, the treatment was promoted. For three groups of nine mice, the tumor genesis was determined by administering 150 μg of 3-Methyl-colantren (MCA) in 0.2 mL corn oil, by injection in the right flank. The treatment was initialized after the tumors reached a volume of 200 mm^3 [26].

Zhang *et al.* (2011) investigated the effect of DNR (Daunorubicin)-Fe_3O_4 MNPs in nude mice (females, 6-week-old) bearing leukemia cells K562, respectively Adriamycin-resistant leukemia cells KA. The team observed suppression in tumor growth for the mice treated with DNR-Fe_3O_4, compared to control mice (untreated). Also, a dramatic growth of apoptotic rate was recorded for KA cells in mice treated with DNR-Fe_3O_4 MNPs, respectively for mice treated with Fe_3O_4 and DNR (separately), simultaneously with the application of an AMF [3].

Gao *et al.* evaluated the efficiency of scAb-PLGA-SPIO/Docetaxel by i.v. injection (50 mg Docetaxel/kg, five times, every three days) in BALB/c nude mice (males, 6–8-week-old), inoculated subcutaneous with antigen-positive cancer stem cells PC3M (3×10^6 cells/100 μL PBS). From the resulting experimental data, a total tumor regression was observed (initial volume was 300 mm^3) after 60–80 days of treatment and a considerably increased survival rate was obtained [19].

Other resembling microspheres were developed by Lv *et al.*, using MPEG-PLGA, to carry the anti-tumor

drug Evodiamine [27]. They evaluated the anti-tumor activity of the resulting system using Kunming mice (males, 20±2 g), inoculated subcutaneous with hepatoma cells H22 (0.2 mL having the concentration of 10^7 cells/mL). The administration of 5, respectively 10 mg/kg MNPs, was made by i.v. injection in tail vein, every two days, for a total period of 12 days. The obtained results showed an improvement of tumor growth suppression for mice treated with MPEG-PLGA-SPIONs-Evodiamine system, compared to the mice treated with Evodiamine, salt solution or MPEG-PLGA-SPIONs. The highest tumor suppression rate was recorded for the highest concentrations from the designed system, compared to Cyclophosphamide as positive control.

For the *in vivo* administration of Doxorubicin (DOX), a system based on Fe_3O_4-CA (citric acid)-chitosan encapsulated in PLGA polymeric microspheres, functionalized with cRGD (cyclo-Arg-Gly-Asp) was developed [28]. BALB/c mice (males, 18–22 g) were inoculated with murine sarcoma cells S-180 (2×10^6 cells/0.2 mL). The administration of the designed MNPs was made by i.v. injection in tail vein (2 mg/kg, every other day, for a period of 12 days); after that, the tumor site was exposed to an AMF for three hours (to facilitate the active targeting), then a laser beam was focused (1 cm diameter for 15 minutes) (to trigger the delivery of the drug in the acid media). The biodistribution and imaging studies proved a preferential accumulation of the designed MNPs in the tumor tissue, but also the inhibition potential of tumor growth and the reduced cardiotoxicity of DOX.

Long-term biodistribution evaluation of Fe_3O_4 MNPs

Regardless the applications of Fe_3O_4 MNPs, it is important to know what happens with the remaining nanoparticles after they fulfill their function. For this purpose, long-term biodistribution studies were elaborated.

Wang *et al.* used imprinting control region IPN-mice to evaluate the biodistribution of Fe_3O_4 MNPs. Forty-two mice were divided in several categories: 36 were divided in six groups for the i.g. (intragastric) administration of 600 mg/kg Fe_3O_4 MNPs and six mice (control group) for the i.g. administration of 0.5 mL SBF (sterile body fluid). Blood samples were harvested at 1, 3, 5, 6, 7 hours after the administration, respectively at 1, 3, 5, 7, 10 days. After 10 days, there were collected samples from the heart, liver, spleen, lungs, kidneys, bone marrow, brain, stomach, small intestine. All mice survived over the monitoring period, the highest MNPs concentration from the peripheral blood being noticed at six hours after the administration (439±28 μg/mL). A decrease of MNPs concentration in peripheral blood was noticed until the 5th day, when a new maximum took place (436±28 μg/mL), followed by a progressive lowering of the concentration. Regarding the MNPs distribution in different organs, an accumulation of Fe_3O_4 MNPs was noticed, the maximum values being reached at six hours after the administration for lungs and kidneys, one day for liver, brain, stomach and small intestine, respectively at three days for heart and spleen. The highest concentrations of MNPs were observed in liver (one-day post-administration) and spleen (three days post administration). Also, there is a narrow correlation between the modifications of MNPs concentrations in blood, respectively in the liver and the spleen, where the MNPs are transported through mononuclear macrophages. Thus, the achieving of the maximum concentration of MNPs in blood is followed by a decrease for the next period, simultaneously with the increasing concentration of MNPs in liver and spleen (at 1–3 days after the administration). Another maximum value of concentration in liver and spleen is noticed at seven days after the administration, which is correlated with the gradual senescence of red blood cells in those organs and with the poor absorption [29].

Mejías *et al.* used MNPs functionalized with DMSA to study its distribution, degradation and long-term toxicity from *in vivo* tests on C57BL/6 mice. For the biodistribution and MNPs transformation study, the team made magnetic susceptibility measurements using alternative current in order to distinguish the MNPs from other Fe species. For this purpose, 12 mice (females) were divided in three groups, which received five i.v. injections with PBS (control group) or five i.v. injections with DMSA-MNPs (15 mg Fe/kg/injection) (testing groups), in two weeks. After the administrations, there were harvested feces and urine samples, and at the end of the treatment period, the mice were euthanized in order to collect spleen liver, lungs, kidneys and blood samples. Histopathological test proved the accumulation of DMSA-MNPs in liver, lungs and spleen at different periods post-administration, but they were transformed in other non-toxic Fe species. A reduction of MNPs agglomerations was observed in liver and lungs, but some liver deposits present an increased dimension; this phenomenon can be due to the occurrence of phagocyte cells clusters in the liver parenchyma. The spleen sections presented several areas with spots in the red pulp (even in the control samples), due to the deposits of Fe degradation products, resulted after the phagocytosis of red blood cells and due to the presence of metallophilic macrophages. The fact that the AC susceptibility signal was reduced for the spleen samples, proved the fact that MNPs were transformed in other Fe species, which can be eliminated by physiological metabolic pathways. No other histopathological modifications were observed [30].

Tate *et al.* realized a study to evaluate the long-term biodistribution of dextran-Fe_3O_4 MNPs. The team used C3H mice (females, 6–10-week-old), which were administered 250 μL MNPs suspension by i.v. injection in tail vein (2 mg Fe/mouse). An equal PBS volume was administered to the control group mice. All animals were sacrificed at 14, respectively 580 days after the treatment and the main organs were harvested for histopathological examination. The 14-day examination revealed the apparition of MNPs deposits in liver and spleen, with modest vacuolization in the liver. The evaluation of hepatic enzymes proved no damage at hepatocellular lever. For 580-day group, a total elimination of MNPs elimination from spleen and liver was noticed, with no histopathological differences, compared with control group [15].

Clinical evaluation of Fe_3O_4 MNPs-based systems

There is a series of SPIONs that already have been approved by *Food and Drug Administration* (*FDA*) to be used as contrast agents in MRI imaging, such as: (1) Lumiren® for small intestine visualization [31]; (2) Feridex IV® for liver and spleen [32]; (3) Combidex® for metastatic lymphatic nodules [33]; (4) Resovist®, reticuloendothelial specific system, for liver lesions [34].

Combidex® [33] was proposed by Harisinghani *et al.* for the imaging of metastatic lymphatic nodules in prostate cancer. The clinical study was made on 80 patients in preoperative stages of prostate cancer (T1, T2 or T3). All patients were examined by MRI imaging 24 hours before the i.v. administration of SPIONs (2.6 mg Fe/kg body-weight) and after the treatment. The MRI results were correlated with histopathological examinations made after resection or biopsy. The proposed method correctly identified all of the patients with metastatic lymphatic nodules. The method proved a higher sensibility compared to MRI imaging with no contrast agent (90.5% comparatively with 35.4%, $p<0.001$).

Other clinical studies were made to prove the hyperthermia effect of SPIONs. Maier-Hauff *et al.* evaluated aminosilane-Fe_3O_4 MNPs on a 14 patients group with multiform glioblastoma, which were exposed to an AMF for intracranial hyperthermia treatment [35]. The same group [36] reported that MNPs mediated hyperthermia followed by radiotherapy assures a higher survival rate for patients with multiform glioblastoma, compared to the control group. The companies which apply MNPs mediated hyperthermia at the moment are MagForce [37] and Dr. Sennewald [38].

Conclusions and perspectives

The development of every Fe_3O_4 MNPs-based system with biomedical applications supposes a process of biological evaluation. Even if many aspects regarding this type of MNPs have already been understood, there are still a lot of insecurity reasons by means of Fe_3O_4 biocompatibility: (1) the small dimensions of these MNPs, but also the large quantities administered can lead to accumulation phenomena, which can determine the impossibility of metabolic elimination; (2) the forming of reactive oxygen species (ROS) can have an impact upon mitochondrial activity or upon the integrity of DNA; (3) the apparition of neurodegenerative diseases; (4) the accumulation of MNPs in organs like spleen, liver, kidney, lung, where they determine histopathological modifications, and (5) foreign body reactions. However, recent studies, which verify the functioning potential of Fe_3O_4 MNPs-based systems in applications, like MRI, magnetic hyperthermia and specific chemotherapeutic treatment, proved insignificant adverse effects that can be overcome by natural defense mechanisms of the human body. Also, there are many attempts to overcome the previously presented disadvantages of magnetite nanoparticles; these use functionalizing biocompatible polymers or surfactants and encapsulating systems to overcome the barriers of the human body and to improve the material biocompatibility. Considering all these studies, we can conclude that Fe_3O_4 MNPs are not cytotoxic at lower concentrations and the removal of MNPs can be made by normal metabolic pathways. Regarding the ROS forming, it can be considerably diminished by cellular defense mechanisms against free radicals. The targeting effect and the antitumor efficiency for the designed systems is valid and the accumulation in different organs (especially in liver and spleen) do not determine significant histopathological modifications, Fe_3O_4 MNPs being transformed in other Fe species, which have no superparamagnetic properties.

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Corresponding author
Alexandru Mihai Grumezescu, Chem. Eng., PhD, Department of Science and Engineering of Oxide Materials and Nanomaterials, Faculty of Applied Chemistry and Materials Science, Politehnica University of Bucharest, 1–7 Polizu Street, 011061 Bucharest, Romania; Phone +4021–402 39 97, e-mail: grumezescu@yahoo.com

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