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Colloidally stable surface-modified iron oxide nanoparticles: Preparation, characterization and anti-tumor activity

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ABSTRACT

Maghemite (γ-Fe_2O_3) nanoparticles were obtained by co-precipitation of Fe(II) and Fe(III) chlorides and subsequent oxidation with sodium hypochlorite and coated with poly(*N*,*N*-dimethylacrylamide-*co*-acrylic acid) [P(DMAAm-AA)]. They were characterized by a range of methods including transmission electron microscopy (TEM), elemental analysis, dynamic light scattering (DLS) and zeta potential measurements. The effect of superparamagnetic P(DMAAm-AA)-γ-Fe_2O_3 nanoparticles on oxidation of blood lipids, glutathione and proteins in blood serum was detected using 2-thiobarbituric acid and the ThioGlo fluorophore. Finally, mice received magnetic nanoparticles administered *per os* and the antitumor activity of the particles was tested on Lewis lung carcinoma (LLC) in male mice line C57BL/6 as an experimental *in vivo* metastatic tumor model; the tumor size was measured and the number of metastases in lungs was determined. Surface-modified γ-Fe_2O_3 nanoparticles showed higher antitumor and antimetastatic activities than commercial $CuFe_2O_4$ particles and the conventional antitumor agent cisplatin.

1. Introduction

Iron oxide nanoparticles show many potential biomedical applications, for instance, in targeted drug delivery and controlled drug release [1], especially in preclinical and clinical oncology as contrast agents for magnetic resonance imaging (MRI) [2], in specific cell labeling and separation, hyperthermia [3], or biocatalysis [4]. Both magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) nanoparticles are often used in these applications. They have to fulfill a range of requirements including a spherical shape, appropriate diameter with a narrow particle size distribution, high saturation magnetization, the presence of functional groups suitable for attachment of target biomolecules and minimal non-specific adsorption [5].

Magnetic iron oxide nanoparticles can be prepared by a number of methods, *e.g.*, alkaline co-precipitation of iron salts [6], thermal decomposition of organometallic precursors [7–11] or hydrothermal process [12]. Since the surface of superparamagnetic nanoparticles plays a key role in their prospective specific application, many reports focus on different modifications of iron oxides [13–17]. Recently, several reports described nanoparticles capable of inducing production of reactive oxygen species (ROS) leading to oxidative stress and cytotoxicity [18]. Examples of such particles include titanium dioxide, carbon black, polystyrene and cobalt-chromium alloy [19]. Cytotoxic response of cancer cells to iron oxide nanoparticles was also investigated [13].

We are reporting here a simple preparation of water-dispersible magnetic iron oxide nanoparticles coated with poly[*N*,*N*-dimethylacrylamide-*co*-(acrylic acid)] P(DMAAm-AA) which is suitable for subsequent modification of the particles. Preliminary model biological experiments are also described envisaging application of such particles in cancer treatment.

2. Methods

2.1. Materials

$FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, *N*,*N*-dimethylacrylamide (DMAAm), 2,2′-azobisisobutyronitrile (AIBN), NaCl, Tris, thiobarbituric acid,

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trichloroacetic acid and copper iron oxide ($CuFe_2O_4$), cell cultivation medium 199 and *cis*-diaminoplatinum(II) dichloride (cisplatin) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium hypochlorite solution was from Bochemie (Bohumín, Czech Republic). Fluorescent ThioGlo thiol reagent was from Calbiochem (San Diego, CA, USA). Acrylic acid (AA) was from Hexion Specialty Chemicals (Sokolov, Czech Republic), other reagents and solvents were from LachNer (Neratovice, Czech Republic). Ultrapure Q water ultrafiltered in a Milli-Q Gradient A10 system (Millipore, Molsheim, France) was used for the preparation of solutions.

2.2. Preparation of γ-Fe_2O_3 nanoparticles modified with poly(N,N-dimethylacrylamide-co-acrylic acid) [DMAAm-γ-Fe_2O_3]

γ-Fe_2O_3 nanoparticles were prepared by alkaline co-precipitation of $FeCl_2$ and $FeCl_3$ (1:2 mole ratio) in aqueous NH_3 solution according to a method described earlier [6]. *N,N*-Dimethylacrylamide (3 g) and acrylic acid (0.33 g) were dissolved in a mixture of toluene (3.5 ml) and THF (3.4 ml) and AIBN initiator (10 mg) was added. Polymerization was performed at 70 °C for 8 h under magnetic stirring. The resulting P(DMAAm-AA) copolymer was precipitated into heptane and dried in vacuum (0.13 Pa). Finally, a solution of P(DMAAm-AA) (5 mg) in water (1 ml) was added to a γ-Fe_2O_3 colloid (1 ml; 50 mg γ-Fe_2O_3/ml).

2.3. Blood serum preparation

Blood (~4 ml) was taken by heart puncture with a polyethylene syringe from intact 250–300 g Wistar rat immediately after euthanasia and transferred into a glass centrifuge tube. The tube was left standing at room temperature for 30 min. The clotted blood was than centrifuged for 15 min at 1500 *g*. The blood serum was collected from the supernatant and stored at −80 °C in polystyrene tubes.

2.4. Blood lipid oxidation

Blood serum (25 μl in 0.9% NaCl and 10 mM Tris, pH 7.4) containing certain amounts of nanoparticles was incubated at 37 °C for 24 h under shaking. Neat blood serum served as a control. Lipid oxidation was monitored by measuring of thiobarbituric acid reactive substances (TBARS), which are formed as a byproduct by the reaction of thiobarbituric acid and compounds resulting from the decomposition of polyunsaturated fatty acid lipid peroxides [20]. After incubation, a mixture of 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl was added to blood serum or malondialdehyde standards at 1:2 (v/v) ratio. The mixtures were incubated at 95 °C for 30 min and protein pellets were separated by centrifugation at 3000 *g* for 15 min. An aliquot was monitored at 540 nm using a MQX 200 BioTek spectrophotometer (Winooski, VT, USA).

2.5. Glutathione and blood protein oxidation

To determine the effect of nanoparticles on the oxidation of a non-enzymatic antioxidant glutathione and of blood protein, protein-oxidation markers were investigated *in vitro* in the absence or presence of nanoparticles using a commercial thiol-specific fluorophore ThioGlo [21, 22]. The incubation mixture contained blood serum (25 μl/ml), γ-Fe_2O_3 or P(DMAAm-AA)-γ-Fe_2O_3 or $CuFe_2O_4$ nanoparticles (10, 25 and 100 mg/l) in 0.9% NaCl solution and 10 mM Tris (pH 7.4). The mixtures were incubated at 37 °C for 24 h under shaking. In order to determine the activity of glutathione, aliquots of the nanoparticle suspensions were incubated in blood serum at 37 °C for 24 h under shaking (20 rpm) and the ThioGlo fluorophore was added to reach 10 μM concentration. To analyze blood protein oxidation, an aliquot of the nanoparticle suspension was treated with ThioGlo fluorophore in 2% sodium dodecyl sulfate solution for 30 min. Fluorescence was detected using a FLX 800 BioTek spectrofluorimeter at excitation and emission wavelengths 388 and 500 nm, respectively.

2.6. Anti-tumor and anti-metastatic activity of P(DMAAm-AA)-γ-Fe_2O_3 nanoparticles

Lewis lung carcinoma (LLC; a metastatic tumor model) in male mice line C57BL/6, body weight of 23 g, was used as an experimental *in vivo* model. 0.2 ml of LLC cells (2×10^6 cells per animal) were injected into femoral muscle in cell culture medium 199. Experimental animals were divided into three groups. First group did not receive any medication (except saline) and served as a tumor control. The second group received 1.2 mg of administered cisplatin per kg intraperitoneally, typically on day 11 after LLC transplantation, when the tumor size reached 2–3 mm. The third group received P(DMAAm-AA)-γ-Fe_2O_3 nanoparticles (30 mg/kg) *per os* into the stomach. In the second and third groups, the animals received six injections during 28 days. At the end of the experiment, the tumor size was measured and the number of metastases in lungs was calculated.

2.7. Ethical issues of animals use

All experiments involving small laboratory animals of non-transgenic strain, such as male mice (C57BL/6 strain), were performed according to the EC regulations on animal experimentation and after approval of the ethical committees of Palladin Institute of Biochemistry and R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology. The animals were housed in a specialized animal facility under appropriate conditions. Euthanasia of animals was performed by necropsy after asphyxiation with carbon dioxide.

2.8. Physicochemical and biological characterization

Morphology of the nanoparticles was investigated using a Tecnai Spirit G2 transmission electron microscope (TEM; FEI; Brno, Czech Republic). Number-average diameters (D_n), weight-average diameters (D_w) and polydispersity indexes PDI$=D_w/D_n$ were calculated using Atlas software (TESCAN Digital Microscopy Imaging, Brno, Czech Republic) by counting at least 500 particles on TEM microphotographs. The D_n and D_w can be expressed as follows: $D_n=\sum D_i/N$; $D_w=\sum D_i^4/\sum D_i^3$, where N is the number of particles. The hydrodynamic diameters D_h, polydispersities PI and zeta potentials were determined by dynamic light scattering (DLS) with an Autosizer Lo-C (Malvern Instruments; Malvern, UK). Elemental analyses were performed on a Perkin-Elmer 2400 CHN apparatus (Norwalk, CT, USA). Size exclusion chromatography (SEC) measurements were performed on a gradient Knauer system (Berlin, Germany) using diode array detection (DAD) and Alltech 3300 evaporative light scattering detection (ELSD). The measurements were performed on a Phenomenex PolySept-GFC-P linear column using an isocratic system of 0.03 M ammonium acetate buffer in CH_3CN/water (20:80).

Blood lipid oxidation analysis was made using a MQX 200 BioTek spectrophotometer (Winooski, VT, USA). Glutathione and blood protein oxidation assays were performed using a FLX 800 BioTek spectrofluorimeter.

Magnetic properties were measured using a SQUID MPMS5 magnetometer (Quantum Design, San Diego, CA, USA) at 300 K.