ANTI-FOULING POLY ETHYLENE GLYCOL BASED COPOLYMER COATED IRON OXIDE NANOPARTICLE PROBES FOR REDUCING NON-SPECIFIC UPTAKE AND IMPROVING CELL TARGETING

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INTRODUCTION

Increasing effort has been focused on the development of biocompatible superparamagnetic iron oxide nanoparticles (IONP) based molecular imaging probes, given the advantages of their superb contrast enhancing effect, capability of surface functionalization, and prolonged blood circulation as well as tumor retention.[1] However, one of the major challenges in applications of IONPs for *in vivo* imaging is the non-specific uptake of the nanoparticles by the macrophages and reticuloendothelial system (RES), e.g. liver and spleen.[2] Non-specific uptake may lead to lower blood circulation time needed for probes reaching the targeted tissue and also introduce interfering background signal, all resulting in a substantial reduction in the efficiency of target-directed imaging. Furthermore, the absorption of macromolecules in blood, such as proteins, on the surface of IONPs, can form a corona that can alter the contrast enhancing effect. To address this problem, we have developed a poly ethylene glycol (PEG)-*b*-poly allyl glycidyl ether (PAGE) copolymer coated IONPs that exhibit anti-biofouling properties and reduced non-specific uptake while can be functionalized with ligands for biomarker targeted molecular MRI.

MATERIALS AND METHODS

Nanoparticles Preparation and Characterization: The PEG-*b*-PAGE copolymer was synthesized starting from substituting one hydroxyl group with azide, followed by the reduction of azide to amine group. Allyl glycidyl ether (AGE) molecules were attached to the other end of the PEG chain. 3-Mercaptopropyltrimethoxysilane was subsequently inserted to the polymer. Obtained co-polymer then was applied to transfer the oleic acid coated hydrophobic IONP (core size of 10 nm) prepared using the thermal decomposition method into aqueous solvent. This process yielded PEG-*b*-PAGE copolymer coated IONPs with $-NH_2$ functional groups available for conjugating targeting ligands. The average hydrodynamic diameter and size distribution of coated nanoparticles were characterized by dynamic light scattering (DLS) instrument (Malven Zeta Sizer Nano S-90) equipped with a 22 mW He-Ne laser operating at $\lambda = 632.8$ nm. The core size of the coated nanocrystals and the thickness of the polymer layers (shown in **Fig. 1**A and B) were measured by transmission electron microscopy (TEM) (Hitachi H-7500 instrument (75 kV)).

Conjugation of Ligands: The IONP was made solution in PBS at the concentration of 2 mg/mL. The solution was then incubated with the linker Sulfo-SMCC (at the concentration of 2 mg/mL) for 1 hr. The solution was purified with a PD-10 column followed by incubation with tumor targeting tri-peptide RGD-SH at the concentration of 0.2mg/mL, or thiolated (using Traut's reagent) transferrin (Trf) at the concentration of 1 mg/mL overnight. The solution of conjugated IONP was obtained after purification with a PD-10 column.

Stability of PEG-b-PAGE copolymer Coated IONPs: The stability study of PEG-*b*-PAGE copolymer coated IONPs was carried out by measuring the average hydrodynamic diameters of the nanoparicles in phosphate buffered saline (PBS), 10% (w/v) NaCl aqueous solution, and 100% fetal bovine serum (FBS) at the concentration of 0.1 mg/mL at different time points.

Cytotoxicity and Cell Uptake and Targeting of PEG-b-PAGE copolymer IONPs: The cytotoxicity of the PEG-*b*-PAGE copolymer coated IONPs was examined using the RAW 264.7 macrophage cell line. A total of 10^5 cells were plated in each well of a 96-well plate for 24 hrs before washing with PBS and adding nanoparticles at selected concentrations. After 12 hrs of incubation, the solutions were removed and cells were washed three times with PBS. Cell viability was then estimated using the MTT conversion test. Raw 264.7 cell line, MCF-7 breast cancer cell line, and MDA-MB-231 breast cancer cell line were used for testing the cell uptake and tumor cell integrin targeting of RGD-conjugated IONPs. Brain cancer cell lines, D556, and Doay with over expressed transferrin receptors, were used for transferrin receptor targeting of transferrin (Trf)-conjugated IONPs. Cells were seeded into an 8-well chamber slide and incubated overnight. The media was then replaced with that containing IONPs at the concentration of 0.2 mg/mL. Cells were incubated at 37 °C for 3 hrs, and then fixed with 4% paraformaldehyde in PBS solution, followed by Prussian blue staining for iron, and counterstaining using nuclear fast red solution.

Blood Half Time of PEG-b-PAGE copolymer coated IONPs: The blood retention time was investigated by measuring the T_2-weighted MR signal intensity of the blood samples collected from the CD1 mice (aged 8 weeks, average body weight around 20 g) at different time points after tail vein injection of PEG-*b*-PAGE copolymer coated IONPs (a dose of 10 mg Fe/Kg of mouse body weight). Blood samples were collected by terminal heart puncture of CD1 mice administered with IONPs.

Measurement of Relaxivities: The transverse relaxation time (T_2) and relaxivity (r_2) were determined using a 3 Tesla MR scanner (Tim/Trio, Siemens, Erlangen, Germany). Solutions of PEG-*b*-PAGE copolymer coated IONPs were prepared with different iron concentrations ranging from 0.0045 to 0.0700 mM. A multi-echo spin echo (SE) sequence was performed with TR of 2520 ms and 20 TEs, starting at 12.2 ms with increments of 12.2 ms. A mean signal intensity values from all ROIs was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA).

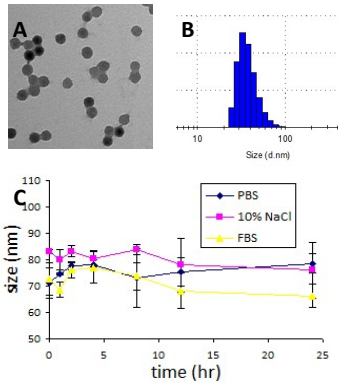

Fig 1. (A) TEM image of PEG-*b*-PAGE copolymer coated IONPs (core size of 10 nm), (B) the hydrodynamic diameter distribution of PEG-*b*-PAGE copolymer coated IONPs (core size of 10 nm) measured by DLS, and (C) stability of PEG-*b*-PAGE copolymer coated IONPs in PBS, 10% NaCl solution, and FBS at the Fe concentration of 0.1 mg/mL.

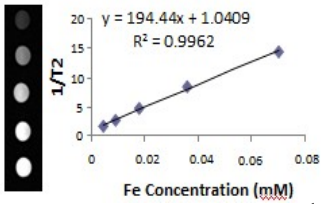

Fig 2. Transverse relaxation rate($1/T_2$, S^{-1}) of PEG-*b*PAGE copolymer coated IONPs as a function of the Fe concentration (mM) with T_2-weighted spin echo MR images (Fe concentration decreases from top).

RESULTS AND DISCUSSION

The transverse relaxivity (r_2) of PEG-*b*-PAGE copolymer coated IONPs at 3T is 194 $mM^{-1}S^{-1}$ calculated from the Fe concentration dependent change of transverse relaxation times, which is directly proportional to the Fe concentrations (**Fig. 2**). A typical hypointense contrast from IONPs was observed in T_2-weighted spin echo images.

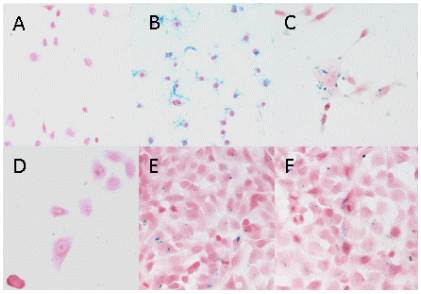

Fig 4. Cell uptake experiments of (A) coated IONPs with Raw 264.7 marcophage cell line, (B) amphiphilic polymer-coated iron oxide nanoparticle (SHP10) having 10 nm core with RAW 264.7 macrophage cell line, (C) RGD-conjugated PEG-*b*-PAGE copolymer coated IONPs s with MDA-MB-231 breast cancer cell line (high expression of integrin), (D) RGD-conjugated coated SPIONs with MCF-7 breast cancer cell line (low expression of integrin), (E) Tf-conjugated coated SPIONs with D556 brain cancer cell line, and (F) Tf-conjugated coated IONPs with Doay brain cancer cell line at the Fe concentration of 0.1 mg/mL.

The PEG-*b*-PAGE copolymer coated IONPs exhibited excellent stability in PBS, 10% NaCl aqueous solution, and 100% FBS with no significant change in the averaged hydrodynamic diameters (**Fig. 1**C), suggesting an excellent antifouling property of PEG-*b*-PAGE copolymer coated IONPs without absorption of serum proteins. Furthermore, cell uptake experiments with macrophage Raw264.7 cells showed reduced non-specific uptake of PEG-*b*-PAGE copolymer coated IONPs by macrophages, comparing to amphiphilic polymer-coated 10 nm iron oxide nanoparticle (**Fig. 3**A and B). After conjugated with RGD (a tumor integrin $\alpha_v\beta_3$ targeting peptide), the RGD-PEG-*b*-PAGE – IONPs can be uptaken by MDA-MB-231 breast cancer cells (high expression of integrin) (**Fig 3**C), but not by MCF-7 breast cancer cells (low expression of integrin) (**Fig. 3**D). After conjugating with transferrin, Trf- PEG-*b*-PAGE-IONPs showed strong uptake by D556 and Doay brain tumor cells with over expression of transferrin receptor (**Fig. 3**E and F). The result from the MTT assay indicated that PEG-*b*-PAGE copolymer coated IONPs has no toxicity to Raw264.7 macrophage cells when incubated at the Fe concentration ranging from 0.0078 to 0.5 mg/mL. At high Fe concentration (over 0.5 mg/mL), the cell survival rate reached 83%, indicating little cytotoxicity at the exceedingly high concentration. It was found that the blood half-time of PEG-*b*-PAGE copolymer coated IONPs was about 6 hrs (**Fig. 3**).

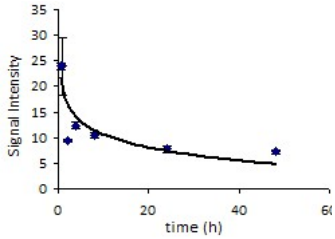

Fig 3. Blood half-life of PEG-*b*-PAGE copolymer coated IONPs after tail vein injection of 10 mg Fe/Kg of mouse body weight.

CONCLUSION

Reported poly ethylene glycol-*b*-poly allyl glycidyl ether copolymer coated IONP exhibits excellent MRI contrast in T_2-weighted imaging. When functionalized with RGD or anti-transferrin as the tumor targeting ligand, PEG-*b*-PAGE copolymer coated IONPs exhibit excellent property of reducing non-specific uptake and improved cancer cell targeting.

REFERENCES: [1] Appl Mater Inter **2009**; *1*:2134-40 [2] Appl Phys **2003**; *36*:R198-R206.