Published in final edited form as:

ChemMedChem. 2014 July ; 9(7): 1356–1360. doi:10.1002/cmdc.201300549.

Development and screening of a series of antibody-conjugated and silica coated iron-oxide nanoparticles for targeting the Prostate Specific Membrane Antigen

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Abstract

The Prostate Specific Membrane Antigen (PSMA) is an established target for the delivery of cancer therapeutic and imaging agents due to its high expression on the surface of prostate cancer cells and within the neovasculature of other solid tumors. Here we describe the synthesis and screening of antibody-conjugated silica-coated iron oxide nanoparticles for PSMA-specific cell targeting. The humanized anti-PSMA antibody, HuJ591, was conjugated to a series of nanoparticles with varying densities of polyethylene glycol and primary amine groups. Customized assays utilizing iron spectral absorbance and Enzyme-Linked Immunoassay (ELISA) were developed to screen microgram quantities of nanoparticle formulations for immunoreactivity and cell targeting ability. Antibody and PSMA-specific targeting of the optimized nanoparticle was evaluated using an isogenic PSMA-positive and PSMA-negative cell line pair. Specific nanoparticle targeting was confirmed by iron quantification with inductively coupled plasma mass spectrometry (ICP-MS). These methods and nanoparticles support the promise of targeted theranostic agents for future treatment of prostate and other cancers.

Keywords

Nanoparticle; PSMA; Iron-oxide; cancer; targeting

Iron-oxide nanoparticles (NPs) have tremendous potential as theranostic agents because of their biocompatibility and responsiveness to magnetic fields, which enables imaging (as MRI contrast agents) and therapy (through hyperthermia or drug delivery).^[1] Making such

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Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cmdc.20xxxxxx>

NPs ‘targeted’ by decorating the surface with affinity ligands can increase the efficiency of NP delivery and retention in target cells and tissues.^[2] However, the surface factors that lead to successful targeting are numerous and may be unpredictable, thus a great deal of optimization is required to generate each desired particle.^[3] For example, recent active NP targeting studies with anti-HER2/neu affibodies found that an intermediate ligand density provided superior targeting efficiency when compared to higher ligand densities.^[4] Similar studies with folate and ICAM (Intercellular Adhesion Molecule-1) as targeting ligands on other NPs have also underscored the benefit of optimized surface coatings and ligand densities for targeting.^[5] It is therefore beneficial to generate and screen multiple NP prototypes, in order to achieve optimal targeting.

The Prostate Specific Membrane Antigen (PSMA) is a cell surface protein that is highly over-expressed in aggressive prostate cancer tumors^[6] and within the tumor neovasculature of other solid tumors.^[7] Unlike Prostate Specific Antigen (PSA), which is a secretory protein and serum biomarker, PSMA is a cell surface displayed integral membrane protein.^[8] It is the target of the only FDA approved prostate-specific imaging agent^[9] and has been studied as a target for the delivery of prostate cancer therapeutic agents for over a decade.^[10] In light of the promising characteristics of PSMA as a cancer target, and iron oxide NPs as theranostic agents, we sought to generate an optimized PSMA-targeted magnetic iron oxide NP (MION) (Scheme 1). Silica was applied for NP surface coating because it is a low-cost and chemically inert material for coating that provides high colloidal stability in aqueous solution. Further, silica has not yet been explored as a coating or NP component for PSMA-targeted agents. Below we describe the synthesis, screening, and validation of these novel PSMA-targeted NPs.

Previously developed citrate stabilized MIONs^[11] were coated with a silica shell of approximately 20 nm thickness by using a modified Stöber growth process.^[12] In this process, various ratios of tetraethyl orthosilicate (TEOS) to (3-aminopropyl) triethoxysilane (APTES) were used to vary primary amine density on the surface of the MIONs (Table 1). Thus five MIONs having the same core, but differing only in surface amine density were generated in parallel. Each of the five silica-coated MIONs were then PEGylated using an excess mixture of 10% maleimide-PEG24-NHS and 90% non-functionalized PEG. Control particles containing 100% non-functionalized PEG were also generated for each coating condition. The maleimide-functionalized NPs were then conjugated to TCEP-reduced humanized anti-PSMA HuJ591 antibody (Ab).^[13] HuJ591 was selected as the targeting agent because of its previous history as a PSMA-targeting ligand in clinical studies.^[14] The zeta potential and hydrodynamic diameter of the five MION systems were measured before and after conjugation with the Ab. From Table 2 it is evident that the zeta potential of the silica-coated MIONs (with or without Ab) were nearly neutral (± 10 mV), which is desired to avoid non-specific charge-based interactions with the cell surface.^[15]

To evaluate the immunoreactivity of the resulting Ab-targeted MIONs, we developed a custom binding assay which utilized the high spectral absorbance of iron. Specifically, individual wells of a 96 well-plate were coated with two Ab-specific (anti-human IgG or Protein A) or non-specific capturing agents (Bovine Serum Albumin, BSA or anti-GFAP Ab). The wells were blocked, washed, and then incubated with Ab-conjugated or non-

conjugated MIONs. After washing, the captured MIONs were quantified by multiwell plate spectrophotometry at an absorbance of 350 nm. Serial dilution of MIONs demonstrated linear detection in low microgram range, with sensitivity to approximately 1 μ g of MION (Figure 1, inset).

All of the J591-conjugated MIONs were captured by the anti-IgG coated wells, when compared to non-conjugated control MIONs or to control coated BSA or anti-GFAP wells (Figure 1, black bars versus grey bars). These results indicate that J591-conjugation was successful for all five coating conditions and that the spectral absorbance assay provided sufficient sensitivity and specificity for evaluating HuJ591-conjugated MION immunoreactivity. The second Ab capturing agent, Protein A, also demonstrated specificity for Ab conjugated MIONS when compared to non-conjugated controls (Figure 1, white bars versus grey bars). However, Protein A only captured MION-1.20-J591, MION-1.40-J591 and MION-1.80-J591 formulations. It is not clear why Protein A and IgG wells had differential MION capture efficiency. We speculate that this difference may be attributed to differential binding affinity or epitope availability of protein A versus anti-IgG. Alternatively, it could reflect differences in Ab conformation on the different MION surfaces. Nevertheless, it is notable that the MIONs having the lowest density of amine (1:80 APTES: TEOS, Table 1), rather than those having the highest, were captured most efficiently by both Protein A and anti-IgG. This result is surprising because MIONs having higher amine densities can be expected to have a greater number of conjugated antibodies and therefore a greater potential for capture. One possible explanation for this unexpected observation is that increased PEG (in MIONs with higher APTES: TEOS) sterically hindered the antigen-Ab interaction, leading to lower immunoreactivity. Conversely, low-to-intermediate J591 surface density may yield a higher percentage of antibodies having correct orientation or reduced steric impedance, thereby enhancing measured immunoreactivity. Regardless, it can be concluded from Figure 1 that J591 was successfully conjugated to the surface of several MIONs and having an orientation that enabled recognition by anti-human IgG and Protein A.

To further characterize the immunoreactivity of each Ab-conjugated MION, we applied a modified ELISA detection method for PSMA-specific cell binding. An isogenic pair of PSMA expressing and non-expressing LMD-MDA-MB-231 cells was used for MION capture. These two cell lines are genetically identical, except one has been engineered to express PSMA at levels comparable to that of prostate cancer cell lines (Figure 2). This model allows assessment of ligand-specific cell binding in a well-controlled manner.

The two cell lines were separately plated to equal density in 96 well-plates. Free J591 Ab or J591-targeted and non-targeted MIONs were then added to each well to allow for cell binding. After rigorous washing of each well, J591 and J591-targeted MIONS were detected by ELISA with HRP-conjugated Anti-human IgG. PSMA specific detection was demonstrated with the positive control, free J591 Ab, with linear sensitivity in the low nanogram range. (Figure 3, inset). The non-conjugated MIONs (J591 -) should not be specifically detected by this assay, although they were included as controls for determining anti-human IgG specificity. Three of the five J591-conjugated MION formulations, MION-1.20, MION-1.40, and MION-1.80, demonstrated PSMA-specific cell targeting

(Figure 3, grey bars versus white bars). These results are consistent with Protein A capture in Figure 1 and indicate that the lower levels of active amine provided superior PSMA-specific immunoreactivity. Interestingly, the level of non-specific cell binding by the J591-conjugated MIONs to the PSMA-negative cells indirectly correlated with the level of functional amines (Figure 3, white bars). This may reflect the lower levels of PEG conjugation and reduced potential for MION surface shielding. Notably, this non-specific binding or “stickiness” was only detected by the cell binding ELISA assay, and was not reflected in the Ab capture spectrophotometric screens of Figure 1. It is likely that reflects the greater level or diversity of non-specific ligands on the surface of a cell when compared to homogenous MION capture by purified proteins.

Collectively, the Protein A capture spectral absorbance assay (Figure 1) and the isogenic cell-based ELISA assay (Figure 3) reveal complementary results for ligand-specific MION targeting. These data indicate that the top candidate particle for PSMA-specific targeting is MION-1.20-Ab, due to its strong PSMA-specific binding and minimal non-specific cell binding. To verify this Ab-specific and ligand-specific targeting we quantified PSMA-specific MION-1.20-Ab cell binding by inductively coupled plasma mass spectrometry (ICP-MS). MION binding, as quantified by iron-content (in ppb), was approximately five fold greater for the PSMA positive cells treated with MION-1.20-Ab, when compared to PSMA negative cells (Figure 4, grey bars versus white bars). In the absence of conjugated Ab, the MIONs bound similarly to PSMA-positive and negative cells.

These studies summarize a successful strategy for generating and evaluating a series of Ab-conjugated iron oxide NPs which target PSMA. There have been previous reports of PSMA-targeted iron oxide nanoparticles with differing surface chemistries and targeting ligands.^[16] Here we selected silica for NP shelling because it is inert, biocompatible, easily modified, and thermally stable. Moreover, silica provided a rigid and stable coating that maintained NP solubility and stability after routine manipulations such as vigorous pipetting or centrifugation. A sequential NP synthesis approach was applied so that a single iron-oxide NP core could be similarly shelled with silica containing variable amine densities for antibody conjugation. Two custom assays were developed to screen the resulting particles for PSMA-targeting ability. The application of multi-well plate spectrophotometry assay enabled evaluation of small μg quantities of modified MIONs for immunoreactivity with low volumes of reagents and standard laboratory equipment. The ELISA-based cell binding assay applied an isogenic cell line pair provided for a well-controlled system for assaying ligand-specific MION-binding. This assay and provided insights into non-specific cell binding or off-target effects of some NP formulations. Both of these assays can be applied in a high-throughput manner to assess the optimal target specificity of a series of nanoparticle in short time. It is notable that the plates can coated and fixed in advance and stored for future use. Interestingly, our results indicate that increased density of PEG and targeting ligands on the NP surface does not necessarily enhance MION targeting. Rather, there is an optimized combination of surface chemistry, PEGylation and ligand density that determines the targeting potential of a NP. These techniques may be applicable to the development of other iron oxide NPs. Future work involving tumor specific imaging and/or therapy of the MION-1.20-Ab formulation will be necessary to determine the in vivo targeting potential of the optimized MIONs.

Experimental Section

Surface coating and antibody conjugation of MIONs

Silica shelling of MIONs was performed using standard silica shelling conditions followed by PEGylation with NHS-PEG-mal. TCEP reduced Ab was reacted with the maleimide group in order to synthesize Ab conjugated MIONs. The details of the process are described in supporting information.

Immunoreactivity assay

Protein A, Anti-GFAP, BSA and Anti-IgG Ab were diluted to 0.2 mg/mL in PBS and 50 μ L of each stock was added to each well of a 96 well plate. After incubating for one hour at 37° C, the solutions were removed and washed 3 times with PBS. Plates were then blocked with 100 μ L of StartingBlock solution (Pierce Biotechnology, Rockford, IL) for one hour. After washing, 50 μ L of various MION (+/- J591) samples in 1:10 dilutions of StartingBlock solutions were added, incubated for one hour at 37° C, washed three times with PBS, and the iron content measured by absorbance at 350 nm using a Molecular Devices Spectramax 250 plate spectrophotometer. Assay sensitivity and linearity were determined using serially diluted MIONs in 100 μ L of PBS.

Cell based ELISA assay

The PSMA positive LMD-MDA-MB-231-PSMA and parental LMD-MDA-MB-231 cells were plated in a 96 well plate at densities of 50,000–60,000 cell per well. The next day the confluent layer of cells were first fixed with 4% para-formaldehyde and then blocked with 3% BSA. After washing with PBS, 50 μ L of different MION (+/- J591) or J591 alone (in different concentrations) were added in triplicate to various wells. The plate was kept at 37° C for 45 min. Sample plates were washed three times with PBS/0.1% tween-20. Anti-human HRP conjugated Ab (1: 20,000 dilutions in PBS) was then added to all wells and kept at 37° C for 45 min. After three PBS washes, 100 μ L of Pierce OPD substrate was added to each well and incubated for 20 minutes at room temperature. After 20 minutes, absorbance at 450 nm was recorded for each well in a Molecular Devices Spectramax 250 spectrophotometer.

ICP-MS

Following the ELISA, plates were washed with PBS (3X) and 20 μ L of concentrated nitric acid was added to each well. After incubating at room temperature overnight, samples were transferred to a 15 mL conical flask. The wells were rinsed with 20 ppb EPA 200.7 water and transferred to the same conical. The total mass of each sample was adjusted to 2.00 g and samples were analyzed by Thermo Scientific X series ICP-MS

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this project was supported by Safeway Foundation and the Prostate Cancer Foundation, a gift from David Koch Foundation and by Award Number U54CA143803 from the NCI. We thank DS Coffey for his suggestions and leadership in this project.

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Figure 1. Iron-absorbance assay to detect Ab-targeted MIONs. (The inset shows linear detection of NP dilutions with absorbance at 350 nm). A 96-well plate was divided into four quadrants and coated with non-specific (BSA or Anti-GFAP), or Ab-specific (anti-IgG or Protein-A) capture agents. The capture of various MIONs (+/- J591) was quantified by iron absorbance at 350 nm. Results are representative of three experiments, each run in duplicate, beginning from citrate-coated MIONs to final product binding analyses. Error bars represent standard deviation

Figure 2. PSMA expression in isogenic cell lines. (A) Western blot detection of PSMA in the isogenic cell line pair LMD-MB-231 and LMD-MB-231-PSMA. LNCaP prostate cancer cells were utilized as a positive control (B) Relative protein quantification of PSMA expression as normalized by GAPDH

Figure 3.

The isogenic-cell based ELISA assay: Isogenic PSMA positive and PSMA negative cells were treated with various MIONs (+/- J591). After washing, MION binding was detected with HRP-conjugated anti-human IgG ELISA by absorbance at 450 nm. (Insert: Assay specificity and sensitivity using free J591). Results are representative of three separate experiments.

Figure 4. Validation of PSMA-specific targeting by MION-1.20-J591: The level of bound MION-1.20-J591 to PSMA +/- cells was quantified for iron by ICP-MS. Results are described in iron parts per billion (ppb). Error bars represent standard deviation.

Scheme 1.

Development and screening of a series of PSMA targeted MIONs.

Table 1

The formulation of various MIONs with varied amine densities

NP	TEOS (μL)	APTES (μL)	APTES: TEOS	Amine density
MION-1.5	329	66	1:5	Highest
MION-1.10	329	33	1:10	High-Med
MION-1.20	329	16.5	1:20	Medium
MION-1.40	329	8	1:40	Low-med
MION-1.80	329	4	1:80	Lowest

Table 2

The average zeta potential and hydrodynamic diameter before and after Ab conjugation and expected relative Ab densities

NP	Before Conjugation		After conjugation		Expected Ab density
	Ave Zeta (mV)	Ave Diameter (nm)	Ave Zeta (mV)	Ave Diameter (nm)	
MION-1.5	1	123	-2	116	Highest
MION-1.10	0	109	-3	102	High-medium
MION-1.20	-2	98	-9	111	Medium
MION-1.40	-7	94	-7	99	Low-med
MION-1.80	-1	139	-5	103	Lowest