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Magnetic Resonance Imaging of Endothelial Cells with Vectorized Iron Oxide Nanoparticles

M. A. Abakumov, A. E. Goldt*, M. Sokolsky-Papkov, Y. A. Zorkina, V. P. Baklaushev, E. A. Goodilin*, A. V. Kabanov**, and V. P. Chekhonin**

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We propose a method for obtaining superparamagnetic nanoparticles based on iron oxide and their water suspensions. The structure and size of nanoparticles were confirmed by transmission electron microscopy, dynamic light scattering, and X-ray diffraction analysis. The nanoparticles also contained a fluorescent dye Dil C18. Cytotoxicity of obtained aqueous suspension was studied by MTT assay; low toxicity of nanoparticles was demonstrated. High T2-relaxivity of nanoparticles allows using them as a contrast agent for MRI. After incubation of cerebellar sections with nanoparticles vectorized with antibodies to antigen AMVB1, specific visualization of blood vessels was detected.

Key Words: *MRI contrast; superparamagnetic nanoparticles; vector delivery*

Superparamagnetic iron oxide nanoparticles (MNP) due to their high specific magnetization and great variety of possible coatings are a promising material for developing tumor-selective contrast agents for MRI [12], as well as different constructions based on MNP for targeted drug delivery [4] or antitumor therapy by local hyperthermia [5].

Colloidal solutions of pure iron nanoparticles are extremely unstable, are easily oxidized on air, and are pyrophoric in dry state, therefore nanoparticles Fe_3O_4 or γ-Fe_2O_3 with similar magnetic properties are often used. Due to low stability of colloidal solutions of iron oxide nanoparticles, their synthesis often includes modification of nanoparticle surface with agents preventing aggregation: macromolecular (dextran [9], chitosan [7], polyethylene glycol (PEG) [6], and pluronics) and low-molecular-weight substances phosphatidylcholine and sodium citrate [14]) are used for this purpose. Since adsorption of molecules is mediated by ionic and electrostatic interactions strictly depending on medium pH and ionic strength, these coatings in most cases can not provide sufficient stability in physiological media. In addition, covalent bonding of the vector or transported molecules to these ligands is often difficult due to the absence of readily available active groups (carboxyl, amine, *etc*.).

For obtaining stable aqueous colloidal solutions, post-synthetic modification of the surface is usually necessary. One of the frequently used approaches is modification of hydrophobic nanoparticles with various amphiphilic pegylated polymers. This approach provides micelle-like structures with nanoparticles internalized inside and protected by PEG crown. The

Department of Medical Nanobiotechnology, Medicobiological Faculty, Russian State Medical University, Ministry of Health Care and Social Development of the Russian Federation, Moscow; *Faculty of Materials Science, M. V. Lomonosov Moscow State University, Russia; **Center for Drug Delivery and Nanomedicine, University of Nebraska Medical Center, Omaha, Nebraska, USA. ***Address for correspondence:*** abakumov1988@gmail.com. M. A. Abakumov

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use of bifunctional PEG molecules allows introduction of any functional group into the structure of these particles for further modifications.

It is well known that poorly differentiated tumors are characterized by high density of blood vessels [1]. This can be explained by active secretion of proangiogenic growth factors (VEGF, EGF) by tumor cells [1]. In the endothelial layer of tumor capillaries, fenestrae 500 nm and more in size are often present. In case of brain tumors, this leads to local violation of the blood-brain barrier integrity. Consequently, macromolecular compounds and large (150-200 nm) nanoparticles can directly penetrate into the interstitial fluid of the tumor through the intercellular spaces and fenestrae bypassing the existing transendothelial transport system. This feature of tumor vasculature makes it possible to use nanoparticles with immobilized antibodies to tumor-specific or endothelium-specific antigens for tumor diagnosis and therapy. One of these antigens is endothelial abluminal membrane antigen AMVB1, identified and described at the Laboratory of Immunochemistry, V. P. Serbskii Research Center for Social and Forensic Psychiatry [2]. This antigen is normally expressed in endothelial cells of the spleen and cerebellar vessels. It is overexpressed in endothelial vascular cells of primary brain tumors [2]. Conjugation of nanoparticles with monoclonal antibodies to AMVB1 offers great opportunities of highly specific tumor visualization, which undoubtedly will facilitate early diagnosis and control over the efficiency of therapy.

The purpose of this study was to obtain a stable aqueous suspension of superparamagnetic vector-oriented biocompatible nanoparticles on the basis of iron oxide.

MATERIALS AND METHODS

Synthesis of magnetic nanoparticles was performed as described in Park's group report [11]. Synthesis of polymer PMAO-PEG was carried out by the method described previously [15]. To obtain stable aqueous suspension, 4 mg DSPE-PEG (distearoylphosphoethanolamine-[methoxy(polyethylene glycol-2000)] or 4 mg of PMAO-PEG, graft copolymer of poly(maleic anhydride-alt-1-octadecane) and methoxy poly(ethylene) glycol 5000 in 1 ml of chloroform, was added to 0.5 ml nanoparticle suspension in chloroform. Then, 5 μl Dil C18 (1,1'-dioctadecyl-3,3,3',3'-tetra- methylindocarbocyanine perchlorate; 1 mg/ml) and 2 volumes of distilled water were added and evaporated on a rotary evaporator (Heidolph Laborota 4000) to complete removal of chloroform. The sample was then sonicated for 20 min at 4°C. The solution was filtered through a 0.22-μ filter. Iron concentration was measured [13]. Cytotoxicity for MCF7 cell culture was evaluated by MTT-assay [11]. The cells were incubated with MNP for 2 and 24 h, washed from the medium with particles, and incubated in the growth medium (DMEM) for 3 days; then, MTT-assay was carried out. Optical density of cell lysate was measured on a Victor X3 plate reader (Perkin Elmer).

The obtained nanoparticles were examined by transmission electron microscopy (TEM) with Phillips 410LS microscope and by dynamic light scattering method (Zetasizer Nano series, Malvern). Relaxivity of MNP was measured by NMR relaxometer (Bruker).

Conjugation of nanoparticles with Mab2B6 antibodies. Water-soluble carbodiimide [N-(3-dimethylaminopropyl)-N-ethylcarbo-diimide hydrochloride; 375 μl, 2.4 mg/ml] and 82 μl water at pH 5.0 were added to 1 ml of nanoparticles solution in water. The mixture was incubated for 30 min at room temperature and then 1375 μl Mab2B6 antibody solution was added (1 mg in 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$, pH 8.6). Vector nanoparticles and unbound antibodies were separated by gel chromatography on Sepharose CL-4B column.

Immunofluorescence analysis of nanoparticles conjugated with monoclonal antibodies to AMVB1 was carried out on 40-μ cryosections of the cerebellum. Nonspecific binding sites of antibodies were blocked by 30-min incubation of sections with 10% equine serum, then MNP-PMAO-PEG conjugated with antibodies and containing fluorescent dye Dil C18 in the hydrophobic inner layer was added in dilutions 1:1000-1:100,000 and incubated for 2 h at room temperature. Similar dilutions of MNP-PMAO-PEG without antibodies to AMVB1 were used as the control. The sections were examined under a Leica DMI 6000 B fluorescent microscope.

RESULTS

Relaxivity, *i.e.* ability to modify the relaxation time of protons, is one of the most important properties of contrast agents. Iron oxide nanoparticles are T2-contrast agents, as by own magnetization in an external magnetic field they create its local heterogeneity thereby reducing the time of T2 relaxation. Particle with a size ≤10 nm are characterized by maximum relaxivity [7]. In our study, particle size was measured by TEM.

The synthesized iron oxide nanoparticles had mean diameter of 7±1 nm (according to TEM; Fig. 1).

To stabilize aqueous colloidal solution of particles, two types of coatings based on DSPE-PEG and PMAO-PEG were used. Coated particles formed a stable aqueous colloidal solution. The mean particle size measured by dynamic light scattering was 108±30 nm for

DSPE-PEG and 131±30 nm for PMAO-PEG. This can be explained by the formation a colloidal solution containing not only individual nanoparticles with a diameter of 7±1 nm, but also their conglomerates consisting of a core formed by nanoparticles and a hydrophilic "crown" formed by PEG. It should be noted that both DSPE-PEG and PMAO-PEG coating yielded stable colloids, but in the case of stabilization with DSPE-PEG the particles were smaller and more homogenous; their polydispersity index was 0.126 (*vs.* 0.22 for PMAO-PEG-coated MNP).

Before relaxivity measurement, the samples were standardized by iron content, which is directly proportional to the number of magnetic centers. Iron content was measured colorimetrically using ferrozine. T2-relaxivity values were 1480±60 ml/(mg/sec^{-1}) for particles coated with DSPE-PEG, and 1370±55 ml/(mg/sec^{-1}) for particles coated with PMAO-PEG. These data suggest that the studied particles have relatively high T2-relaxivity and can be used as MRI-contrast. We present the dependence of R2, inversely proportional to the relaxation time of protons T2, on the concentration of particles, normalized to iron content (Fig. 2).

Biocompatibility is another key characteristics (apart from relaxivity) of any contrast agent intended for clinical use. Evaluation of cytotoxicity of the obtained nanoparticles by MTT test showed high viability of cells incubated with particles coated with both PMAO-PEG and DSPE-PEG (Fig. 3). The particles coated with PMAO-PEG were 20-fold less toxic than particles coated with DSPE-PEG. In the first case, high cell viability (80%) was observed up to nanoparticle concentration of 20 mg/ml, while toxicity of MNP-DSPE-PEG was noted at a concentration of 1 mg/ml. The higher toxicity of DSPE-PEG coating can explained by the presence of lipid fragment in the molecule, which probably increases membranotropic properties of these particles.

Evaluation of the specificity of magnetic nanoparticles vectorized with monoclonal antibodies carried out *ex vivo* on frozen sections of the cerebellum demonstrated their high specificity for endothelial antigen AMVB1. Specific visualization of blood vessels indicating interaction of antibodies on the surface of the nanoparticles with the target antigen exposed on the membrane of endothelial cells was observed up to nanoparticle dilution of 1:100,000 (Fig. 4).

Thus, low-toxic biocompatible iron oxide nanoparticles with high relaxivity were obtained, that can be used as T2 contrast agent for MRI. Immunofluorescence detection of AMVB1 with nanoparticles conjugated with the corresponding antibodies showed that the antibodies retain their immunochemical activity after conjugation and can be used for visualization of

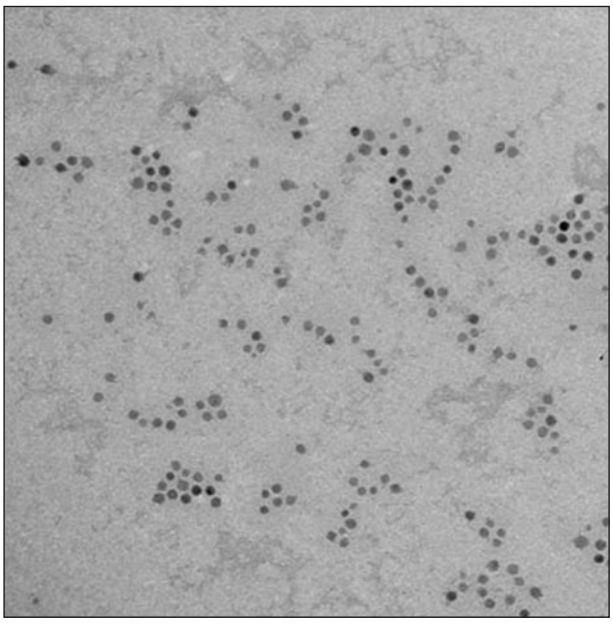

Fig. 1. Aqueous suspension of iron oxide nanoparticles obtained by decomposition of iron pentacarbonyl. Scale 100 nm.

AMVB1$^+$-endothelial cells. According to our previous findings, the antigen AMVB1 is most actively produced in pathological microvessels of glial tumors [2]. Since this antigen is present only on the abluminal surface of the endothelium, it is inaccessible for antibodies entering the bloodstream with intact blood-brain barrier. Hence, we can assume with high probability that magnetic nanoparticles with immobilized antibodies to AMVB1 will selectively visualize tumor microvessels characterized by increased permeability. The use of these targeted magnetic nanoparticles as MRI-contrast agents can greatly increase the impor-

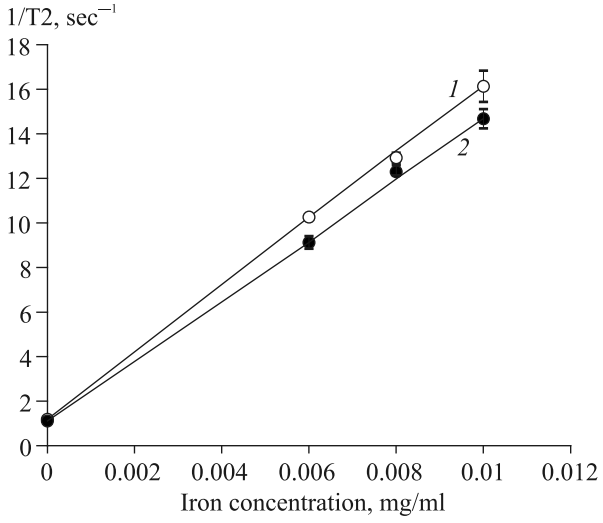

Fig. 2. Relaxivity of iron oxide nanoparticles coated with DSPE-PEG (MNP-DSPE-PEG; *1*) and PMAO-PEG (MNP-PMAO-PEG; *2*).

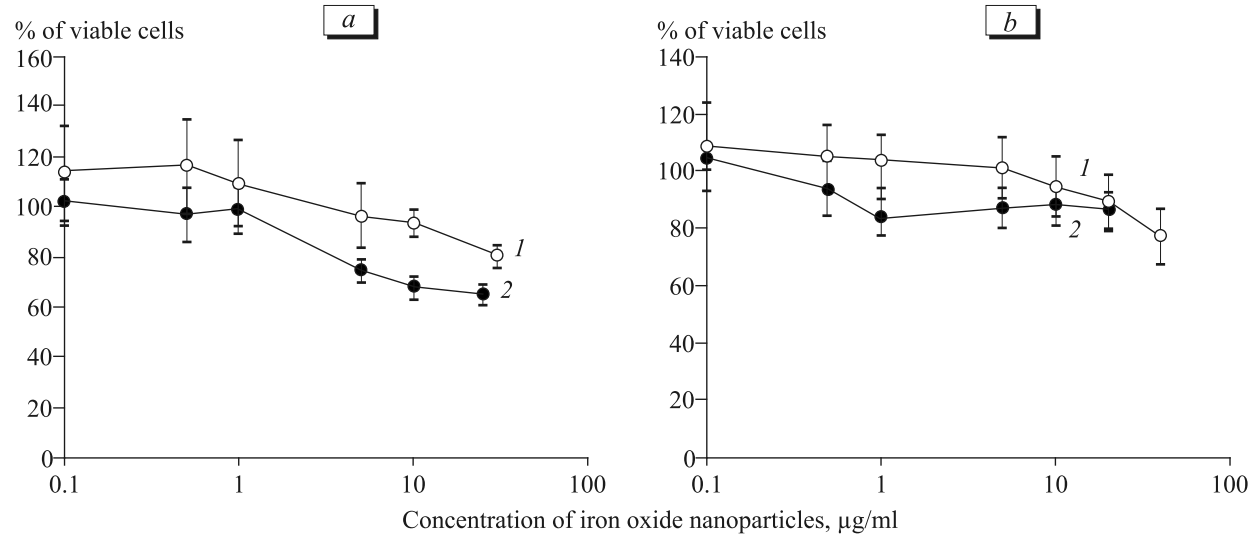

Fig. 3. Cytotoxicity of iron oxide nanoparticles coated with DSPE-PEG (*a*) and PMAO-PEG (*b*) against cultured breast carcinoma MCF7 cells after 2- (*1*) and 24-h (*2*) incubation.

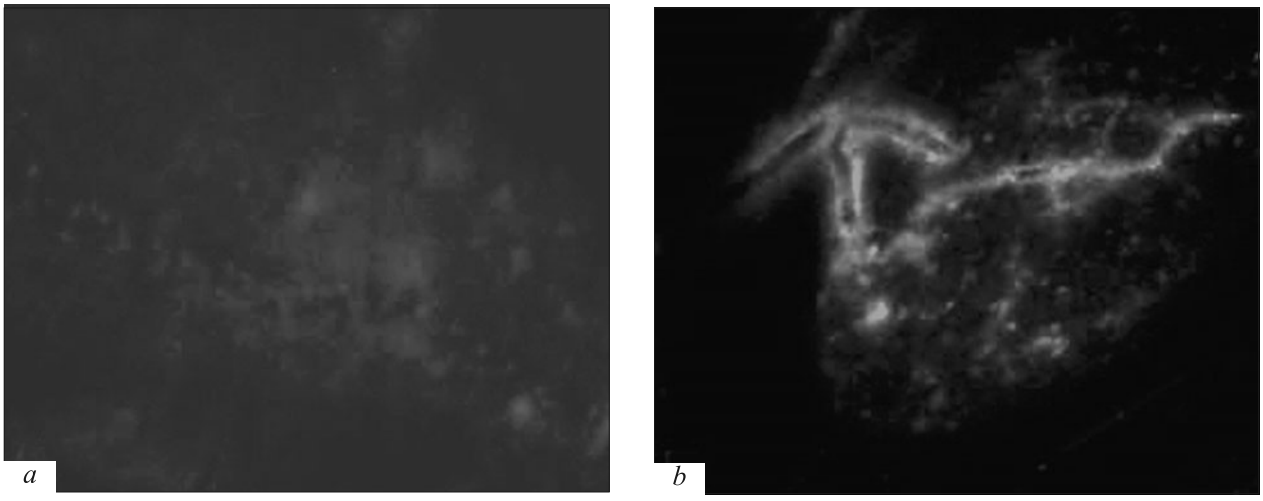

Fig. 4. Immunofluorescent visualization of cerebellar microvessels using vector iron oxide nanoparticles with immobilized antibodies to endothelial antigen AMVB1, ×200. *a*) nanoparticles not conjugated with antibodies to AMVB1; *b*) nanoparticles conjugated with antibodies to AMVB1.

tance of MRI-studies in the early diagnosis of poorly differentiated glial tumors.

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