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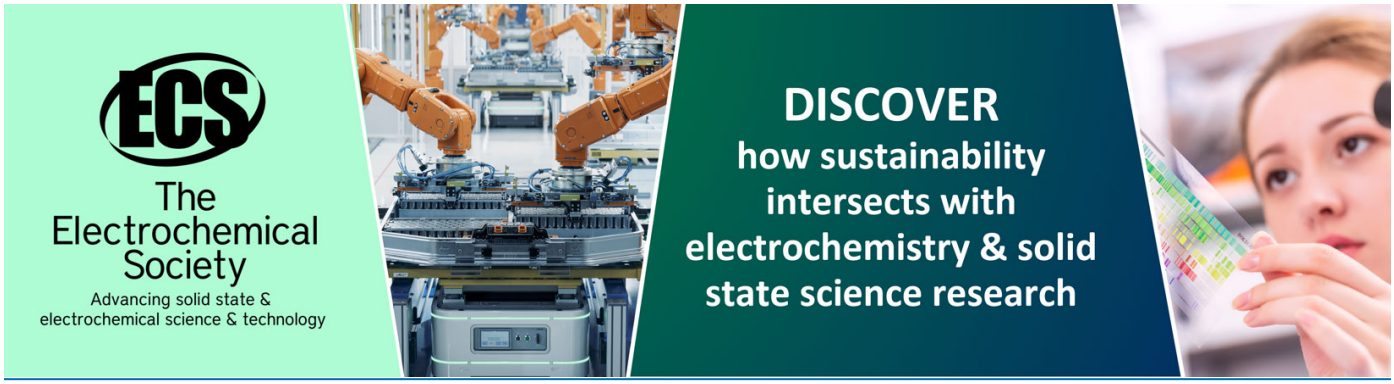

Superparamagnetic nanopreparations in early diagnostics and treatment of cancer

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Abstract. The superparamagnetic nanoparticles of magnetite citrate (SNMCs) consisting of nanospheres aggregates with the hydrodynamic diameter being between 15 to 35 nm with a magnetite core diameter of 3 to 12 nm, coated with of citrate anions was synthesized. A water 2-5% sol of SNMC was successfully tested as magnetic resonance imaging (MRI) contrast agent. The same 40% sol was then combined with Inox and used at regional magneto-thermo-chemo-therapy (RMTCT) of Lewis lung carcinoma (LLC) and Ehrlich carcinoma (EC), in female C57Bl / 6j mice. In the early stages of carcinoma development, the proliferation centers of LLC and EC cells were visualized and, during treatment, they obtained a significant increase in the efficiency of regional magneto-thermo-chemo-therapy.

1.Introduction

The aim of the study was the synthesis of superparamagnetic magnetite citrate nanoparticles (SNMCs), the assessment of early contrast enhanced MRI (CEMRI) visualization of the centers of proliferation of malignant cells (CPMC) by SNMC and combinations of the SNMC-Magnevist® [1 - 4]. CPMCs are formed during the latent period of development of malignant tumors [5].

The determination of anticancer activity (ACA) Inox during regional chemo-therapy (RCT), anticancer activity 40% of water sol SNMC during regional magneto-thermo-therapy (RMTT), and also a ACA combination 40% of water sol of superparamagnetic magnetite citrate nanoparticles-Inox (SNMC-Inox) at regional magneto-thermo-chemo-therapy (RMTCT) of Lewis lung carcinoma (LLC) and Ehrlich carcinoma (EC), in female C57Bl / 6j mice [5, 6]. Taking into account the instability to the oxidation of stoichiometric magnetite molecules, which leads to a decrease in the saturation magnetization (***M***s) the synthesis of activated magnetite was derived from nanocrystals with a diameter of 5 to 16 nm of fresh non-stoichiometric magnetite with a surplus of Fe^{2+} cations. Process of the synthesis of activated superparamagnetic non-stoichiometric magnetite nanoparticles were performed by stirring of water sol of cristals of non-stoichiometric magnetite from 500 to 1000 rpm from 2 to 3 h with 36% hydrochloric acid. Get activated superparamagnetic nanoparticles with a diameter of from 3 to 13 nm, of the formula $(Fe^{2+}_{2.1}O_3Fe^{3+}_{3.9}O_5)m(Fe^{3+}OCl)n$ (**1**), where: differing in the structure of spinel a rounded shape, and a surface consisting of Cl^- anions, protecting them from sticking. The coating of nanoparticles of the general formula (1), with citrate anions by replacing Cl^- anions is carried out at +

80 - + 90 ° C and stirring from 500 to 1000 rpm, pH from 3.5 to 6.9, until the formation of an aqueous sol of the compound general formula $(Fe^{2+}_{2.1}O_3Fe^{3+}_{3.9}O_4)m\cdot(Fe^{3+}O)n\cdot(C_6H_7O_7)r$ (**2**), where: m from 30 to 90; n from 40 to 60; r from 40 to 60, consisting of nanospheres aggregates with the hydrodynamic diameter being between 15 - 35 nm with a magnetite core diameter of 3 to 12 nm, coated with of citrate anions [5]. Its specific saturation magnetization (σ) value was 18 $A\cdot m^2$ / kg, LD_{50} 1.3 g / kg. The 40% SNMC magnetic sol appeared as dark-brown solution ***pH*** 7.4, ***M***s ranged from 4.3 kA / m to 8.9 kA / m, zeta-potential (ζ) - 40 ± 5 mV; relaxivity 4550 ± 90 ml $mg^{-1}s^{-1}$, the specific power absorption rate (***SAR***) 230 W / g Fe, produced heat up to 0.5 ºC / mg Fe per minute; Fe content 7.6%. Transmission electron microscopy of SNMC was performed using an EM-400, resolution 1.4 Å (Philips) at 300000x magnification. The images were analyzed by a Contron SEM IPS image analyzer.

2. Materials and methods

90 female mice C57Bl/6j that were 6-10 weeks old and weight 18-20 g was prepared and distributed by laboratory of experimental biology. To prepare CPMC-bearing mice, the solid malignant cells form of The Ehrlich carcinoma (EC) was implanted subcutaneously into the right femoral region of 30 female C57Bl/6j mice injection of 10^6 viable CPMC cells in 0.1 ml of saline at pH 7.4. Lewis lungs carcinoma was implanted into the femoral muscle of 5 mice and 39 mice were implanted subcutaneously into the right femoral region. 30 mice with Ehrlich carcinoma and 44 mice with Lewis lung carcinoma underwent the first, non-enhanced MRI with T1-weighted (W) {500/15 [repetition time m sec/echo time m sec]} and T2 W (1900/80) spin-echo and T2 W gradient-echo (GRE) (500/15) sequences. From 6 to 15 minutes before the second MRI measurement the mice received 4-10 µl of Magnevist® into their caudal vein and T2 W GRE sequences were performed. Signal intensity increase was measured, and visual analysis was performed. From 24 to 30 hours before the third MRI measurement 0.01-1.0 ml of SNMC were injected in the mice' caudal vein and SNMC-enhanced MRI was performed by measuring the decrease of the signal intensity [5, 6].

Pathomorphological analyses were also performed of the small pieces of tissues from C57Bl/6j mice, which were expected to contain the metastases. The analyses results were compared to the MRI findings and RMTCT planning was modified accordingly.

After inoculation of 10^6 malignant cells (MC) to mice on the basis of SNMC and Magnevist® according to the method developed by us: after intravenous administration of the combination of SNMC-Magnevist® for the first time, an early (after 3 days) contrast magnetic resonance imaging was performed visualization of MR images of the development of the CPMC. Malignant cells that endocytized SNMCs continued to proliferate, each of the daughter cells contained ~ 1/2 maternal SNMCs. We tested the SNMC as a negative contrast MRI agent. To evaluate the contrasting MRI properties of SNMCs, 2 ml / kg of 5% aqueous sol SNMC or a combination of SNMC-Magnevist® was intravenously injected into caudal vein. During the early investigations of SNMC [5, 6], it was also found that SNMC enhances the potential for metastases detection by enhancing contrast in magnetic resonance imaging (MRI). Since the detection of metastases is a huge problem in the early detection of invasive CPMC, we also investigated the usefulness of SNMC for the detection of metastases and small CPMC which cannot be palpated yet by physical examination. The second aim of this paper is thus to evaluate SNMC for metastases detection by MRI and comparing it to other currently used contrast agents.

Malignant cells take up magnetic nanoparticles as present in SNMC sol in high concentrations. Based on these observations, we explore in this paper the possibilities of treatment optimization of CPMC and metastases, of the Ehrlich carcinoma (EC) and the Lewis lung carcinoma (LLC) in C57Bl/6j mice. One part of this treatment optimization includes the use of CPMC necrotic slime aspiration. During the treatment interaction of the Inox magnetic sol with the CPMC cells, the destroyed CPMC tissue turns into a slimy, liquid substance, which mast then removed by aspiration.

The magnetite nanoparticle cores had a diameter of between 6 to 13 nm, with the hydrodynamic diameter being between 15 to 35 nm. The $\boldsymbol{M_S}$ of the evaluated Inox containing magnetic sol was from 7.8 to 8.2 kA / m. The samples of Inox 10 mg containing SNMC 4 ml 40% sol were placed inside a 60x200 mm air-cooled inductor with a matching high-Q-resonator fed with RF power (the device was

developed in the All-Russian Radio-technical Research Institute and operated at ***f*** 0.88 MHz). The sample temperature was monitored using an organic liquid thermometer. We tested a 2% sol of SNMC for contrast enhanced MRI; 10% water solution of Inox; 40% water sol SNMC; combination of 10% water solution of Inox containing 40% water sol SNMC for its anticancer properties. In order to prepare 10% water solution of Inox, containing 40% water sol SNMC a water 20% solution of Inox was mixed with SNMC in appropriate proportions immediately before injection.

The determination of ACA Inox during RCT, anticancer activity 40% of the SNMC sol during RMTT, and also a ACA combination of 40% sol (SNMC-Inox) at RMTCT of Lewis lung carcinoma (LLC) and Ehrlich carcinoma (EC), in female C57Bl / 6j mice [5, 6]. The determination of ACA was performed in 48 mice with LLC separated into four groups (1, 2, 3, and 4). The mice CPMC volumes ranged from 12.0 to 14.0 mm^3. The first control group (12 mice) received only 0.2 ml saline injection. 6 mice from each of the 2^{nd} group received of Inox; 6 mice from 3^{ed} group received of SNMC 40% water sol and 4^{th} group received of SNMC 80 mg, Inox 10 mg injected into multiple CPMC sites and adjacent tissues. Simultaneously with the injections there was a non-uniform magnetic field applied from a $SmCo_5$ magnet (50x50x20 mm, 0.2 T induction, and gradient 0.015 T/cm) in order to concentrate the nanoparticles in the desired region CPMC.

For anesthesia of animals injected intraperitonealy, in sterile conditions, 0.01 g/kg «Zoletil 100», (Virbac). Mice were placed in a field of 7 T, "BioSpec BC 70/30 USR biospectrograph" ("Bruker"). SNMC tissue concentrations were determined by mice MRI mapping. The malignant cells inoculation location was scanned in T1 weighted modes (W) {600/15 [repetition time, ms / echo time, ms], T2 W (1950/85) spin echo, T2 W gradient echo (600/13) and T*2 W gradient echo (550/15)} image. Early contrasting MRI images of a CPMC coated with a multi-layered capsule membrane were visualized at the site of inoculation of the malignant cells. The sizes of the CPMS were determined by the formula: $V = ZP \cdot 2 (ZT + M)$ (**3**). The volume of the CPMS was 12 ± 4.0 mm^3.

Within 6-7 hours 6 mice from 3^{rd} group were placed inside a 60x200 mm air-cooled inductor with RF power for 30 min of RMTT and 6 mice of group 4 for RMTCT. SNMC-cell interactions were investigated by MTT assay. Nanoparticle-cell thermo-interactions were carried out 3 times for 30 minutes every 3 days in the group 3, and thermo-chemo-interactions 3 times for 30 minutes every 3 days in the group 4. Moreover, in groups 3 nanoparticles-cells thermo-interactions and in groups 4 nanoparticles-cells thermo-chemo-interaction was associated with CPMC necrotic slime aspiration. This slime is toxic, and it was thus aspirated in groups 3 and 4.

In an additional group of 9 mice with metastases, 0.2 ml of a solution containing 0.1 mg cyclophosphamide was injected into the caudal vein 3-6 days after thermo-chemo-therapy was carried out.

30 mice with EC CPMC volumes ranging from 36.0 to 41.0 mm^3 were selected and separated into four groups with either 12 (group 1') or 6 mice each (groups 2', 3' and 4'). The above experiment was repeated with mice of the 1', 2', and 3' groups. Thermo-chemo-therapy associated with CPMC necrotic slime aspiration was carried out 3 times for 30 min every 3 days in group 4'.

3. Results and discussion

The SNMC-enhanced MRI detected the metastases of LLC in the lymph nodes in 6 mice from 20 with CPMC from 21 to 34 mm^3. Besides, it made feasible the detection of metastases of the LLC in the backbone, kidney and urinary bladder of 5 mice (figure 1), and metastases of the LLC in the lungs, spleen, liver and urine bladder of 25 mice. It must be mentioned here that such micrometastases were not detected with non-enhanced or Magnevist®-enhanced MRI.

From 24 to 30 hours after intravenous injection of SNMC a strong enhancement of MRI and the metastases was clearly detected: in positive CPMC, as clear spots on dark background and with negative CPMC as inverse spots. The pathomorphological and histopathological analyses of lymph nodes and other organs revealed the presence of metastases. SNMC-enhanced MRI was thus the method of choice for the differefigurentiation of benign and malignant lymph nodes and the metastases of the rest organs. From 30 to 60% of SNMC and from 15 to 36% of Inox were targeted in the CPMC tissue after the first

injection and nanoparticle-cell thermo-chemo-interaction in groups 4, 4'. SNMC particles aggregates accumulated in CPMC tissue were visible in Prussian blue stained sections.

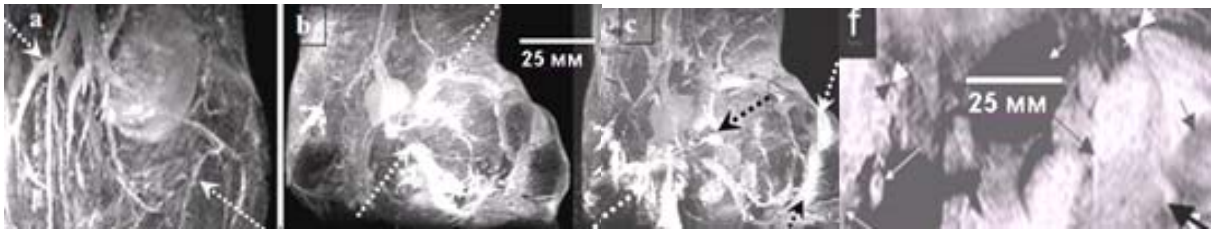

Figure 1. MR image (a, b, c, f). Angio mode. 4 to 7 days after inoculation of 10^6 LLC cells into the muscle of the thigh of mice, contrast MR images of the vessels feeding the CPMC are visualized: (a) a network of malignant vessels and capillaries feeding the CPMC, covered with a membrane (between dashed arrows); (b) enlarged maligniti vessels that feed the CPMC (between dashed arrows); (c) the bright bands emerging from the tumor are invasions of malignant cords and cells into normal tissues (between black and white dotted arrows); 14 - 17 days after inoculation of 10^6 LLC cells into the muscle of the thigh of mice, (f) invasion of malignant cells in the bladder (white thin arrow), start of invasion of the lungs and liver (between the ends of the white arrows), CPMC of LLC at the site of the invasion (between black arrows).

A temperature of 45 ºC for groups 3, 4, 3', 4' was reached after 15 minutes of hyperthermia treatment when an alternating current (AC) magnetic field (0.88 MHz, 7.3 kA/m, 150 W) was used.

RMTCT treatment demonstrated significant CPMC response over 36 days in groups 3 and 4 and in groups 3' and 4' comparatively to control groups 1 and 1' or to treatment in groups 2 and 2'. The complete CPMC (~13 mm^3) regression in C57Bl / 6j mice before metastases appeared was 30% and an increase in life span of 280% was achieved. For larger CPMC of ~50 mm^3, the increase in life span was slightly lower at 60% ($\rho>0.05$).

All experimental results are represented as mean ± standard deviation. Statistical analysis was done as described in [7].

4. Conclusions

SNMC-enhanced MRI was able to visualize the metastases in lymph nodes, backbone, brain, liver, spleen, urine bladder, throat, lungs, kidney and rectum which were not palpable upon physical examination and could not be visualized using the non-enhanced MRI. SNMC-enhanced contrast in MRI thus has a considerable effect on the planning of therapy intensity. The advantages of the presented regional thermo-chemo-therapy in combination with CPMC necrotic slime aspiration and regional Inox chemo-therapy were confirmed and resulted in increased life spans of mice with CPMC.

Aqueous 40% sol of the SNMC was tested as structured by a 0.2 T heterogeneous permanent magnetic field (HPMF), a gradient of 0.001 T / cm magnetic carrier of ACA, retention Inox in the spaces that was formed by the SNMC in HPMF. Moreover, aqueous 40% sol of the SNMC was tested as an interstitial nano-heat-generator operating at 0.88 MHz, 7.3 kA / m, 150 W. The concentration of water sol of SNMC from 0.1 to 50%, specific energy absorption was from 230 to 270 W / g Fe. In the radio frequency fields, an aqueous 40% sol of SNMC heated from + 37 to + 45 ºC for 15-20 minutes.

Inox is active against both resting and rapidly dividing malignant cells. It has been found that Inox’s activity can be increased by combining it with a water-based SNMC magnetic sol. In addition, SNMC can be used to an alternating current (AC) locally heat CPMC by externally applying AC magnetic field. We call this therapy that combines hyperthermia and chemotherapy, RMTCT and are working on making it available to the treatment of CPMC and metastases. Inox showed in vitro cytotoxicity and ACA with I / V injection in doses from 30 to 100.0 mg / kg. RMTT was performed on 6 mice of 3 group; regional chemo-therapy on 6 mice of 2 group and regional magneto-thermo-chemo-therapy on 6 mice

of 4 group with LLC.

We have developed a combination of a 40% aqueous sol of SNMC-Inox, which in mice caused a decrease in the volume of CPMC to 1.0 ± 0.3 mm^3 in RMTCT, increased the lifespan of mice by 280%. In all groups of mice the life time was compared, mean ± standard deviation ($\rho \leq 0.05$). In the early stages of CPMC development, the proliferation centers of Lewis lung carcinoma and Ehrlich carcinoma cells were visualized and, during treatment, they obtained a significant increase in the efficiency of regional magneto-thermo-chemo-therapy.

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