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Quantitative [Fe]MRI Determination of the Dynamics of PSMA-Targeted SPIONs Discriminates Among Prostate Tumor Xenografts Based on Their PSMA Expression

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Abstract

Background—There is a need for a quantitative MRI method for iron concentration magnetic resonance imaging suitable for measuring the delivery of targeted superparamagnetic iron oxide nanoparticles (SPIONs) to tumors.

Purpose—To apply our newly-developed [Fe]MRI method to the quantitative imaging in both space and time of the iron dynamics of anti-prostate specific membrane antigen (PSMA) conjugated SPIONs within human prostate tumor xenografts in nude mice.

Study Type—Longitudinal

Animal Model—45 Harlan Sprague Dawley athymic nude mice bearing xenografts from PSMA-positive LNCaP, C4-2 and PSMA-negative DU145 tumors from human prostate tumor cell lines.

Field Strength/Sequence—1.0 Tesla/ T₁ and T₂ weighted spin echo

Assessment—Image intensity and contrast measurements

Statistical Tests—Student's t-test

Results—The SPION diffusion coefficient within tumors was $D = 44.8 \pm 2.4 \times 10^{-6} \text{ mm}^2/\text{sec}$. The iron taken up by PSMA-positive LNCaP and C4-2 tumors was proportional to the tail-vein injected dose from 60 nmoles to 1.6 micromoles; injection of 1 micromole of iron in anti-PSMA conjugated SPIONs resulted in a tumor [Fe] of 76 μM . Even at the highest iron dose of 1.6 micromoles, the PSMA-negative DU145 tumors took up no significant iron from the anti-PSMA conjugated SPIONs. A similar lack of non-specific uptake was observed when the antibodies against PSMA were omitted from the injected SPION preparation. The fraction of the initial iron dose that was taken up by PSMA-positive tumors was $2.32 \pm 0.75 \%$ ($n = 10$); uptake by the PSMA-negative DU145 tumors and for SPIONs without anti-PSMA antibodies was $0.16 \pm 0.34 \%$ ($n = 7$) giving a ratio of [Fe] in PSMA+ vs. PSMA- tumors greater than 15:1 ($p = 0.01$).

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Conclusion—Quantitative [Fe]MRI of anti-PSMA conjugated SPIONs discriminated between PSMA-positive LNCaP and C4-2 and PSMA-negative DU145 human prostate tumor xenografts *in vivo*.

Introduction

Applications of magnetic resonance imaging (MRI) in clinical medicine have benefitted from the discovery and deployment of targeted nanomagnetic agents that produce *qualitative* alterations in image contrast *via* perturbations of the relaxation rates of nearby water protons. One popular type of targeted MRI contrast agent, superparamagnetic iron oxide nanoparticles (SPIONs) [1, 2] is available in a range of sizes from 10 nm to 5 μ m and produces contrast changes due to their large induced magnetic moments, which are propagated over long distances. Because targeted SPION preparations can also carry cargoes, such as drugs, siRNAs, microRNAs, or enzymes, and can therefore be both diagnostic and therapeutic [3] it is important to measure the *quantitative* delivery of SPIONs to tumors. The delivery of drugs to tumors could then be studied by first measuring the ratio of iron to drug in the SPIONs and then using the iron concentration in the tumor as a surrogate for the initial drug concentration in the tissue in order to determine whether the drug actually achieves a therapeutic concentration in the tissue of interest.

However, the non-linear nature of the effects of SPIONs on water relaxation presents a unique set of challenges with respect to quantitation. SPIONs are primarily transverse relaxation (T_2 , T_2^*) enhancers that produce negative image contrast, but they can also provide positive contrast through longitudinal relaxation (T_1) enhancement; the final effect on tissue MRI contrast is a function of the iron concentration in the tissue. By taking both transverse and longitudinal relaxation effects into consideration we have found that for biologically-relevant concentrations of SPION iron, in the [Fe] range $\sim$ 1-100 μ M, there is a monotonic relationship between iron concentration and the *difference* between T_1 -weighted and T_2 -weighted MRI contrast [6]. We have used this to develop [6] a general theory of iron-concentration magnetic resonance imaging ([Fe]MRI; pronounced *fem-ree*), based on specific, SPION-induced, transverse *and* longitudinal MRI contrast changes for the non-invasive, quantitative molecular magnetic resonance imaging of targeted magnetic nanoparticles or other magnetic contrast agents in tumors and other biological sites. [Fe]MRI can be implemented on the myriad of MRI instruments available around the world because it requires only a small set of easily measured MRI parameters and the acquisition of pairs of T_1 -weighted and T_2 -weighted images.

In order to use targeted SPIONs for tumor detection by MRI a tumor cell-surface epitope must be identified that discriminates cancer cells from normal, non-malignant cells. A large body of research [7-9] on prostate cancer specific cell markers has led to the identification of prostate specific membrane antigen (PSMA), the folate receptor, as a cell-surface protein highly-overexpressed on prostate tumors in a grade and stage dependent fashion. For example, aggressive, androgen-independent C4-2 prostate cancer cells [10] make more than one million PSMA molecules per cell [11] making PSMA a good candidate for monitoring the presence and progression of prostate cancer. A humanized antibody to PSMA, J591, has been successfully used to target prostate cancer in clinical trials [9]. We have shown that

antibodies to PSMA can be attached to SPIONs which specifically bind to prostate tumor cells [12] and have already used [Fe]MRI to measure the iron concentration, [Fe], in tumor cell preparations *in vitro* [6]. Here, we sought to extend these studies to determine whether quantitative [Fe]MRI of PSMA-targeted SPIONs could discriminate among human prostate tumor xenografts in nude mice on the basis of their expression of PSMA. We also sought to monitor the diffusion, dynamics and targeting efficacy of these SPIONs and to derive their lifetimes and kinetics in xenografts.

Materials and Methods

Materials

MACS® (µMACs) streptavidin superparamagnetic iron oxide nanoparticles (SPIONs: ~50 nm diameter, 57 % iron oxide w/w, dextran coating, 1.67×10^{-17} g Fe/nanoparticle) were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Fully-humanized anti-PSMA antibody J591 was purchased from N. Bander (Weill Cornell Medical College, NY). Anti-PSMA antibody (clone 3C6) was purchased from Northwest Biotherapeutics (Bothell, WA). These antibodies were used interchangeably and were biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin from Pierce (Rockford, IL) according to the manufacturer's instructions. LNCaP and DU145 human prostate cancer cell lines were purchased from the American Type Culture Collection (Manassas VA). The C4-2 prostate cancer cell line was a kind gift from Dr. G.N. Thalmann (University of Bern, Switzerland) [10].

Iron Assays

Solutions containing SPIONs were dissolved in 0.5 ml of 6 N HCl (Sigma-Aldrich, St. Louis, MO) at 95 °C overnight in sealed tubes. Aliquots (0.1 ml) of the resulting solution were assayed for iron spectrophotometrically using the Ferrozine assay (Sigma-Aldrich, St. Louis, MO) and reading the absorbance at 562 nm. Standard curves (n = 7) prepared using 1.00 mM FeCl₃ (Sigma-Aldrich, St. Louis, MO) gave an extinction coefficient of $27,300 \pm 971$ liters/mole-cm. Note that here we use the chemist's notation for the iron concentration as [Fe].

Cell culture

Human LNCaP, C4-2, and DU145 prostate cancer cells were cultured in T-medium with 10% fetal calf serum (Hyclone, Logan, UT) [8] at 37 °C in a humidified 5% CO₂ atmosphere. Upon reaching 90% confluency, cells were collected either by detachment in 0.5% trypsin containing 0.02% EDTA or detached in cold phosphate buffered saline (PBS; pH 7.41) using cell scrapers. Cells (6.5×10^6) were concentrated by centrifugation at $5000 \times g$ for 10 min, and resuspended in 1% matrigel in PBS (100 µl) for sterile injection into the flanks of athymic nude mice (*vide infra*). Prior studies [11] have shown that LNCaP and C4-2 cells expressed $(1.05 \pm 0.10) \times 10^6$ and $(1.67 \pm 0.28) \times 10^6$ PSMA molecules per cell, respectively, while DU145 cells expressed 100-fold less at only $(1.35 \pm 0.52) \times 10^4$ PSMA molecules per cell. Subcutaneous xenografts in nude mice used LNCaP and C4-2 cells as PSMA positive samples, and DU145 cells as negative controls based on these measurements in cultured cells [6].

Antibody conjugation and SPION labeling

Sulfo-NHS-LC-Biotin groups were attached to primary amines of MAb 3C6 using the EZ-Link™ Sulfo-NHS-LC-Biotin kit according to the manufacturer's protocol. Conjugated MAb 3C6 was separated from low molecular weight compounds using a Millipore Ultrafree®-MC Centrifugal Filter Unit at a force of 3000 g in a fixed angle microfuge rotor. Antibody concentration was determined using the Pierce Protein Assay Reagent Kit, and the ratio of biotin/antibody was determined with a Pierce HABA colorimetric assay, both according to the manufacturer's protocol. Later experiments utilized J591, which was biotinylated using the same method as 3C6 and attached to streptavidin-conjugated SPIONs by incubation for 30 min. The free antibody was removed by washing the beads with the aid of a magnetic bead separation device (Miltenyi, Inc.). The ratio of antibody to SPION was determined from the protein and Fe assays; a typical SPION contained ~50 antibody molecules. Control SPIONs were also prepared by omitting the antibody conjugation step.

Animals

Forty five 5 to 6-week old athymic nude male mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis IN). All animal procedures were approved by our local animal use committee. Tumors were produced by injecting 100 µl of the cell:matigel mixture (containing 6.5×10^6 cells) in PBS subcutaneously into the abdominal flank skin of nude mice. Tumors were allowed to grow while the animals were monitored on a daily basis and the tumor volume was measured weekly with a caliper. The animals were used for MRI when the resulting tumors reached a volume of ~1 ml, which took 55-65 days. A total of 53 tumors were grown consisting of 21 LNCaP tumors, 15 C4-2 tumors and 17 DU145 tumors. The number of tumors differed from the number of animals because tumors did not reach sufficient size within 2 months in 8 mice, and sixteen mice were injected with tumor cells in both flanks, producing dual-tumor animals in which direct comparisons among tumors types could be made in the same animal; these dual-tumor mice were distributed into five groups (using the abbreviations L = LNCaP; C = C4-2; and D = DU145) containing four L/L mice; one D/D; three D/L; 3 C/C and 5 D/C. No difference in tumor growth rates were found when the growth in dual-tumor mice was compared with the growth in single-tumor mice.

Anesthesia

For the MRI studies, the tumor-bearing mice were anesthetized with 1 % isoflurane mixed with oxygen. Pre-injection images were obtained in order to measure the necessary tumor water relaxation times and the relaxation-weighted tumor MR signal backgrounds. Then aliquots of the SPION preparations were either directly injected into the tumors, or into the tail veins of the mice. Time courses of the tumor MR intensities were acquired for durations of up to ~2 hours, after which the mice were removed from the magnet, allowed to recover, and later reanesthetized for the determination of additional time points. For studies requiring MR scans the next day, the mice were removed from the magnet, allowed to recover overnight, and the next day reanesthetized for the determination of additional time points. For measurement of the dose-response curve for PSMA-conjugated SPIONs the [Fe] in the injections was varied from 0 to ~1 mg/mL.

Magnetic resonance imaging

MR imaging was carried out using a horizontal bore instrument at 1.0 T (MRT Systems, Tsukuba, Japan) with a custom 50 mm dia. 10-turn solenoid coil. All 2D images were acquired with a 32 mm FOV, a 128×128 image matrix, 0.25 mm in-plane resolution, 9 slices each 2.5 mm-thick, and $nex = 8$. T_2 -weighted (T_2w) images were acquired using a spin echo (SE) sequence with $TE = 23.5$ ms and $TR = 1000$ ms. T_1 -weighted (T_1w) images used $TR = 500$ ms and $TE = 4$ ms. The spin-lattice relaxation time (T_1) for tumor water was determined using an inversion-recovery sequence of spin echo images with an echo time (TE) of 10.7 ms and variable recycle time (TR) ranging from 0.01 to 10 sec. Regions of interest (ROIs) were selected corresponding to the psoas muscle and the implanted tumors. The signal intensity S of an ROI was plotted as a function of recovery time TR, and T_1 was determined by a 3-parameter fit to the function $S(TR) = S_{\infty}(1 - P \exp(-TR/T_1))$, where S_{∞} was the fully-recovered signal intensity and P was a measure of the level of inversion achieved ($P = 2$ for perfect inversion). Using a series of SE images with different TE values, T_2 decay curves were generated for each of the tissue components by selecting an ROI corresponding to each component (psoas muscle and tumor) and plotting the mean signal intensity, S , of the ROI as a function of TE. T_2 was determined by fitting the decay curves with an exponential, $S(TE) = S_0 \exp(-TE/T_2)$, where S_0 is the signal intensity for $TE = 0$, and T_2 was the desired time constant.

SPION Relaxivities

The relaxivities of the SPIONs were determined by dissolving them in 1 % agarose gels and measuring the water relaxation times (T_1 , T_2) for various [Fe] values; these relaxation times were inverted to produce relaxation rate enhancements ($R_1 = 1/T_1$, $R_2 = 1/T_2$) which were then plotted as a function of [Fe]. Linear least squares fits to these data gave the relaxivities, r_1 and r_2 as the slopes of the fitted lines.

SPION Diffusion within Tumors

The SPIONs locally altered the intensity of the tumor's T_1w MR signal, but did not immediately distribute themselves uniformly within the tumor parenchyma after direct intra-tumoral injection, we were able to monitor the diffusion of the SPIONs away from the injection site. The pixel area of the altered MR signal was converted to real space using the calibrated gradient value of 0.25 mm/pixel and measured over time in T_1w images; these areas, A , were then converted to an effective diffusion radius using $r = \sqrt{A/\pi}$ and plotted *versus* $\sqrt{t}$, where t was the elapsed time after injection. The slope of the resulting linear least squares fit to the data was $\sqrt{2D}$, where D was the diffusion coefficient.

Image Processing

The complex, time-domain MRI datasets were baseline-corrected, Fourier-transformed and co-added using Mathematica version 10.0.2 (Wolfram Research, Urbana, IL) to produce the frequency domain images of each MRI slice. The average and standard deviation of the pixel intensities in the regions of interest within the tumors were measured with ImageJ Software [14]. Qualitative image contrast was judged visually by comparing the intensity of a given region on an MR image with the intensities of other organs or regions (reference regions)

and was reported as equal to (isointense) less than (hypointense) or greater than (hyperintense) with respect to the reference region. Quantitative tumor image contrast was defined as $C(x, y) = [A(x, y) - B(x, y)]/B(x, y)$, where $B(x, y)$ was the background MR signal intensity from the pixel at (x, y) in the tumor region of interest before the injection of SPIONs, while $A(x, y)$ was the MR signal intensity of the tumor pixel within the same slice after the injection of SPIONs (either directly into the tumor, or by injection into the tail vein). Note that contrast defined here can range from $C = +\infty$ to $C = -1.0$, with positive (negative) contrast indicating that the object of interest was hyperintense (hypointense) with respect to the background, pre-injection, signal from the tumor.

Summary of the [Fe]MRI Method

The details of the [Fe]MRI method for the quantitative MR imaging of the added iron concentration in cells are given in reference 6 (*q.v.*) and are briefly summarized as follows. Pairs of T_1 -weighted and T_2 -weighted MR spin-echo images were converted into contrast images, C_1 and C_2 , and these were subtracted to produce a contrast difference image, $C = C_1 - C_2$. The theoretical dependence of [Fe] on the image contrast difference, C , between T_1 -weighted and T_2 -weighted MR images, its inversion to produce [Fe] as a function of contrast difference, as well as code to convert the MR images into [Fe] images were developed in Mathematica [6.] The [Fe]MRI procedure was as follows:

1. The r_1 , and r_2 relaxivities of the SPIONs were measured. Here: $r_1 = 40.6 \pm 1.1$ Hz/mM and $r_2 = 890 \pm 15.9$ Hz/mM (mean $\pm$ S.D.).
2. The T_1 and T_2 relaxation times of the tissues of interest were measured. Here, for the tumors, $T_1 = 720$ ms and $T_2 = 100$ ms.
3. A pair of control images with T_1 and T_2 weighting were taken to give the tumor backgrounds, $B_1(x,y)$ and $B_2(x,y)$ in the region of interest.
4. The SPIONs were either directly injected into the tumor, or into the tail vein.
5. Another pair of SE MR images was taken with T_1 and T_2 weighting to give MR signal intensities $A_1(x,y)$ and $A_2(x,y)$, respectively.
6. Each of these images was converted into contrast maps, $C(x,y)$, using the measured T_1 w, or T_2 w, backgrounds, $B_1(x,y)$ and $B_2(x,y)$ producing:

$$C_1(x, y) = (A_1(x, y) - B_1(x, y))/B_1(x, y)$$

and

$$C_2(x, y) = (A_2(x, y) - B_2(x, y))/B_2(x, y)$$

7. The difference between these two contrast maps was a contrast difference map $C(x,y) = C_1(x,y) - C_2(x,y)$ whose intensity was proportional to the Fe concentration, $[Fe] = g(C)$. The function g was calculated in Mathematica® from the relaxivities, the relaxation times and the acquisition parameters, using

our published method [6] as follows: The contrast difference (C : the green curve in Fig. 2) was numerically computed using the equations in [6] giving C as a function of $[Fe]$ as a Table with 242 values of C vs. $[Fe]$ over the range 0 $[Fe]$ 0.12 mM (= femax). These Table data were fitted to a third order polynomial with the result that $C = f([Fe]) = -0.01672 + 28.41 [Fe] - 224.6 [Fe]^2 + 565.8 [Fe]^3$. Although this function could be used in a look up table to provide the $[Fe]$ for a particular C value, that approach did not readily lend itself to the direct conversion of C images to $[Fe]$ values. For this, one needed to invert this function, $C = f([Fe])$, to give $[Fe] = g(C)$ where $g = f^{-1}$. The Mathematica tool that accomplished this was the Interpolation function: `[Fe] = Interpolation[Table[{ C, [Fe] }, {[Fe], 0, femax, 0.002}][,]` and then this interpolation function was fitted to a third order polynomial used in the direct conversion of C images into $[Fe]$ images. In this case $[Fe] = g(C) = -0.0004935 + 0.05250 C - 0.04408 (C)^2 + 0.05275 (C)^3$. The units of $[Fe]$ were mM because the relaxivities were measured in Hz/mM. Note that in the absence of SPIONs, $C = 0$, and the difference image was essentially zero giving an $[Fe] \sim 0.49 \mu M$. This was smaller than the background noise limit in the $[Fe]$ MR images of $[Fe] \sim 2 \mu M$ (*vide infra*) a value which set the lower limit of iron detection with this method [6]. The Mathematica code also served as an essential guide for planning experiments in order to achieve the optimal sensitivity of contrast to $[Fe]$. We would be glad to provide this Mathematica notebook to the interested reader via email.

8. The intensity of this difference image, C , was converted, using the computed relationship between $[Fe]$ and contrast difference, $[Fe] = g(C)$, to an image whose voxel intensity directly gave the $[Fe]$ within the voxel. The reported errors are the standard deviations of the average $[Fe]$ values within the tumors.

A technical note to be aware of is the fact that MR images are commonly presented as magnitude data calculated from the acquired complex data sets. The result is that the image noise in otherwise signal-free regions is also presented as magnitude data and hence acquires a positive, non-zero mean. While this is of little concern for most MR imaging applications, its impact here was to produce images of the iron concentration ($[Fe]$ MR images) that appeared to contain a background non-zero $[Fe]$ in image regions that contained no MR signal. We have therefore measured this noise background of $[Fe] \sim 2 \mu M$ in the $[Fe]$ MR images and subtracted it from the reported $[Fe]$.

Results

MRI Contrast in C4-2 Xenografts after Intratumoral Injections of SPIONs

MR images of the human C4-2 prostate tumor xenografts, prior to SPION injection, visually showed the tumors as regions isointense (See Methods) with the psoas muscle in T_1w images (Fig. 1A) but hyperintense (Fig. 1B) in T_2w images. Moreover, in both types of MR image the tumors visually appeared uniform in intensity with little pixel to pixel variation in the MR signal. Direct injection of anti-PSMA-conjugated SPIONs containing 0.24 mM iron into the tumor was followed by local alterations in the MR signal in both types of image

with an area of hypointensity observed in the T₂w image (Fig. 1 D) and a smaller hypointense area in the T₁w image (Fig. 1 C). The region of hypointensity in the T₂w image expanded with time as the SPIONs diffused throughout the tumor (Fig. 1F&H). On the other hand the initial hypointense region seen at early times in the T₁w image (Fig. 1C) became isointense by 177 minutes (Fig. 1E) and became hyperintense in the T₁-w image taken 416 min later (Fig. 1G).

Theoretical Dependence of Contrast on [Fe]

Our theory, using the measured relaxivities of the μ MACs SPIONs at 1.0 T and the stated acquisition parameters, predicted that the T₂w image contrast was negative for all the [Fe] values, while the T₁w image contrast was positive up an [Fe] of $\sim 200 \mu\text{M}$ (Fig. 2). Note that the contrast difference was always positive, and was a monotonic, increasing function of [Fe], within the physiological range of iron concentration from 1 to $\sim 150 \mu\text{M}$.

Measurement of the SPION diffusion coefficient from boundary movement

A control T₁w MR image, taken prior to anti-PSMA-conjugated SPION injection, of a mouse bearing a C4-2 tumor xenograft (Fig. 3A) displayed the uniform MR signal intensity distribution seen earlier (Fig. 1A) for these tumors. Intratumoral injection of 20 μL of the SPION preparation containing 0.4 mM iron (Fig. 3B) altered the MR image obtained 41 min later by producing a visually hypointense central region in the tumor where the [Fe] was so high that even in this T₁w image the MR signal was almost completely quenched. As the SPIONs diffused outwards from this central reservoir into the tumor parenchyma they became diluted by about 4-fold and their effect on the MR signal changed to one of visual hyperintensity (Fig. 3 B&C) congruent with our theory (Fig. 2). By measuring the number of bright pixels in these images and converting the area to a radius using the known gradient calibration (See methods) the time dependence of the boundary was used determine the SPION diffusion coefficient $D = 44.8 \pm 2.4 \times 10^{-6} \text{ mm}^2/\text{sec}$ within the tumor.

[Fe]MRI After Intratumoral Injections of SPION

Control [Fe] MR images of a C4-2 tumor (Fig. 4A) taken prior to the injection of the PSMA-targeted SPIONs gave an average tissue background [Fe] of $1.6 \pm 1.3 \mu\text{M}$ ($n=5$), a value statistically indistinguishable from zero and congruent with our previously determined limit of detection of $\sim 1\text{-}2 \mu\text{M}$ [6] for this [Fe]MRI method. A [Fe] MR image of this mouse, taken 1420 minutes after intratumoral injection of $2 \times 20 \mu\text{L}$ of anti-PSMA-conjugated Macs SPIONs, containing 13.4 μg Fe ([Fe] = 0.24 mM), showed the injected SPIONs as a visually hyperintense region within the tumor parenchyma (Fig. 4D) as both 3D surface plots, using a rust-colored scheme (Mathematica's "SunsetColors" reversed) for mapping [Fe] to color (Fig. 4B&E), and as 2D plots using the same color scheme (Fig. C&F). Here, the iron signal was uniformly positive since it was derived from the positive contrast difference between the C₁ and C₂ MR images (See Methods and Fig. 2, green curve).

Time course of the [Fe] after Intratumoral Injections of SPIONs

Quantitative measurements of the [Fe] within a tumor in real-time after direct injection 9.6 nmoles of SPION iron (Fig. 5) showed that the average [Fe] within the tumor increased

within 30 minutes to 9.05 ± 0.90 nmoles (or 94 % of the injected dose) and remained constant for up to a day post-injection.

[Fe]MRI After Tail Vein Injections Injections of anti-PSMA Conjugated SPIONs

Both single-tumor (Fig. 6A) and dual-tumor (Fig. 6B) mice (See Methods) were used for tail-vein injections. An anatomical spin-echo image taken through the midsection of a mouse in which a LNCaP tumor was grown on one flank and a DU145 (Fig. 6B) on the other showed (Fig. 6D) both tumors within the same MRI slice. A 3D plot of an [Fe]MR image generated prior to SPION injection (Fig. 6C) showed that the [Fe] image contained no iron signal, above the background noise, from either tumor. Eleven hours after the administration of anti-PSMA-conjugated SPIONs the [Fe] MR image of this mouse revealed (Fig. 6E) uptake of these SPIONs by the PSMA-positive LNCaP tumor to a concentration of $\sim 100 \mu\text{M}$, but essentially no uptake by the PSMA-negative DU145 tumor in the same animal. We confirmed this iron uptake post-mortem by excising both tumors and staining them for iron with the result that the LNCaP tumor stained (Fig. 6F) for iron with Perl's reagent, but the DU145 tumor did not (Fig. 6G). This optical image of the LNCaP tumor also showed that the SPIONs were taken up by the $\sim 12 \mu\text{m}$ dia. cells because the cytoplasm stained blue in agreement with the fact that anti-PSMA antibody binding to the PSMA molecule on the tumor cell surface triggered internalization of the complex [12,13]. Control injections of SPIONs lacking anti-PSMA antibodies into mice bearing LNCaP, C4-2 or DU145 tumors produced [Fe]MR images that were devoid of an [Fe] signal greater than background (*vide infra*).

The time course of SPION uptake in prostate tumor xenografts

[Fe] MR images (Fig. 7 A&C) taken prior to tail-vein injection of anti-PSMA conjugated SPIONs showed no added iron within either a DU145 tumor (Fig. 7A) or an LNCaP tumor (Fig. 7C). An anatomical reference T_2w MR image of the same slice through the mouse (Fig. 7B) showed the locations of the tumors. 3D surface plots (Fig. 7 D&E) showed the lack of added iron within each tumor as well as the background noise prior to the injection of the SPIONs.

After the injection of anti-PSMA conjugated SPIONs we observed a rapid uptake of iron in only the PSMA-positive LNCaP and C4-2 tumors, but essentially no uptake by the PSMA-negative DU145 tumors. For example, Fig. 8A shows a typical [Fe] MR image of a DU145 tumor taken 123 minutes post-injection as a 3D surface plot from the region of interest (Fig. 8C; yellow rectangle) whose anatomical MR image is shown in Fig. 8B. This [Fe]MR image showed no iron uptake. At later times (666 minutes, Fig. 8E; and 1242 minutes, Fig. 8I) there was still no iron uptake by this PSMA-negative tumor (See also Fig. 8 G&K). The resulting uptake of iron by the LNCaP tumor, on the other hand, was observed as early as 123 minutes post-injection (Fig. 8 C&D). This continued at 666 minutes and diminished at 1242 minutes (See also Fig. 8 G&K). The average [Fe] within the LNCaP tumor between 123 and 666 minutes post-injection was $\sim 150 \mu\text{M}$. The 3D surface plots also showed that the distribution of iron within the tumor was not completely uniform (Fig. 8 D&H) congruent with the optical findings (Fig. 6F).

The time course of iron uptake by both PSMA-negative DU145 and PSMA-positive C4-2 and LNCaP tumors as a result of the injection of 60 – 160 nmoles of iron in the form of anti-PSMA conjugated SPIONs (Fig. 9) showed that the [Fe] rose, reaching a maximum within 30 minutes in the PSMA-positive tumors, but never rose above the background limit of detection of $\sim 2 \mu\text{M}$ for the PSMA-negative tumors. The results in Fig. 9A also showed that the iron uptake by PSMA-positive tumors increased in proportion to the amount of injected iron. As controls we used, in addition to the PSMA-negative DU145 tumors, SPIONs to which no antibody was attached in both PSMA-positive and PSMA-negative tumors and these results from $n = 17$ measurements (the blue line in Fig. 9A) showed that without a targeting agent directed at the tumor there was no iron uptake. The injection of larger amounts of iron, up to 1.6 μmoles , did not appear to alter the [Fe] dynamics (Fig. 9B) with the maximum [Fe] occurring around 10-12 hours post-injection. Even at this high dose of iron the DU-145 tumors did not appear to sequester amounts of iron higher than the background detection limit. The iron from the injected SPIONs appeared to be metabolized by 26 hours because the [Fe]MRI signals returned to essentially their pre-injection values by this time (Fig. 9 A&B).

The Quantitative Dose-Response for [Fe] discriminated Prostate tumors on the basis of their PSMA expression

The response of the tumor's [Fe] to the tail-vein injection of varying amounts of iron contained in SPIONs was determined over an injection range from 60 nMoles to 1.6 microMoles with the results shown in Fig. 10. The uptake of iron by PSMA-positive tumors was roughly proportional to the amount injected; the slope of the line fitted to the data indicated that an injection of anti-PSMA conjugated SPIONs containing 1 micromole of iron resulted in a tumor [Fe] of 76 μM , which resulted in the delivery of ~ 76 nmoles of iron to a tumor with a volume of 1.0 mL. It is also seen in Fig. 10 that even at the highest iron dose of 1.6 micromoles, the PSMA-negative DU145 tumors did not non-specifically take up iron from the anti-PSMA conjugated SPIONs in the circulation (Fig. 10 B; green and blue curves). A similar lack of non-specific uptake was observed when the antibodies against PSMA were omitted from the injected SPION preparation (Fig. 10 A; blue curve).

We measured the fraction of the initial iron dose that was taken up by the PSMA-positive tumors to be $2.32 \pm 0.75 \%$ ($n = 10$), while that for the DU145 tumors and for the SPIONs lacking anti-PSMA antibodies to be $0.16 \pm 0.34 \%$ ($n = 7$) from which we calculated a ratio of iron uptake by PSMA+ to PSMA- tumors of at least 15:1 ($p = 0.01$).

Discussion

We have previously developed and validated a general, quantitative MRI theory for the measurement of nanoscale magnetic objects introduced into biological systems [6]. This method, which we refer to as iron concentration magnetic resonance imaging, or [Fe]MRI, uses only the $T_1\text{w}$ and $T_2\text{w}$ spin-echo images available on any MRI system, and was applied here for the determination of the iron concentration in human prostate tumor xenografts after the injection of anti-PSMA-conjugated SPIONs. One potential use for these PSMA-targeted SPIONs is for the specific MR imaging and detection of prostate tumors, but it first needed

to be demonstrated that these SPIONs discriminated among prostate tumors *in vivo* on the basis of their expression of this important cell-surface marker, as we previously found for prostate tumor cells [6]. Our primary goal then was to apply [Fe]MRI to the quantitative imaging of the iron concentration, in human prostate tumor xenografts *in vivo* in nude mice, in order to determine whether measurements of the dynamics of anti-PSMA conjugated SPIONs discriminated PSMA-Positive from PSMA-negative Prostate Tumors.

In intratumoral injections of these SPIONs we observed [Fe]-dependent MRI contrast changes *in vivo* that developed in time in a manner congruent with our quantitative [Fe]MRI theory. The contrast calculations shown in Fig. 2 for an injection with an initial [Fe] of 0.24 mM predicted an initial contrast of -0.16 and -0.98 for the T₁w and T₂w images, respectively, while the measured contrast from the images (Fig. 1 C&D) gave the closely-related values of -0.21 ± 0.05 and -0.88 ± 0.31 . The reversal of the sign of the contrast seen in the T₁w images (Fig. 1C, E, G) as time progressed after the SPION injection was also congruent with the theory (Fig. 2) because, as the SPIONs diffused away from the injection site, the local [Fe] decreased to ~ 0.2 mM at 3 hours where Fig. 2 showed that the T₁w contrast was zero. By 7 hours post-SPION-injection the calculations indicated that the [Fe] decreased further to ~ 0.16 mM where the T₁-w contrast became slightly positive at 0.24 ± 0.04 , while the T₂-w contrast remained negative (-0.79 ± 0.12).

These contrast changes were used to measure the SPION boundary movement into the tumor parenchyma after direct intratumoral SPION injection to give the diffusion coefficient of the SPIONs. The value found of $D_{\text{SPION}} = 4.48 \pm 2.4 \times 10^{-7} \text{ mm}^2/\text{sec}$ was four orders of magnitude smaller than that for pure water at 37 °C ($D_{\text{H}_2\text{O}} = 2.9 \times 10^{-3} \text{ mm}^2/\text{sec}$). The diffusion coefficient for water in tumor cells was known to be smaller ($\sim 5 \times 10^{-4} \text{ mm}^2/\text{sec}$; [15]) than that for pure water due to the increased viscosity of the cytoplasm and from restrictions imposed by organelles. The ~ 50 nm dia. SPIONs used here had a mass of approximately 10^7 Da (1.7×10^{-17} g) and diffused much like spherical viruses. For example, bushy stunt virus has a similar mass ($\sim 10^7$ Da) and diffusion coefficient of $\sim 1.3 \times 10^{-7} \text{ mm}^2/\text{sec}$ [16]. However, since the density of iron oxide in our SPIONs was much higher ($\sim 5.5 \text{ g/cm}^3$) than that of viral particles, a better comparison was with 100 nm polystyrene spheres ($D \sim 6 \times 10^{-6} \text{ mm}^2/\text{sec}$ [17]) or metal nanoparticles ($D \sim 2 \times 10^{-6} \text{ mm}^2/\text{sec}$ [18]). The diffusion of the SPIONs appeared to be isotropic within the tumor tissue in three separate experiments.

Although the direct injection of SPIONs into tumors could be used to gather diffusion data simply from the observed boundary movement, further analysis required that the actual concentration of iron within the tumor be determined. As a guide in this direction, we used our theory of the MRI contrast which we found to correctly predict the contrast for various concentrations of iron. When applied to tumors with direct SPION injection we could measure the actual iron concentration and follow it as a function of time *in vivo*. We found that we could account for greater than 90% of the injected iron by means of [Fe]MRI and that the injected iron remained within the tumor for up to 24 hours. However, one of the drawbacks of our [Fe]MRI method was that the high iron concentration at the injection site could saturate the MRI response of the tissue water and render the tumor region completely black, again understandable on the basis of our calculations. Direct injection was also not

particularly meaningful from a physiological point of view since drugs, such as those used for chemotherapy, are often administered intravenously.

We therefore undertook a series of studies of the dynamics of our anti-PSMA conjugated SPIONs using tail vein injection into the circulation. [Fe] MRI was used to quantitatively monitor the uptake and release of the SPIONs in human prostate tumor xenografts. We were aided in the discrimination of tumors based on their cell-surface expression of PSMA by the development of our dual-tumor model in which separate tumors were introduced into each flank of a nude mouse. This was particularly useful when these two tumors differed in PSMA expression, such as for the pairs DU145/LNCaP or C4-2/DU145 where we generated both a PSMA-positive and a control, PSMA-negative tumor within the same animal. Then our [Fe] MRI technique enabled the separate measurement of the time dependence of the [Fe] simultaneously in each tumor.

By these means we showed that iron uptake by the tumors depended on their expression of PSMA and that the iron content of the PSMA-positive tumors reached a maximum within ~30 minutes after tail vein injection of the SPIONs, a time that was determined by the delivery of the SPIONs to the tumors *via* the circulation and the speed with which the mice could be reimaged after injection. The [Fe] in PSMA-positive LNCaP or C4-2 tumors remained elevated for 11-12 hours and then decreased so that by ~24 hours later it had essentially returned to pre-injection levels. Numerous control experiments in which we used SPIONs that were prepared without antibodies supported this conclusion because when these were injected into the circulation of animals bearing PSMA-positive C4-2 and LNCaP tumors we observed no iron uptake in the absence of a targeting ligand. No iron uptake was observed in PSMA-negative DU145 tumors, either using anti-PSMA-conjugated SPIONs or SPIONs containing no anti-PSMA antibodies.

Our quantitative [Fe]MRI measurements enabled us to estimate the iron content per tumor cell. For the highest injected dose of 1.6 micromoles of iron, we found that the average tumor [Fe] was 150 μM . If a tumor cell was assumed to be a cube 10 microns on a side and that the tumor volume was 1 mL, then a tumor would contain 10^9 cells. The amount of iron in this volume (150 nMoles) then corresponded to about 1.4×10^{-14} g of iron per cell, or about 850 SPIONs per tumor cell. This value is about 100-fold smaller than previously determined for cell suspensions *in vitro* of 1.74 ± 0.12 pg Fe/cell [6]. This difference could reflect the much higher cell density in tumors *in vivo* compared with cell suspensions *in vitro*.

Because we could measure the actual [Fe] within the tumors in real time, we were also able to determine the dose-response of tumor iron and to measure the fraction of the injected dose that was delivered to the tumors. It is interesting to note that we observed no difference in the iron uptake between LNCaP and C4-2 tumors. This was likely due to the fact that the cells in neither type of tumor would have been saturated with SPIONs because the number of PSMA molecules per tumor cell was on the order of a million while the number of SPIONs per cell was approximately 1000-fold lower; we did not administer sufficient SPIONs to saturate the cells binding sites so that the tumor iron concentration would have been solely determined by the initial dose.

Several areas of future interest for [Fe]MRI include the ability to quantitatively determine the delivery of material incorporated within nanoparticles, such as drugs, RNA or other biological agents and to directly measure the targeting efficiency of nanoparticles *in vivo*. For example, our earlier studies on the treatment of prostate cancer utilized superparamagnetic FePt nanomicelles [19] into which chemotherapeutic drugs were bound [3]. Iron assays and HPLC were used to determine the iron to drug ratio, and by using [Fe]MRI could potentially use the iron measurements as a surrogate *in vivo* for the concentration of drug delivered to the tumor. One advantage of the [Fe]MRI method is that it only shows the added iron because the background tumor signal prior to iron addition is subtracted out in the analysis and is therefore insensitive to the background tissue [Fe].

Among the many other potential applications of this method, an important one would be to measure the macrophage density in inflamed tissues by [Fe]MRI. We are currently pursuing a human clinical trial of the application of MRI of SPIONs in the diagnosis of diabetic osteomyelitis [21,22] where large numbers of macrophages invade the tissue. We have used the fact that macrophages take up ferumoxytol (a similar SPION to the ones used here) and home to the site(s) of infection in the bone marrow to specifically image the lesions in the marrow. The macrophage density, and by inference, the severity of the infection could then be judged with the aid of [Fe] determinations in the affected tissues. Other potential applications of [Fe]MRI could be to the measurement of amyloid plaque density or microglial activation in Alzheimer's Dementia [20], or the quantitation of magnetically-labeled stem cells [23,24].

The limitations of [Fe]MRI include the fact that the initial studies of prostate tumors and cells were not yet optimized so the present lower iron concentration that could be reliably measured was $\sim 2 \mu\text{M}$. However, as we have shown for the detection of magnetic nanoparticles in Alzheimer's studies, the MRI detection parameters could be optimized [20] and that this limit of detection could probably be reduced. The upper limit on the iron concentration for the [Fe]MRI method using the MACs SPIONs was in the range $0.7 - 1.0 \text{ mM}$ as determined by the reduction in the contrast change to zero. For the SPIONs used here, the MR signal would be entirely quenched at these iron concentrations. The studies reported here also used average values for the tumor T_1 and T_2 relaxation times. We have found that these vary from point to point within a tumor and among tumors by $\sim 5\%$ so that a more accurate way of computing the [Fe] would take these individual variations into account in the theory.

In conclusion, we have shown that our novel, quantitative [Fe]MRI method can be used to determine the diffusion of SPIONs within tumors, to measure the targeting and delivery of anti-PSMA-labeled SPIONs to tumors, and to ascertain the dynamics of the [Fe] in human prostate tumor xenografts in nude mice. Furthermore, this method can be used to differentiate prostate tumor types expressing large amounts of PSMA from those that do not.

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Figure 1.

T₁-w and T₂-w MR images at 1.0 T before and after the direct injection of anti-PSMA SPIONs into a C4-2 tumor. *Before SPION injection:* (A) T₁-w and (B) T₂-w MR images of a C4-2 human prostate tumor xenograft in a nude mouse. The numbers in the lower right of each image are the times in minutes with respect to the SPION injection. The tumor is shown within the red ellipses in both pre-injection images (A & B). *After SPION injection:* Twenty microliters of anti-PSMA-conjugated Macs SPIONs containing 13.4 μg Fe ([Fe] = 0.24 mM) were injected directly into the tumor. (C) A T₁-w MR image taken 24 minutes post-injection and (D) a T₂-w MR image taken 42 minutes post-injection. (E & G) T₁-w MR images taken 177 and 416 minutes post-injection. (F & H) T₂-w MR images taken 193 and 432 minutes post-injection. As the SPIONs are diluted by diffusion into the tumor parenchyma (E & G) the initial darkening in the T₁-w image (C) changes to hyperintensity. In these and subsequent images the bright circle in the lower left hand corner of (A) and all the other images is from a gadolinium-doped water standard contained in a 1.0 mL syringe.

Figure 2. The theoretical [Fe]-MRI relationship between MR image contrast and the concentration of Fe

The red curve represents the contrast in T₂-w MR images; the blue curve is for T₁-w MR images, and the green curve is the difference in contrast. These curves were calculated from the equations in [Ref 8] using the parameters given in the Methods.

Figure 3. The Diffusion of anti-PSMA conjugated SPIONs into a C4-2 prostate cancer xenograft (outlined in red)

The numbers in the lower right of each image are the times in minutes with respect to the SPION injection. T₁-w MR images (A) Prior to SPION injection. (B) Forty-one minutes post injection. (C) T = 371 min post injection. Note the bright ring within the tumor in (B) that expands in (C) surrounding the injection site. (D) Direct measurement of the diffusion coefficient of the anti-PSMA SPIONs by boundary displacement: $D = 44.8 \pm 2.4 \times 10^{-6} \text{ mm}^2/\text{sec}$.

Figure 4. Tumor [Fe] Quantitation using [Fe]-MRI

(A) This grayscale [Fe] image from the mouse shown in Fig. 1 was derived from the pair of T₁-w and T₂-w axial MR images in Fig. 1 (A&B) using the mathematical analysis outlined in the Methods. The C4-2 tumor region is outlined in red. Since this was an initial [Fe] image prior to the injection of any SPIONs, the tumor appeared black because it contained no added iron. (B) A quantitative 3-dimensional surface plot of the tumor region outlined in red in (A) showed an absence of added iron within the tumor. (C) An extracted [Fe] image of the tumor from (A) was colored to reflect the iron intensity scale shown in the legend to the right. (D) A [Fe] MR image of the mouse 1420 minutes after 20 μ L of anti-PSMA-conjugated Macs SPIONs containing 13.4 μ g Fe ([Fe] = 0.24 mM) were injected directly into the tumor. The injected SPIONs appeared as a hyperintense region within the tumor parenchyma (outlined in red). (E) A quantitative 3-dimensional surface plot of the [Fe] distribution in the tumor region in (D) from which one can readily determine the [Fe] in each voxel. (F) An extracted [Fe] image of the tumor from (D) colored to reflect the iron intensity scale shown in the legend to the right. Note that the other bright areas in images (A) and (D) are artefacts due to the incomplete nulling of the tissue backgrounds in these regions (See Methods).

Figure 5. Time dependence of the [Fe] in a C4-2 prostate tumor injected with anti-PSMA SPIONs

The total amount of measured iron within the tumor as a function of time after the injection of 9.6 nmoles of iron. The errors were derived from the signal to noise ratios of the [Fe] MR images.

Figure 6. Images of Single and Dual Tumor Mice

(A) Image of a mouse bearing a single DU145 tumor on its flank at 55 days post inoculation of tumor cells. (B) Image of a mouse bearing two LNCaP tumors on its flanks, 57 days post inoculation. (C) Three-dimensional [Fe]MR image of a dual tumor (DU145, left; LNCaP, right) mouse whose anatomical T_2w MR image is shown in (D). The image in (C) shows that prior to the injection of anti-PSMA conjugated SPIONs, there was no discernable added iron within either of the tumors. (D) Anatomical T_2w MR image of this dual-tumor mouse. The Du145 tumor (left) is outlined in green while the LNCaP tumor is outlined in red. (E) Three-dimensional [Fe]MR image of this dual tumor mouse taken 11 hours after the tail vein injection of 1.58 μ moles (88 μ g) of iron in the form of PSMA-targeted SPIONs. Note the uptake of iron solely by the LNCaP tumor (right). (F) Hemotoxylin and eosin, and Perl's stained section from the post-mortem excised LNCaP tumor showing robust iron staining. (G) A similarly stained section from the DU145 tumor showing a lack of iron staining in this PSMA-negative tissue.

Figure 7. Control [Fe]MRI of a Dual Tumor mouse prior to SPION injection

(A) [Fe]MR image calculated using the signal background from the DU145 tumor (which was ~10 % less intense than the LNCaP background signal) outlined in yellow. (B) A T2w MR image of the same slice for comparison of the [Fe] MR image with the anatomy. (C) [Fe]MR image calculated using the signal background from the LNCaP tumor outlined in red. (D) Three-dimensional plot of the yellow outlined region of the [Fe] image showing a lack of added iron in the DU145 tumor. (E) Three-dimensional plot of the red-outlined region of the [Fe] image showing a lack of added iron in the LNCaP tumor.

Figure 8. Time course of [Fe] in a dual tumor Du145, LNCaP mouse after anti-PSMA conjugated SPION injection into the tail vein

The times in minutes post-injection are shown in the lower right-hand images in C, G, and K. (A, E, I) Three-dimensional plots of the [Fe]MR images of the DU145 tumor outlined in yellow in (B) and (C) taken at 123, 666, and 1242 minutes post-injection of 1.6 micromoles of iron showing no iron uptake by this PSMA-negative tumor. (B, F, J) Anatomical T₂w MR images of the same slices as in (A, E, I) in this mouse at the indicated times. (C, G, K) [Fe]MR images of the same slices as shown in (B, F, J) at the indicated times. (D, H, L) Three-dimensional [Fe] plots of the LNCaP tumor outlined in red in (B) and (C) at the indicated times post-injection showing the time course of robust iron uptake by this PSMA-positive tumor. The large signal at the edge of the tumor in (A) was an artifact from the tumor edge, seen as the bright line at the upper right in (C).

Figure 9. Time courses of the uptake of iron after anti-PSMA conjugated SPION injection into the tail vein

The lines through the data are meant to guide the eye. (A) *Yellow line and squares*, 60 nanomoles iron injected, LNCaP tumor; *Orange line and squares*, 120 nanomoles iron, LNCaP tumor; *Red lines and squares*, 160 nanomoles iron, dual LNCaP tumors showing data from four slices from each tumor separately from the same animal; *Blue line and colored circles*, Control data (n = 5 mice; up to 1.68 micromoles of iron injected) showing no iron uptake taken from DU145 tumor-bearing mice injected with anti-PSMA conjugated SPIONs and SPIONs without antibody, C4-2 tumors with no antibody on the SPIONs, and

LNCaP tumors without antibodies on the SPIONs. (B) Dual-tumor mouse after the Injection of 10 times more iron (1.6 micromoles) than in (A); *Blue* and green *Circles*, DU145 tumors, two slices showing no iron uptake. *Red Squares*, LNCaP tumors, two slices showing robust iron uptake in each slice.

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Figure 10. Response of the tumor Iron Concentration to the injected Iron Dose of anti-PSMA conjugated SPIONs

Blue line and circles, PSMA-positive LNCaP and C4-2 tumors. The slope of the fitted line was 76.5 micromolar Fe/micromole of iron injected. *Red line and squares*, Control PSMA-negative DU145 tumor-bearing mice injected with anti-PSMA conjugated SPIONs and SPIONs without antibody, C4-2 tumors with no antibody on the SPIONs, and LNCaP tumors without antibodies on the SPIONs.

[Fe] ~units of [Fe] are mM because the relaxivities are measured in Hz/mM. 1 min post injection. Note the For the MRI studies.