Intracellular uptake of folate receptor targeted superparamagnetic nanoparticles for enhanced tumor detection by MRI

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ABSTRACT

Folic acid (FA) was conjugated to superparamagnetic iron oxide nanoparticles to develop a tumor specific contrast agent for magnetic resonance imaging (MRI). In this scheme a bifunctional poly(ethylene glycol) (PEG) linker was utilized to both increase biocompatibility and reduce nanoparticle agglomeration. The uptake of nanoparticle-PEG-FA conjugates by folate-receptor (FR) positive MCF-7 cells was compared against that of nanoparticles coated with only PEG over a range cell incubation times. Significant intracellular uptake was observed for MCF-7 cells cultured with nanoparticle-PEG-FA conjugates compared to those incubated with non-specific control nanoparticles. In addition, the preferential uptake of nanoparticle-PEG-FA conjugates by MCF-7 cells, as compared to FR-negative HT1080 cells, was observed through MRI. These results suggest that the synthesized FA superparamagnetic nanoparticle conjugates can potentially be used to target specific tumor cells for detection by MRI.

Keywords: magnetite nanoparticles, PEG, folic acid, MRI, tumor imaging

1 INTRODUCTION

Superparamagnetic iron oxide nanoparticles have been extensively studied as contrast agents for magnetic resonance imaging (MRI) due to their unique magnetic properties [1-3]. Currently, iron oxide particles of various sizes and polymeric coatings are approved for clinical usage in a variety of MRI applications [4]. Intracellular uptake of these nanoparticles within the tissue of interest is primarily the result of non-specific uptake by cells such as macrophages of the mononuclear phagocytic system (MPS) [5].

One approach to specifically target tumor cells with nanoparticles is through the use of ligands that can preferentially bind to the receptors expressed on the cell membrane. In this study, we examine the vitamin folic acid (FA) as a targeting agent for delivery of superparamagnetic nanoparticles to specific tumor cells *in vitro.* FA and folate-conjugates are known to penetrate cells via receptor-mediated endocytosis facilitated by the folate receptor (FR) [6, 7], which is significantly overexpressed on many human cancer cells (e.g. ovarian, lung, breast, endometrial, renal, and colon) [8, 9].

To be effective *in vivo*, iron oxide nanoparticles must remain dispersed within the bloodstream long enough to reach their target cells. However, upon intravenous injection, nanoparticles tend to agglomerate due to their large surface area/volume ratio. These clusters of nanoparticles are subject to opsinization and subsequent clearance by the macrophages of the MPS. To prevent the nanoparticle agglomeration and increase their blood circulation time, coating nanoparticles with biocompatible polymeric films have been extensively investigated. A majority of the current applications of nanoparticle-based MRI contrast agents utilize dextran or its derivatives. In many cases, these polymers significantly increase the overall size of the nanoparticle therefore limiting its tissue distribution and penetration. In this study, we utilize a low molecular weight bifunctional poly(ethylene glycol) (PEG) linker to both increase biocompatibility and reduce nanoparticle agglomeration [10].

In our previous work, we have identified the immobilization of folic acid on the surface of magnetite (Fe_3O_4) nanoparticles as an effective means of targeting human breast cancer BT20 cells [11]. It was also found that coating PEG on magnetite nanoparticles improved nanoparticle uptake by BT20 cells while decreasing uptake by macrophage cells [11]. In this study, we use a PEG-FA conjugate to further improve cellular uptake of nanoparticles by cancer cells.

Uptake of nanoparticle-PEG-FA conjugates by target cells was compared with that of nanoparticles coated with only PEG. *In vitro* experiments were performed using FR expressing human adenocarcinoma MCF-7 cells over a range of incubation times. Cellular uptake studies were also performed using MCF-7 and non-FR expressing human fibrosarcoma HT1080 cell lines. Intracellular uptake of the nanoparticle conjugates was determined by change in signal intensity of MRI phantom images. An increase in cellular uptake of superparamagnetic nanoparticle conjugates corresponds to a decrease in signal intensity (negative contrast) under T2-weighted MRI pulse sequences. Preferential binding of nanoparticle-PEG-FA

conjugates to FR-positive MCF-7 cells, as compared to FR-negative HT1080 cells, was observed through MRI. Significant negative contrast enhancement was also observed for MCF-7 cells cultured with nanoparticle-PEG-FA conjugates compared to those incubated with non-specific control nanoparticles. These results suggest that the synthesized FA superparamagnetic nanoparticle conjugates can be used to target specific tumor cells for detection by MRI. Together with optimized MRI techniques, the use of such nanoparticle conjugates as targeted-contrast agents may prove to be a powerful tool for the enhanced detection and diagnosis of cancer.

2 MATERIALS AND METHODS

2.1 Materials

Folic acid (FA), dicyclohexylcarbodiimide, anhydrous dimethyl sulfoxide (DMSO), toluene and pyridine were purchased from Sigma-Aldrich, St. Louis, MO. 2-methoxy (poly ethyleneoxy)-propyl trimethoxy silane was purchased from Gelest, Morrisville, PA. RPMI-1640 cell culture medium, fetal bovine serum (FBS), penicillin/streptomycin, phosphate buffered solution (PBS), trypsin-EDTA and trypan blue were purchased from Invitrogen, Carlsbad, CA. Human fibrosarcoma HT1080 cells were purchased from American Type Culture Collection, Manassas, VA. Human adenocarcinoma MCF-7 cells were provided by Dr. James Olson, Fred Hutchinson Cancer Research Center. Agarose was purchased from BioRad, Hercules, CA.

2.2 Synthesis of nanoparticle-PEG conjugates

Magnetite nanoparticles were synthesized by a co-precipitation method reported previously [12]. Approximately 100 mg of as-produced nanoparticles was isolated with a rare-earth magnet and washed twice with ethanol and twice with dry toluene. The nanoparticles were then dispersed in 17.9 mM 2-methoxy (poly ethyleneoxy)-propyl trimethoxy silane in 100 ml of dry toluene with sonication. The mixture was purged with nitrogen and sonicated at 60ºC for 4 hrs. The resulting PEG coated nanoparticles (NP-PEG) were then washed twice in ethanol and resuspeneded in 20 mM sodium citrate pH 8.0.

2.3 Synthesis of nanoparticle-PEG-FA conjugates

One hundred milligrams of as-produced nanoparticles was washed with dry toluene as stated above. The nanoparticles were then coated with PEG and functionalized with terminal amine groups as reported previously [10]. The amine-PEG coated nanoparticles were washed twice in ethanol and twice in DMSO. The nanoparticles were then resuspended in 50ml of anhydrous DMSO. A 50ml solution of 23mM folic acid in DMSO with equimolar dicyclohexylcarbodiimide, and 10µl pyridine was then added to the nanoparticle solution. The resulting mixture was protected from light and allowed to reacted overnight. The folic acid conjugated nanoparticles (NP-PEG-FA) were washed twice in ethanol and resuspeneded in 20 mM sodium citrate pH 8.0.

2.4 Cell culture

Human adenocarcinoma MCF-7 cells and Human fibrosarcoma HT1080 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. Cells were grown in 75cm^2 cell culture flasks with 10 mL of culture medium with medium changes occurring every second day.

To evaluate the uptake of NP-PEG-FA conjugates as compared to control NP-PEG by MCF-7 cells, flasks containing approximately 5 x 10^6 cells were washed twice with phosphate buffered solution (PBS) and incubated at 37˚C with either 0.01 mg Fe/ml NP-PEG or NP-PEG-FA for 0.25–4.0 hrs. Cells were then washed twice with PBS and incubated with trypsin-EDTA solution (0.25% trypsin, 1mM EDTA) for 5 min at 37˚C to detach them from the flask. The cells were then resuspended in medium for characterization. Cell density and viability were determined through staining with trypan blue, and cells were counted using a hemocytometer with 0.9 mm^3 counting chambers.

To quantify the intracellular uptake of nanoparticles, a known quantity of cells from each sample was analyzed for iron content by inductively coupled plasma emission spectroscopy (ICP). ICP samples were prepared by centrifuging down the cells and dissolving the cell pellet in 37% HCl at 70°C for 1hr. The samples were then diluted upto a volume of 3ml for analysis.

To evaluate the preferential binding of nanoparticle-PEG-FA conjugates to FR-positive MCF-7 cells, as compared to FR-negative HT1080 cells, flasks of each cell line were washed twice with PBS and incubated with various concentrations (0.001, 0.005 and 0.01 mg Fe/ml) of NP-PEG-FA in RPMI-1640 for 2 hrs. Cells were then washed, trypsinized and counted as stated above.

2.5 MR imaging

Samples for MR phantom imaging were prepared in microcentrifuge tubes by suspending 5 x 10^6 cells in 1.0 ml low-melting 1% agarose gel. Cell suspensions were allowed to solidify at 4°C for 10 min.

MR images were acquired using a 4.7-T Varian Spectrometer (Varian Inc., Palo Alto, CA) with a Bruker magnet (Bruker Medical Systems, Karlsruhe, Germany) equipped with a 5 cm volume coil. A spin-echo multisection pulse sequence was selected to acquire MR phantom images. Repetition times (TR) of 2000 msec and 3000 msec were used with variable echo times (TE) of 15-

120 msec. The spatial resolution parameters were as follows: an acquisition matrix of 256 × 128, field of view of 40 × 40 mm, section thickness of 1 mm, and 2 averages.

3 RESULTS & DISCUSSION

Figure 1 shows the schematic of the surface modification of magnetite nanoparticles and subsequent internalization of nanoparticles by cancer cells. In this study, we have immobilized PEG and FA onto magnetite nanoparticles to improve their biocompatibility and ability to preferentially target cancer cells. Here the folic acid serves as a targeting agent while PEG serves to improve intracellular uptake of the nanoparticles by preventing particle agglomeration and adsorption of plasma proteins.

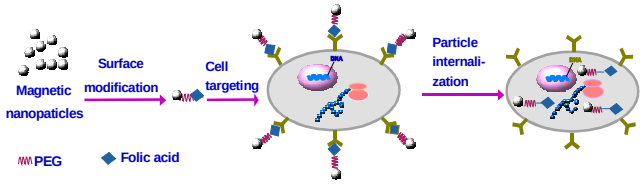

Figure 1: Schematic diagram of the surface modification process and subsequent uptake of magnetite nanoparticles into target cells.

Intracellular uptake of NP-PEG and NP-PEG-FA by MCF-7 cells was quantified by measuring the iron content in cells using ICP. Figure 2 shows the iron uptake by cells cultured with the nanoparticle conjugates over various time intervals.

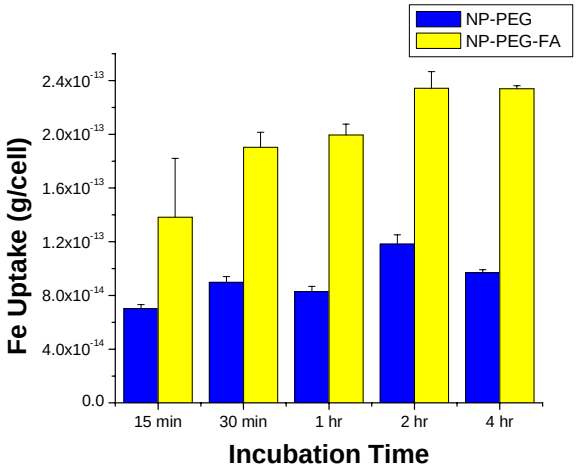

Figure 2: Intracellular uptake of NP-PEG and NP-PEG-FA conjugates by MCF-7 cells a function of incubation time.

The FR-positive MCF-7 cell line showed increased uptake of the NP-PEG-FA conjugate as compared to NP-PEG over all the incubation times tested. The uptake of NP-PEG-FA increases with incubation time up to 2 hrs upon which nanoparticle internalization plateaus, most likely due to saturation of the folate receptors. NP-PEG conjugates were also taken up by MCF-7 cells due to the high solubility of PEG in the cell membrane. However, no correlation between NP-PEG uptake and incubation time was observed.

MR imaging was used to evaluate the preferential uptake of NP-PEG-FA conjugates by MCF-7 cells. Figure 3 shows MR phantom images of control cells and cells incubated with the nanoparticle conjugates for 2 hrs at a concentration of 0.01 mg Fe/ml.

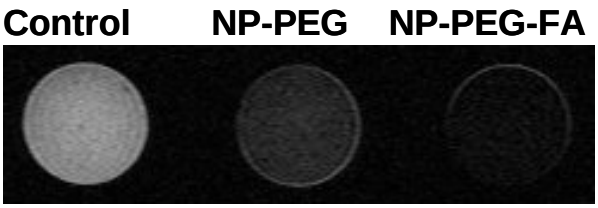

Figure 3: T2-weighted MR phantom image (TR 3000ms, TE 90ms) of MCF-7 cells alone and cells cultured with NP-PEG and NP-PEG-FA for 2hrs.

The increased uptake of superparamagnetic nanoparticle conjugates corresponds to a decrease in signal intensity (negative contrast) under T2-weighted MRI pulse sequences as shown by both cell samples incubated with nanoparticle conjugates. In correlation with the ICP data, MCF-7 cells cultured with NP-PEG-FA exhibited significant negative contrast as compared to control cells and cells cultured NP-PEG.

Figure 4A shows T2-weighted MR phantom images of MCF-7 and HT1080 cells cultured with various concentration of NP-PEG-FA for 2 hrs. Decreased signal intensities were observed for both cell lines as the concentration of NP-PEG-FA increased, as shown in Figure 4B. However, at concentrations of 0.005 and 0.01 mg Fe/ml a statistically significant difference in signal intensity was observed for the two cell lines. The increased negative contrast of the MCF-7 cells corresponds to the higher uptake of the folic acid bound nanoparticles by these FR-positive cells. Uptake of the NP-PEG-FA conjugate by the FR-negative HT1080 cells can be attributed to nonspecific internalization probably facilitated by the PEG coating.

These results show that superparamagnetic nanoparticles can be effectively conjugated to folic acid to increase the intracellular internalization of these contrast agents by target tumor cells that overexpress the folate receptor. As shown in the previous figures, increase accumulation of such superparamagnetic nanoparticles in tumor cells resulted in the enhanced detection of the target cells by MRI.

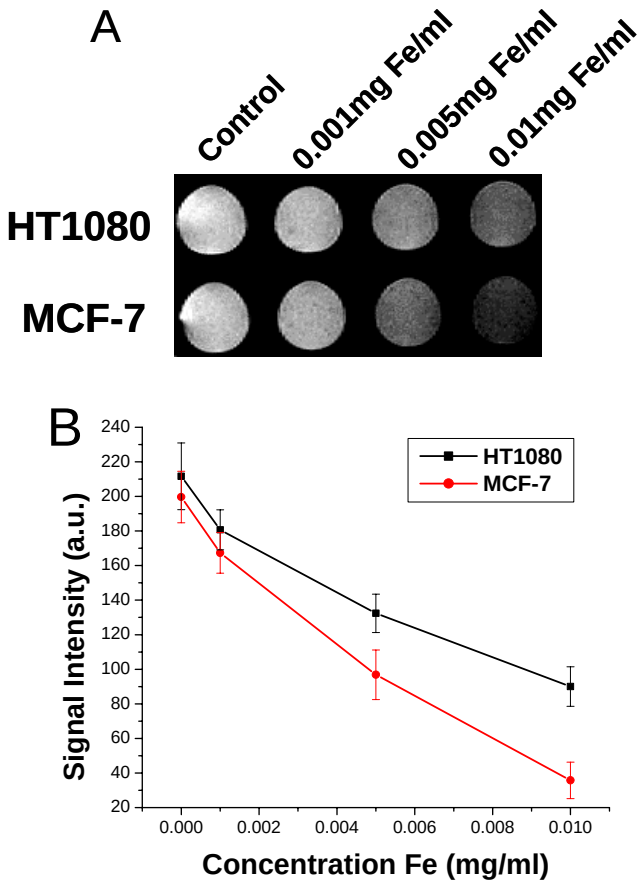

Figure 4: (A) T2-weighted MR phantom image (TR 2000ms, TE 60ms) of HT1080 and MCF-7 cells culture with various concentrations of NP-PEG-FA. (B) Signal intensities of MR image (A).

4 CONCLUSIONS

Tumor specific superparamagnetic nanoparticle conjugates are currently being developed as targeting-contrast agents for MRI. In this study, folic acid was conjugated to superparamagnetic magnetite nanoparticles through a bifunctional PEG linker to serve as a targeted MRI contrast agent for tumor cells overexpressing the folate receptor. By combining the biocompatibility and dispersion properties of PEG with the specific cell targeting capability of folic acid we take advantage of a synergistic effect which results in greatly increased nanoparticle uptake. The application of such "smart" molecular imaging probes could have a significant clinical impact as a non-invasive method for cancer detection and diagnosis.

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