

ANTI-HER2 ANTIBODY AND SCFV OF EGFR CONJUGATED "STEALTH" MAGNETIC IRON OXIDE NANOPARTICLES FOR TARGETING AND MAGNETIC RESONANCE IMAGING OF BREAST CANCER

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INTRODUCTION

Magnetic iron oxide nanoparticles (IONP) functionalized with biomarker targeting ligands, such as antibodies against cell surface receptors, offer promising applications as novel and more sensitive magnetic resonance imaging (MRI) contrast enhancing agents for biomarker specific detection. In order to achieve an effective concentration of nanoparticles in the targeted tissue or tumor site after systemic delivery, the nanoparticle accumulation, retention and eventually cellular internalization should entail the targeted nanoparticles navigating from the circulating blood to the tissue of interest and binding to their molecular target. However, many types of systemically delivered nanoparticles are rapidly cleared from the blood stream by the reticuloendothelial system (RES) and the mononuclear phagocytic system (MPS) in the liver, spleen, and bone marrow, resulting in reduced bioavailability of the targeting agents, a low therapeutic index and potential toxicity to normal organs. Here we report the further development of antibiofouling poly(ethylene oxide)-*block*-poly(γ -methacryloxy propyl trimethoxysilane) (PEO-*b*-PyMPS) coated magnetic nanocrystals for targeting and imaging of breast cancer using HER2 antibody or single chain antibody (scFv) against EGFR-conjugated magnetic IONPs. Reported IONPs exhibit "stealth" properties and long circulation time, enabling active targeting of breast cancer cells and receptor targeted imaging of xenografted human breast tumors in nude mice using MRI.

MATERIALS AND METHODS

Nanoparticles and Properties: Combining PEG with crosslinkable polysiloxane coating, antibiofouling copolymer PEO-*b*-PyMPS coated IONPs with 10 nm core size shown in **Figure 1** were prepared using the method that we reported previously.^[1] Overall hydrodynamic size of the PEO-*b*-PyMPS coated IONPs was measured at 23 nm by dynamic light scattering (DLS). The hydrolyzed silanol groups from copolymer coating result in slight negative surface charge with ξ potential of -9.1 mV at pH 6.5. Humanized anti-HER2 monoclonal antibody Herceptin® (trastuzumab) and the scFv of EGFR were conjugated to IONPs as breast cancer targeting ligands. Conjugation was carried out after successful amine group grafting onto the surface of IONPs which was confirmed using FITC labeling.

Receptor Targeted Cellular Uptake of IONPs: For testing receptor mediated uptake of anti-HER2 or anti- scFvEGFR conjugated IONPs to breast cancer cells with over-expression of HER2 (SK-BR-3) or EGFR (MDA-MB-231), cells were incubated with nanoparticles. 100 nM of antibody conjugated IONPs or non-conjugated IONPs in cell culture medium were added into the culture chamber, and the incubation was performed at room temperature for 6 h. Prussian blue staining was used to determine the presence of iron in all the cells followed by counterstaining with nuclear fast red solution. For HER2 or scFvEGFR inhibition experiment, before incubating with anti-HER2 or scFvEGFR-IONPs, cells

were treated with 100 times excess of free HER2 or scFvEGFR (molar) for 1 h. For testing non-specific uptake by macrophages, anti-HER2-IONPs, scFvEGFR-IONPs and non-conjugated IONPs were incubated with macrophages (RAW 264.7). Electron microscopy was used to examine uptake of scFvEGFR-conjugated IONPs in MDA-MB-231.

MRI of Tumor Bearing Mice Administered with IONPs: 1×10^7 of 4T1 mouse mammary tumor cells with EGFR over-expression were inoculated subcutaneously in the upper back of 4 to 6-week-old female Balb/c mice. After 10–14 days post-inoculation, tumor-bearing mice were scanned using a 3T MRI scanner (Siemens Healthcare) with a wrist coil to collect pre- and post-contrast enhanced MRI data. Mice were imaged before and 24 h after being injected with scFvEGFR-IONPs or non-targeted IONPs (0.2 mg Fe/mouse) in PBS (100 μ L) through the tail vein. Images from pre- and post-contrast administration were compared to evaluate the contrast enhancement by the target-specific contrast agent. The imaging sequences include: T₁ and T₂ weighted spin echo or gradient echo imaging. A multi-echo T₂ weighted fast spin echo sequence (8 different TE values ranging from 10–120 ms) was used to obtain T₂ relaxometry of the whole mouse. The averaged signal intensity of whole tumor was calculated manually using ImageJ for comparing the signal intensity before and after injection of scFvEGFR-IONPs. The region of interest (ROI) method was used to evaluate and quantify the contrast agent induced signal or T₂ value changes in the tumor and other organs.

RESULTS

The PEO-*b*-PyMPS coated antibody-conjugated IONPs has 12 hours blood half time in mice with lower liver and spleen uptake comparing to other IONPs coated with conventional polymers, such as PEG. Receptor mediated uptake of scFvEGFR-IONPs (10 nm core size) enables the MRI probes internalized in the targeted cancer cells as shown in TEM images (**Figure 1**). Strong uptake of anti-HER2-IONPs to HER2 positive SK-BR-3 cells or scFvEGFR-IONP in EGFR-positive cell line, MDA-MB-231 was demonstrated by the positive Prussian blue staining (**Figure 2**). Inhibiting the HER2 receptor with 100 times excess of free HER2 antibody effectively reduced the amount of blue staining (**Figure 2**), suggesting that the uptake of these IONPs is specifically mediated by HER-2 receptors of the cancer cells. Additionally, Prussian blue staining was negative in MCF-7 cancer cell lines without HER2 over-expression after treatment with anti-HER2-IONPs. Furthermore, HER2 antibody-conjugated IONPs and scFvEGFR-conjugated IONPs do not increase any non-specific cellular uptake by macrophages. This reduced non-specific binding may potentially increase the targeted imaging contrast. Similarly, improved receptor targeting and reduced non-specific uptake by macrophages were observed in EGFR targeted IONPs. T₂ weighted MRI showed the significant signal drop in the various areas of the tumor in the mice 24 h post administration of scFvEGFR conjugated IONPs (**Figures 3a and b**), suggesting the accumulation of scFvEGFR antibody conjugated IONPs in the tumor. This indicates that scFvEGFR-IONPs can penetrate deep throughout tumor tissue, actively target to EGFR-expressing tumor cells. The presence of scFvEGFR conjugated IONPs in the tumor tissue was also confirmed by positive Prussian blue staining after sacrificing animals (**Figure 3c**).

CONCLUSION

Antibiofouling polysiloxane-contained PEO-*b*-PyMPS copolymer coated IONPs exhibit a long blood circulation time with reduced non-specific uptake by the liver and spleen. With conjugation of the antibody against HER2 or scFvEGFR, HER2 and EGFR targeted IONPs exhibited efficient targeting of breast cancer cells that over-express HER2 or EGFR. Furthermore, T₂ weighted MRI of mice bearing EGFR positive tumor xenografts 24 h after injection of scFvEGFR-IONPs shows the signal reduction in tumors as the result of the contrast induced by the accumulation of EGFR targeted IONPs

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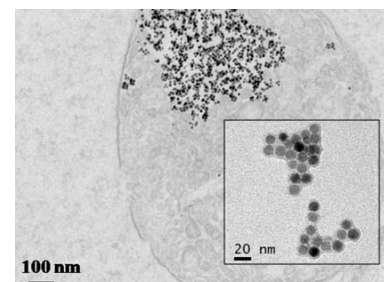


Fig 1. TEM images of the uptake of scFvEGFR-IONP. Inserted image: PEO-*b*-PyMPS coated IONPs with a core size of 10 nm.

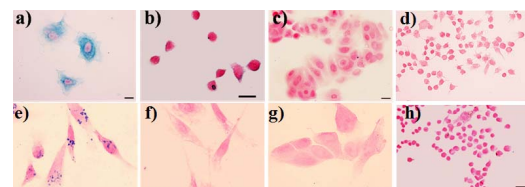


Fig 2. Prussian blue staining of HER2 positive SK-BR-3 cancer cells (a), 100 times free pre-HER2 blocked SK-BR-3 cancer cells (b), negative MCF-7 (c). MDA-MB-231 cancer cells bound with scFvEGFR-IONPs (e), 100 times free pre-scFvEGFR inhibited the binding of the EGFR-positive MDA-MB-231 cancer cells (f), negative MDA-MB-453 cancer cells (g). Mouse macrophage RAW 264.7, after treatment with anti-HER2-IONP, scFvEGFR-IONPs and non-conjugated IONPs (d,h)

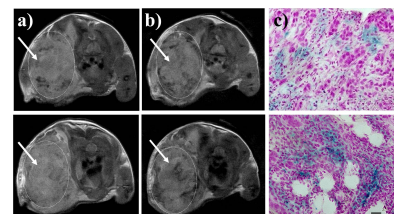


Fig 2. (a, b) T₂-weighted MR images of a mouse bearing 4T1 mammary tumor before and 24 h after i.v. injection of scFvEGFR-IONPs. Prussian blue stained tumor tissue after 24 h injection of scFvEGFR conjugated IONPs (c). Scale bar is 50 μ m. The signal intensity of tumor was reduced after 24 h injection of scFvEGFR-IONPs compared to that before injection