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Preparation of poly-L-lysine functionalized magnetic nanoparticles and their influence on viability of cancer cells

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ABSTRACT

This study was aimed at development of biocompatible amino-functionalized magnetic nanoparticles as carriers of specific antibodies able to detect and/or target cancer cells. Poly-L-lysine (PLL)-modified magnetic nanoparticle samples with different PLL/Fe_3O_4 content were prepared and tested to define the optimal PLL/Fe_3O_4 weight ratio. The samples were characterized for particle size and morphology (SEM, TEM and DLS), and surface properties (zeta potential measurements). The optimal PLL/Fe_3O_4 weight ratio of 1.0 based on both zeta potential and DLS measurements was in agreement with the UV/VIS measurements. Magnetic nanoparticles with the optimal PLL content were conjugated with antibody specific for the cancer biomarker carbonic anhydrase IX (CA IX), which is induced by hypoxia, a physiologic stress present in solid tumors and linked with aggressive tumor behavior. CA IX is localized on the cell surface with the antibody-binding epitope facing the extracellular space and is therefore suitable for antibody-based targeting of tumor cells. Here we showed that PLL/Fe_3O_4 magnetic nanoparticles exhibit cytotoxic activities in a cell type-dependent manner and bind to cells expressing CA IX when conjugated with the CA IX-specific antibody. These data support further investigations of the CA IX antibody-conjugated, magnetic field-guided/activated nanoparticles as tools in anticancer strategies.

1. Introduction

Recent advances in understanding mechanisms of cancer development and progression have facilitated development of new treatment modalities, strategies for patients' stratification and identification of novel promising molecular targets. The development of targeted therapy has now become more tailored to the individual patient and to specific tumor types. Personalized treatment can reduce side effects associated with current nonspecific cancer therapies. Sensitive tumor detection and effective targeted therapy are two important challenges in successful treatment of cancer patients.

Thus, combinations of the molecular approaches with new nanotechnologies and materials offer new opportunities for improvements of cancer therapy. Magnetic nanoparticles (MNPs) possess unique properties that make them highly attractive to medical applications.

The applicability of magnetic fluids (MFs) containing MNPs requires special properties of particles such as superparamagnetic behavior at room temperature, colloidal stability [1], which depends on the size of the MNPs and biocompatibility of polymer coating [2,3], respectively. Coating of the MNPs by biocompatible polymers (such as dextran or protein) [4] makes it possible to prevent the formation of large aggregates and to restrain the biodegradation caused by the influence of biological systems. The surface of the polymer also has adsorbing ability mediated by covalent attachments [5]. Excellent properties of magnetic fluids predispose them for diagnostic and therapeutic applications [6], e.g. as contrast agents for magnetic resonance imaging, for local magnetic hyperthermia treatment, or for targeted drug delivery with the potential use in oncology. Due to their properties, MNPs can be located at any desired place by localized magnetic field gradients, kept in place for as long as required by the particular therapy process, and consequently removed from the body [7].

These aspects of the MNPs utilization are undoubtedly highly positive, however, it is necessary to choose the optimal method of the synthesis of nanomaterials that determines the shape, size, and surface chemistry of the particles, and hence their magnetic properties.

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Moreover, most of these applications require well-dispersed biocompatible particles. One of the effective ways to prevent particle agglomeration and confer biocompatibility is coating of nanoparticles with polymers and/or other targeting agents.

Biocompatible Poly-L-Lysine (PLL), is a synthetic polymer composed of the positively charged amino acid lysine that is widely used in the pharmaceutical industry. Its sub-products, monomeric amino acid lysine units, are characterized by no toxicity, no antigenicity, good biocompatibility and biodegradability [8].

In this paper we focused on detection and targeting of tumor cells using an antibody coupled to MNPs. Solid tumors are often hypoxic, because the growing tumor mass is inefficiently vascularized by aberrant blood vessels, which fail to deliver sufficient amount of oxygen to certain tumor tissue areas. It has been well documented that hypoxic tumors are more metastatic and resistant to chemo/radiotherapy [9]. Therefore, targeting hypoxic tumor cells can be achieved through hypoxia-induced molecules. Carbonic anhydrase IX (CA IX) is one of the best intrinsic markers of tumor hypoxia due to its very strong induction by low oxygen expressed in different types of solid tumors and is frequently associated with aggressive tumor phenotype [10]. Here we show that CA IX specific antibody coupled to MNPs can be used for selective detection of the CA IX-positive cancer cell lines. For this purpose, we employed our highly specific monoclonal antibody M75 binding to the extracellular domain of CA IX. The antibody was coupled to nanoparticles and utilized for targeting of the CA IX-expressing cells.

2. Materials and methods

2.1. Materials

Poly-L-Lysine (PLL, molecular weight Mw =150 000–300 000), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich. All solutions were prepared using an ultrapurified water. Human CA IX PG domain-specific mouse monoclonal antibody M75 was described previously [11].

2.1.1. Cell culture

For the experiments described in this paper, we used human cervical cancer cells C33a permanently transfected with the full-length CA9 cDNA in pcDNA3.1+ plasmid (C33 CA IX). Mock-transfected cells (C33 neo) were used as a negative control. We also used MDCK canine kidney cells permanently transfected with the full-length human carbonic anhydrase (CA9) cDNA in the pSG5C plasmid (MDCK CA IX). Mock-transfected cells (MDCK neo) were used as a negative control. Human tumor-derived cell lines with endogenous CA IX expression, such as cervical carcinoma HeLa cells, colon carcinoma RKO cells, renal carcinoma ACHN *cells,* lung adenocarcinoma A549 cells*, as well as CA IX-negative HEK 293* human embryonic kidney cells, MRC5 human lung (fetal) fibroblast, NIH 3T3 mouse embryonic fibroblast cell line, CHO Chinese hamster ovary cells and BHK baby hamster kidney fibroblasts were used for in vitro experiments. The cells were routinely cultivated in DMEM medium with 10% FCS (BioWhittaker) at 37 °C in 5% CO_2 in air.

2.2. Synthesis of Fe_3O_4 MNPs and their surface modification with PLL

Magnetic fluids containing magnetic nanoparticles modified by PLL (MFPLLs) were prepared as follows. First, magnetite nanoparticles were formed by precipitation of Fe(2+) and Fe(3+) ions in aqueous solution with a dropwise addition of ammonium hydroxide. The black precipitate was washed 4 times and put in a glass vial with defined volume of water. Then, the ultrasonication was applied using an immersed probe (horn tip) of a sonicator (BRANSON - Model 450) for 5 min at 70% of maximum power (280 W) in the water bath. In the next step, a modification of magnetite nanoparticles with PLL was done. The suspension of nanoparticles was mixed with Poly-L-Lysine solution (0.1%) at the theoretical PLL/Fe_3O_4 weight ratio from 0.1 to 3.9 (corresponding to PLL loading 0.02–0.9 mg/mL). The mixture was sonicated for 5 min at 70% power in the ice bath. Consequently, the samples were subjected to ultracentrifugation at 44,000*g* for 2 h at 4 °C in order to increase their concentration. Then the supernatants were removed and the sediments were thoroughly dispersed in ultrapure water and collected. By this procedure, the MFPLLs with the PLL/Fe_3O_4 weight ratio values from 0.1 to 3.9 were obtained.

2.3. Antibody conjugation to MFPLLs

Binding of the antibodies to amino-modified MFPLL was carried out using the carbodiimide procedure described elsewhere [12]. The coupling reaction was conducted under different sets of condition to determine the optimum conditions for the antibody (Ab) immobilization. To study the effect of various MNPs to Ab ratios on the Ab immobilization, the reaction was carried out at pH 7 using 1 mM carbodiimide in PBS at constant weight ratio of carbodiimide (CDI) to Ab, whereas the ratio between MNPs and Ab varied from 1 to 5. Shortly, the reaction mixtures containing Ab, CDI and MFPLL were stirred 24 h at room temperature. Then the samples were centrifuged at 32,000*g* for 1 h at 4 °C and the amount of unbound Ab in the supernatant was estimated by Bradford's dye binding methods [13]. For each studied sample a corresponding blank comparative sample with no MNPs was prepared. Each sample with one of the ratios mentioned above was prepared in triplets.

2.4. Cell viability assay

To assess the toxicity of different nanoparticles and PLL on various cell lines, the Cell Titer Blue viability assay (Promega Madison, WI) was used. The assay was performed according to the manufacturer's instructions. Briefly, the cells were plated in 96-well plates (at 2000 cells/well, and at 10000 cells/well for MRC5) and allowed to grow for 24 h. MNPs were suspended in the culture medium at different concentrations (100, 50 and 10 μg/mL) and added to wells. The cells cultured in the medium without nanoparticles served as controls. After 24 h, 48 h and 5/6 days incubation, 20 μl of the Cell Titer Blue solution was directly added to the wells and incubated for 4 h at 37 °C. The fluorescence was recorded with 530 nm/590 nm (excitation/emission) filter set using Bio-Tek Synergy HT microplate reader. The samples were run in triplicates for each concentration of nanoparticles.

2.5. Immunofluorescence assay

Cells grown on glass coverslips were gently washed with PBS and fixed in ice-cold methanol at −20 °C for 5 min. Nonspecific binding was blocked by incubation with PBS containing 1% BSA for 30 min at 37 °C. Then, the cells were incubated with the free M75 antibody (5 μg/mL), M75 (5 μg/mL) conjugated to MNPs or with MFPLL without conjugated antibody diluted in 1% BSA in PBS for 1 h at 37 °C followed by an anti-mouse ALEXA 488–conjugated antibody (Invitrogen) diluted 1:1000 in the blocking buffer for 1 h at 37 °C to detect the M75 antibody. The nuclei were stained with DAPI (Sigma). Finally, the coverslips were mounted onto slides in the Fluorescent Mounting Media (Sigma), and analyzed by the confocal laser scanning microscope Zeiss LSM 510 Meta.

2.5.1. Immunofluorescence-based internalization assay

The immunofluorescence-based internalization assay was performed using the established MDCK cell model. MDCK CA IX cells (700 000 cells per Petri dish) were plated on sterile glass coverslips 24 h before the experiment. Subsequently the live cells were incubated with the free M75 antibody (5 μg/mL) or the M75 (5 μg/mL) con-

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