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pH-Sensitive MR Responses Induced by Dendron-Functionalized SPIONs

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Abstract

We report a series of investigations of the pH-sensitive magnetic resonance (MR) responses of various surface-functionalized SPIONs (superparamagnetic iron oxide nanoparticles). First, functionalization of ~12 nm highly monocrystalline SPION cores with three different generations of melamine-dendrons was optimized to give agents with high molar relaxivities (e.g. $R_2^m \sim 300 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 7 T and $R_1^m \sim 20\text{--}30 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 0.5 T) and excellent aqueous stabilities. Molar relaxivities were found to exhibit great sensitivity to pH at physiologically-relevant ionic strengths, with sharp inflections observed at pH values near the pK_a of the melamine monomer. The strength of the effect was observed to grow with increasing dendron generation (with concomitant shift in the position of the main pH inflection). Opposing behavior in R_2^m and R_2^{m*} trends may be exploited to provide a ratiometric MR response to pH. Combined with TEM and corresponding MR measurements from solutions of varying ionic strengths, these results are consistent with the pH-sensitive behavior originating from transient, reversible SPION clustering modulated by an interplay between SPION surface charge density and solution ionic strength. Studies of SPION cellular uptake and MR response in HeLa cell cultures are also presented. Finally, comparisons with the MR responses of SPIONs with alternative functionalities—derivatives of nitrilotriacetic acid or poly(1-vinylimidazole)—indicate that these types of pH-sensitive MR responses can be highly dependent upon the chemical composition of the surface species (and thus amenable to modulation through rational design).

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*Portions of this work were presented previously in Refs. 40–44.

†A similar preparation was used to successfully synthesize poly-imidazole SPIONs linked with carboxylic acid moieties instead of dopamine groups, but these SPIONs were found to have lower aqueous stabilities and inferior MR properties.

Supporting Information Available: A brief synthetic procedure for PEGylated G2-SPIONs, determination of SPION surface-coverage of melamine dendrons from elemental analysis (and comparisons with our previous work), comparisons of relaxivities of the various melamine-dendron and PEGylated SPIONs, and supplementary information regarding HeLa cell culture samples and preliminary biocompatibility studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

NMR; MRI; contrast agent; dendrimer; superparamagnetic; iron oxide

1. INTRODUCTION

By combining molecular-scale knowledge of local physiological conditions with the increasing anatomical resolution of conventional imaging modalities, molecular imaging techniques promise to provide dramatically improved detection, diagnoses, and tracking of pathological conditions, as well as new insights into the underlying mechanisms of various diseases.¹ While many such approaches target specific biomolecular markers of disease, a more general biochemical parameter of increasing interest is pH. Because mammalian energy metabolism results in the production of acids (e.g., lactic acid and $\text{CO}_2/\text{H}_2\text{CO}_3$), the body must actively regulate pH in order to maintain normal healthy physiological conditions. Correspondingly, local variations (or reductions) in extra- or intracellular pH are associated with the heterogeneous blood flow and nutrient supply concomitant with a number of altered physiological states and pathological conditions—including injury, ischemia, and inflammation, as well as various cancers.^{2–7}

Given the promise of *in vivo* pH mapping, a number of magnetic resonance (MR) modalities using either endogenous^{8,9} or exogenous agents^{10–30} have been developed over the years to provide a more complete, less-invasive alternative to microelectrode-based pH measurements.⁷ In particular, increasingly elaborate exogenous agents have been developed that exploit pH-sensitive NMR chemical shifts (e.g. ^{31}P or ^{19}F)^{10–16} chemical-exchange saturation transfer (CEST) effects,^{9,17,18,20,21} or Gadolinium (Gd)-based R_1 relaxivity changes^{19,22–25,27} to spectrally probe or image pH variations. While considerable progress has been made, the development of MR agents that provide sufficient spectral sensitivity to the modest local variations in pH across different tissue types and physiological conditions—while also yielding sufficient detection sensitivity for high-resolution imaging, sufficient chemical stability and lifetime, low minimum dose, and high biological compatibility (including low deposition of rf radiation into tissues)—has been a persistent challenge. Moreover, for many potential modalities the pH-sensitive MR effects may be strongly concentration-dependent, and it can be difficult to control (or even know) the local concentration of such agents *in vivo*. Alternatively, Gallagher *et al.*^{26,28} have demonstrated the use of hyperpolarized ^{13}C -bicarbonate for MRI pH mapping *in vivo*; while this exciting approach avoids many aforementioned challenges, its application may be limited by the inherently short (tens of seconds) lifetime of the highly non-equilibrium nuclear spin magnetization induced by the dynamic nuclear polarization (DNP) process, and by its nature the approach requires specialized instrumentation and capabilities not generally available in hospitals and imaging clinics.

Another class of MRI contrast agents—based on superparamagnetic iron oxide nanoparticles (SPIONs)—has generated increasing attention due to their high biological tolerability and large magnetic moments, giving rise to high (usually transverse) molar relaxivities (with R_2^m or R_2^{*m} typically up to $\sim 10^2 \text{ mM}^{-1} \cdot \text{s}^{-1}$ per Fe ion, e.g., Refs. ^{1,31–37}). SPIONs are often synthesized with surface modifications to improve aqueous solubility/stability, limit aggregation, and modulate biological uptake (e.g. Refs. ^{32,33,38}). Such surface functionalizations have also been designed to improve the information content of the SPIONs' MR response by binding specific ions³⁹ or biological molecules⁴⁰, thereby targeting specific tissue types or altering the SPIONs' transverse relaxivities (e.g., via analyte-modulated aggregation) to yield molecular 'switch'-based contrast^{36,41}—not unlike demonstrations of colorimetric responses from analyte-induced aggregation of gold

nanoparticles (e.g., Ref. ⁴²). Yet also of interest are the fundamental aspects that determine what roles such surface functionalities can play in determining different types of SPION MR behavior.

Here we provide a full report* of our studies of highly pH-sensitive MR responses induced by a series of surface-functionalized SPIONs in aqueous media. The surface-functionalization of highly monocrystalline SPION cores (~12 nm) with three different generations of melamine-dendrons was optimized to give agents with high molar relaxivities (e.g. R_2^m 300 mM⁻¹.s⁻¹ Fe at 7 T and R_1^m ~20–30 mM⁻¹.s⁻¹ Fe at 0.5 T) and excellent aqueous stabilities—allowing the sensitivity of their MR responses to the local chemical environment and the effects of dendron-generation number to be investigated in detail. R_2^m and R_2^{m*} values at 7 T were found to exhibit great sensitivity to pH at physiologically-relevant ionic strengths, with sharp inflections observed at pH values near the pK_a of the melamine monomer (~5.0⁴³). Opposing behavior in the R_2^m and R_2^{m*} trends may be exploited to provide a ratiometric MR response to solution pH—potentially allowing such SPIONs to act as concentration-independent pH-sensors for generating MRI contrast. Moreover, it was observed that the strength of the effect grows—and the position of the main pH inflection shifts—with increasing dendron generation, and the pH sensitivity was also manifested at lower field (0.5 T), including a strong R_1^m (longitudinal molar relaxivity) dependence. When combined with studies of dendron-SPION MR responses to variances in salt concentration and TEM results, the observed SPION pH-sensitivities are consistent with transient, reversible SPION clustering modulated by an interplay between SPION surface charge density and solution ionic strength. In addition, we also present results of cellular uptake and MR response of these dendron-functionalized SPIONs in HeLa cell cultures. Finally, pH-dependent MR studies using SPIONs with alternative functionalities—dopamine-coupled derivatives of nitrilotriacetic acid and poly(1-vinylimidazole), respectively—are reported, demonstrating that the nature of these SPION MR responses to pH are highly sensitive to the chemical composition of the surface moieties. Taken together, these results^{44–48} have demonstrated the utility of surface-functionalized SPIONs as MR pH sensors, and point towards the development of SPION-based agents capable of providing highly pH-sensitive MRI contrast in physiologically relevant regimes and environments. Here, we also note more recent relevant work by Crayton & Tsourkas⁴⁹ and Minehara *et al.*,⁵⁰ who presented studies of SPIONs coated with a glycol chitosan polymer (which exhibit pH-sensitive cellular uptake, including *in vivo*) and an organic arsenic derivative (which exhibit aggregation via pH-dependent hydrogen-bonding networks), respectively.

2. EXPERIMENTAL SECTION

2.1. SPION Preparation/Materials Characterization

2.1.1. Synthesis

Simanek-type ^{51,52} melamine dendron-functionalized SPIONs: (Chart 1) were prepared by modifying our previously described procedure.⁵³ Briefly, oleate-coated maghemite (γ -Fe₂O₃) nanoparticles⁵⁴ (~12 nm) and dopamine-linked melamine dendron molecules of three different generations (“G1”, “G2”, and “G3”) were each synthesized as previously described. As before, for each dendron-SPION conjugate, the dendron molecules were dissolved in 2.5 mL MeOH and added to oleate-SPIONs in 2.5 mL CHCl₃ (2 mg/0.05 mL) and sonicated under Ar. For the current syntheses, the dendron-to-SPION-core ratio and sonication time were optimized; it was found that using a higher dendron-to-SPION-core ratio (~0.06 mmol melamine dendrons to 25 mg of SPION cores) and an 8 hr sonication time yielded dendron-SPIONs with the best properties (dendron surface loadings, stabilities, and MR properties). Dendron-SPIONs were collected by permanent magnet (LifeSepTM 50sx) and washed with MeOH and CHCl₃ three times each, and stored in MeOH. Finally,

we also report here the successful synthesis of PEGylated G2-SPIONs ('PEG' = poly(ethylene glycol), PEG-750; see Supporting Information for details).

Dopamine-linked poly-imidazole ("I-SPIONs"; Chart 1inset): The same oleate-coated SPION cores used above (5 mL of a 0.00579 g/0.1 mL solution) were added to acetonitrile (1 mL), collected using a magnet, then washed with EtOH. TMAOH solution (10 mL of a 0.1 M solution) was added; the solution was then sonicated for 10 minutes and stirred at room temperature for 1 hour. NaCl solution (2 mL) was added to precipitate the nanoparticles, which were collected using the permanent magnet and washed 2x with distilled water. The sonication process was then repeated (except the solution was stirred for 1 day before NaCl precipitation). The aqueous SPIONs were dispersed in 5 mL of water at a concentration of 7 mg/mL.

0.2 mmol dopamine was added to 0.4 mmol sodium bicarbonate, dissolved in MeOH (2 mL), and purged with Ar for 5 min. TMAOH-treated nanoparticles (50 mg suspended in CHCl₃) were added, and the resulting mixture was purged with N₂ gas for 2 minutes, sonicated for 7 hours, then kept at room temperature for three days and washed with DMF and CHCl₃ three times to make an iron-oxide-OH-dopamine nanoparticle solution. Iron-oxide-OH-dopamine nanoparticles (30 mL of 10 mg/mL stock solution in CHCl₃) were added to a mixture of: AIBN carboxylic acid (0.36 mmol), EDCI (0.72 mmol), and di-isopropyl ethylamine (55.8 mg) and stirred for 24 hr at room temperature. Nanoparticles were collected using a permanent magnet and washed with CHCl₃ and DMF. The resulting iron-oxide-OH-dopamine-AIBN carboxylic acid nanoparticles were dispersed in 5 mL DMF as a stock solution. Iron-oxide-OH-dopamine-AIBN carboxylic acid nanoparticles (5 mL of 15.6 mg/mL stock solution) were degassed for 5 minutes while stirring. Vinyl-imidazole monomer (18 mmol) was added and stirred at 900 rpm while heating to 60 °C for 6.5 hr. The resulting DI-SPION nanoparticles were collected using a permanent magnet, washed with DMF and distilled water (twice), and stored in 5 mL of water as stock solution.[†]

Nitrilotriacetic-acid functionalized SPIONs ('C-SPIONs'; Chart 1inset): Iron-oxide-OH-dopamine nanoparticles (250 mg in CHCl₃) were added to a mixture of: nitrilotriacetic acid (69 mg), EDCI (138 mg), and di-isopropyl ethylamine (55.8 mg) at room temperature in CHCl₃ (30 mL). After 12 hours, nanoparticles were collected using a permanent magnet and washed sequentially with CHCl₃, DMF, and Milli-Q water. The resulting C-SPIONs (where the 'C' stands simply for carboxylic acid) were dispersed in 5 mL of Milli-Q water as stock solution before use.

2.1.2. Elemental Analysis—Elemental analyses of the dendron-functionalized SPIONs were performed by Galbraith Laboratories, Inc. (Knoxville, TN). The Fe wt% for each type of SPION was pre-determined by elemental analysis of dry SPIONs (prepared under high vacuum at 80 °C overnight) taken from each stock solution, allowing the Fe concentration (in mM Fe) of a given sample to be determined based on the SPION loading (in µg/mL). Corresponding C-H-N elemental analysis data provided the percentage of nitrogen atoms on the SPIONs' surfaces, allowing determination of the molar surface loading of dendron molecules for each GX-SPION type. More details of the elemental analyses are provided in the Supporting Information section.

2.1.3. TEM Imaging—SPION size distribution and structural integrity were checked via TEM (e.g., Fig. 1(a))⁵³ before and after surface functionalization. SPION average core dimension was 12 nm, with size distributions typically <10%. TEM images were also taken of G3-SPIONs following preparation in 150 mM buffer solutions at pH~3 and pH~8 (using acetic acid/acetate and phosphoric acid/phosphate buffers, respectively). For each pH a 20 µg/mL G3-SPION solution was prepared and sonicated for 30 s before loading onto a

hydrophilic plasma-treated TEM plate for imaging. All images were obtained with a Hitachi 7100 TEM (SIUC IMAGE Center) operating at an accelerating voltage of 75, 100, or 300 KV. Images were captured using a Gatan 789 digital camera. Magnification was calibrated using a MAG*I*CAL high-resolution magnification standard accurate to 1×10^6 X.

2.2. In Vitro Cellular Studies

Studies of cellular uptake, compatibility, and MR response were performed on different subsets of identically-prepared plates of HeLa cells (human cervical adenocarcinoma cell line; ATCC CCL-2) for each type of SPION studied. HeLa cells were cultured in MEM (minimum essential media) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO₂. When cell growth was ~60% confluent (i.e., ~60% plate coverage), G1-, G2-, or G3-SPIONs suspended in MEM (25 µg/mL) were added to the cells and incubated for 24 hours. Following incubation, cells were then rinsed 3 times with phosphate buffered saline (PBS, pH=7.4) to remove SPIONs that were not uptaken by the cells, and used in the subsequent studies.

For cellular uptake studies, the cells from one subset of plates were fixed with 4% formaldehyde for 30 minutes followed by staining with Prussian-blue in a 2% potassium ferrocyanide and 6% HCl solution for an additional 30 minutes. The cells were examined under a light microscope and optical micrographs were obtained to qualitatively characterize SPION uptake. For cellular compatibility studies, the cells were removed from plates with trypsin and counted using a Beckman Coulter counter (see Supplementary Information). Finally, for MR studies (using G1- or G3-SPIONs), cells were removed from the plates with trypsin, centrifuged to form cell 'plugs', and loaded into NMR tubes for relaxivity measurements. Following MR measurement, the cell plugs were acid-digested and sent for Fe-quantification via elemental analysis as described above.

2.3. MR Relaxivity Studies

2.3.1. Sample preparation—For a given type of SPION and experiment, an array of gelatin 'phantoms' (4% w/v) with varied SPION concentrations (0–50 µg/mL) were prepared in standard 5 mm NMR tubes from pre-measured SPION stock solutions diluted with DI water, a selected pH buffer, or NaCl stock solution to give 500 µL total volume at the desired final concentrations. For pH-dependent experiments, buffer solutions containing either acetic acid/acetate (for phantoms with pH 3–6.5) or phosphoric acid/phosphate (for samples with pH 6) were used; unless stated otherwise, all pH experiments involving GX-SPIONs used 150 mM final buffer concentrations, whereas 20 mM final buffer concentrations were utilized for C-SPION and I-SPION experiments. pH measurements were performed before and immediately after sample preparation using an Oakton ION 510 pH meter and single-junction pH probe prior to placing the samples in cold storage (4 °C) overnight to set the gelatin suspensions. SPION-loaded HeLa cell samples were prepared and characterized as described above.

2.3.2. MR measurements—Aqueous ¹H longitudinal ($R_1=1/T_1$), transverse ($R_2=1/T_2$), and inhomogeneous dephasing ($R_2^*=1/T_2^*$) relaxation rates were measured at either 7.05 T (at 18 °C, using a 300 MHz Varian Inova NMR spectrometer) or 0.5 T (at 40 °C, using a 23 MHz Oxford Maran Ultra spectrometer located at Washington University Medical School, St. Louis, MO). Relativities exhibited little or no sensitivity to temperature over the investigated range (15–40 °C; data not shown). T_1 and T_2 relaxation times were measured via inversion-recovery and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences (nominal spin echo time $\tau=1$ ms) respectively; high-field (7 T) R_2^* values were estimated from line widths following a standard 90°-acquire sequence using the relationship $\Delta\nu_{FWHM}=(\pi T_2^*)^{-1}$ (R_2^* values were not obtained at 0.5 T owing to the high magnetic field

inhomogeneity of that type of relaxometer). Samples were stored at 4 °C when not in use; for all SPIONs studied, spin relaxation rates were not observed to change significantly over several months. For a given SPION type and set of experimental conditions, corresponding molar relaxivity values (respectively denoted R_1^m , R_2^m , and R_2^{*m} for clarity) are given by the slope obtained by linearly fitting the experimentally determined spin relaxation rates plotted versus the molar Fe concentration.

3. RESULTS AND DISCUSSION

3.1. Nominal Relaxivity Measurements of Melamine Dendron-Functionalized SPIONs

Dendrons, and their larger brethren, dendrimers, provide high aqueous stability to molecular imaging contrast agents, in addition to having low toxicity, the ability to modulate biological uptake, and in principle, the potential to be bio-functionalized to target specific tissues.⁵⁵ Dendrons have been utilized in a variety of applications including: Gd-based MRI contrast agents,⁵⁵ synthetic matrices for novel SPION-dendrimer conjugates,^{32,33} and cellular transfection agents for conventional SPIONs.³⁵ Unlike most polymers, dendrons have well-defined chemical structures with precisely scalable and tunable physical properties, leading to our interest in dendron-functionalized SPIONs^{53,56} as a springboard for better understanding the effect of SPION surface properties on MR behavior and how such effects can be exploited to improve MR sensitivity to changes in the local chemical environment.

Figure 1(b–f) shows measurements of aqueous ^1H spin relaxation rates at 7 and 0.5 T from buffer-free gelatin-phantoms prepared with variable concentrations of G1-, G2-, or G3-dendron-SPIONs; the corresponding nominal relaxivities are provided in Table 1. As expected, for buffer-free phantoms, R_1^m is poorly sensitive to SPIONs at 7 T due to reduced susceptibility to dipolar contributions at high field, as well as the presence of bulky surface groups hindering the surface accessibility of water to the SPION cores. However, due to the superparamagnetic nature of the γ Fe_2O_3 cores, R_2^m and R_2^{*m} values are highly sensitive to the presence of the functionalized SPIONs (Fig. 1). These relaxivities are also mildly dependent on dendron generation, and the effects on R_1^m and R_2^m are consistent with the steric crowding of dendron branches thus resulting in the reduced accessibility of water molecules to the magnetic SPION core. Larger R_2^{*m} values (specifically, greater R_2^{*m}/R_2^m ratio) for G1-SPIONs would be consistent with a slightly greater degree of clustering (see below). At lower field, R_2^m values are relatively high but reduced by ~40% compared to high-field values, which follows qualitative expectation based on the reduced (but still nearly saturated) field-induced magnetic response expected at 0.5 T. However, R_1^m values are ~35-fold higher at 0.5 T, which is consistent with reduced suppression of dipolar contributions to T_1 at lower fields and comparable to the relaxivities of available Gd-based T_1 contrast agents used in clinically relevant magnetic fields.^{22–25} High relaxivities (e.g. R_2^m 300 $\text{mM}^{-1}\cdot\text{s}^{-1}$ Fe), R_2^{*m}/R_2^m ratios approaching unity (implying magnetically homogeneous samples), highly linear responses, excellent aqueous stabilities, and terminal group variability of these dendron-functionalized SPIONs indicate an excellent platform for the development of MR contrast agents. Finally, PEGylation has previously been exploited to suppress unwanted uptake of agents (including nanoparticles (see, e.g. Refs. 57–59)) by the reticuloendothelial system following introduction to the body (otherwise resulting in potentially poor *in vivo* agent delivery efficiency); here, the successful preparation of PEG-G2-SPIONs yielded agents with good aqueous stability and only modest reductions in the transverse ^1H relaxivities compared to standard melamine G2-SPIONs (see Supporting Information). While outside of our current scope, future efforts will thus include investigating the effects of PEGylation on the pH-sensing capabilities of such dendron-SPIONs—particularly in tissue environments.

3.2. Sensitivity of GX-SPION MR Responses to pH

The melamine dendron-functionalized SPIONs were observed to have MR responses that are highly sensitive to the solution environment. For example, both R_2^m and R_2^{*m} exhibit enhanced sensitivity to solution pH at 7 T and at physiologically-relevant ionic strengths, with responses varying by over an order of magnitude (Fig. 2). As shown in Fig. 2(a), the large R_2^m values observed in the absence of buffer—and at low pH in 150 mM buffers—decrease significantly at higher pH values; sharp inflections are observed just below pH values near the pK_a of melamine monomer (~ 5.0).⁴³ Moreover, the magnitude of the effect grows—and the position of the main pH inflection shifts—with increasing dendron generation. It is worth noting that such pH-sensitive responses are not typical for conventional SPIONs (see, e.g., Ref. 60).

Next, for all three generations of SPIONs, R_2^{*m} values were observed to follow R_2^m values at low pH; however, at higher pH R_2^{*m} values were observed to grow significantly—exhibiting the opposite trend of the R_2^m behavior. Correspondingly, the R_2^{*m}/R_2^m ratio (Fig. 2(b)) demonstrates significant, dendron-generation-dependent sensitivity to pH: The position of the inflection points of R_2^{*m}/R_2^m for these experiments increases with each generation (Fig. 2(b)), and the magnitude of the observed change is larger for G1 compared to G2 and G3. Thus, the R_2^{*m}/R_2^m ratio is one example of an MR response that could (in principle) be determined independently from SPION loading to provide a concentration-independent pH sensor of the local environment.

Corresponding spin-relaxation studies at 0.5 T showed that the pH effect was also clearly manifested at low field—including a strong R_1^m dependence (varying by ~ 5 -fold; Fig. 2(a)*inset*)—pointing to another potential approach for generating (positive) contrast. Of course, while the low-field R_2^m values roughly track those of R_1^m effects, they are much larger in magnitude; indeed, at 0.5 T, R_2^m values are similar to those at 7 T, but merely scaled down by $\sim 40\%$ (as would be expected from the smaller field-dependent magnetic response of the SPION cores—see above).

One effect that is not yet fully understood is the apparent deviation from sigmoidal behavior observed at low pH (i.e., the initial *rise* in molar relaxivity (R_2^m) to very high values—even higher than the nominal (buffer-free) measurements for G2, G3-SPIONs—just before the main inflection, Fig. 2(a)); this effect is reproducible and also appears to scale with dendron generation. And while the low-field R_1^m curve closely follows that of R_2^m over most of the pH range studied, we note that R_1^m did not exhibit the same initial rise at low pH and instead more closely follows traditional sigmoidal behavior (see further discussion below in Section 3.3). In any case, while the pH region of greatest sensitivity for these SPIONs is below the relevant range for most potential biomedical applications, these tantalizing observations nevertheless have significant implications for future efforts.

3.3. Sensitivity of GX-SPION MR Response to Solution Ionic Strength

Based on the above observations, it was hypothesized that the observed SPION pH sensitivity may primarily result from transient clustering governed by the interplay of charge repulsion forces vs. aggregation tendencies: At lower pH values, the dendron surface functionalities should be more positively charged, causing the SPIONs to repel from each other, thereby allowing greater surface access of water molecules to the superparamagnetic cores—which in turn gives rise to high R_2 values (and relatively high R_1 values at low field). On the other hand, if the surfaces were poorly charged, and/or if the surface charges of different SPIONs were effectively screened from each other (as would be expected at higher ionic strengths), the SPIONs would tend to cluster, reducing the access of water

molecules to the SPION surfaces and greatly increasing the microscale magnetic inhomogeneity of the sample, which give rise to very high R_2^* values.

To gain insight into this clustering hypothesis, R_2 values of G1, G2, and G3s were measured with fixed SPION loading (40 $\mu\text{g/mL}$) but varying ionic strength (i.e., NaCl concentration) at the natural pH of the gelatin-phantoms prepared with DI water (no buffer, pH ~ 5.8). As predicted, for all three generations of SPIONs, dependences of R_2 and R_2^* on ionic strengths (Fig. 3) qualitatively followed the behavior observed with varying pH (Fig. 2). More specifically, R_2 values initially increased as a result of increasing salt concentration then sharply fell at higher salt concentration (Fig. 3(a), mimicking the high pH results). In contrast, R_2^* values increased with rising solution ionic strength, giving rise to R_2^*/R_2 ratios that were flat at low ionic strengths but grew substantially as the ionic strength was increased (Fig. 3(b)). Moreover, a dendron-generation-dependent effect was once again observed. R_2 values peaked at higher ionic strengths for the G2- and G3-SPIONs compared to G1-SPIONs, and correspondingly, the R_2^*/R_2 ratio for the G1-SPIONs exhibited much greater sensitivity to ionic strength than the G2- and G3-SPIONs (Fig. 3(b) *inset*). These observations also support the clustering hypothesis (Fig. 3(c)), as higher-generation dendrons should give rise to larger SPION surface-charge densities at a given pH value—charges that would require increasingly high ionic strengths to be effectively screened (*cf.* the dendron surface loadings of these SPIONs as determined by elemental analysis in the Supporting Information section).

As mentioned above, an initial rise in R_2 values with increasing ionic strength (Fig. 3(a)) was observed that mimicked the initial rise in R_2^m relaxivities recorded below the inflection (Fig. 2(a)). Thus, the origin of this ‘secondary’ effect is likely to be the result of changes in the collective SPION response to the environment, rather than (say) an irreversible chemical alteration of the SPION surfaces caused by a pH change. However, the near absence of this effect in the low-field (0.5 T) R_1^m results (Fig. 2(a) *inset*) suggests that the dipolar contribution to spin-relaxation—and hence, the water accessibility to the surfaces of the SPION cores—is largely unaffected as the pH is increased over the pre-inflection regime. In any case, the origins of this secondary effect will be the subject of future studies.

3.4 Demonstration of Reversibility of MR Response and Imaging of pH-Modulated SPION Clustering

Another series of experiments were performed to probe the reversibility of the “environment-sensing” MR responses; for experimental simplicity, these experiments were performed using changes in ionic strength (rather than pH). Figure 4 shows R_2^m measurements obtained (at 7 T, 15 °C) from a series of 400 mM and 100 mM [NaCl] aqueous gelatin phantoms containing varying amounts of G3-SPIONs—along with a corresponding measurement from a series of samples that were first placed in a 400 mM salt environment for 10 minutes, diluted with DI water until the salt concentration of the samples was 100 mM, allowed to equilibrate in the low-salt environment, and sonicated for 30 seconds before being loaded into gelatin phantoms. SPIONs exposed to high ionic strength conditions (where MR (e.g. R_2^m) results consistent with greater clustering are observed) followed by dilution to lower ionic strengths (where reduced clustering would otherwise be expected) give MR responses similar to those observed under low-clustering/low ionic-strength conditions. These results are consistent with transient, reversible dendron-SPION clustering under our conditions and further support the conclusion that the high, generation-dependent sensitivity of the SPIONs to their environment is not likely the result of irreversible chemical modification of the surfaces or irreversible aggregation.

For further support of the clustering model (and after some initial failures), we obtained TEM images that display differential clustering behavior in different pH regimes. Figures

5(a) and 5(b) show TEM images obtained with G3-SPIONs prepared in solutions with ‘low’ pH (~3) and ‘high’ pH (~8), respectively. While the sample preparation that was required to observe this result (here, solution deposition onto plasma-treated TEM plates followed by evaporation) is inherently a step removed from the ambient aqueous conditions relevant to the MR results, the images fit well with our qualitative expectations: small SPION clusters were observed at low pH, whereas considerably more clustering was observed at higher pH—with a wide distribution of cluster sizes appearing in the high-pH micrograph.

When combined with the TEM results and the studies of MR responses to variances in salt concentration, the observed SPION pH-sensitivities are consistent with transient, reversible SPION clustering modulated by an interplay between surface charge density and solution ionic strength. A more detailed theoretical discussion of these results (and other experiments studying these effects) is beyond the scope of the present work and will be provided elsewhere⁶¹; however, a few additional comments can be made here. Briefly, a given SPION cluster may be considered as a large magnetized sphere (with an overall magnetic moment aligned to the external field and with a magnitude that follows Langevin’s classical law⁶²). The effects of the SPION clustering are manifested in the integrated MR responses (R_1 , R_2 , and R_2^*) of the ^1H spins of water molecules interacting with and (diffusing about) these clusters (see, e.g., Refs. ^{63–66}): First, longitudinal relaxation (R_1) effects can be considered to arise primarily from chemical exchange between (fast-relaxing) water ^1H spins interacting with SPION surfaces within the clusters and (slowly-relaxing) water spins in the bulk solution; modulation of the cluster sizes via changes in solution conditions affects both the accessibility of SPION surface sites and effective exchange rates. Second, while “inner-sphere” effects (resulting from transient binding of water molecules to surfaces) may contribute to transverse relaxation (R_2 , R_2^*), such relaxation is generally dominated by so-called “outer-sphere” effects (resulting from water molecules diffusing past magnetic clusters and dephasing as they pass through the local field inhomogeneities). The magnitudes and the spatial extents of the field inhomogeneities can be expected to vary dramatically with the degree of SPION clustering in solution. Thus, depending on a given cluster size distribution (and experimental parameters), the observed transverse relaxation behavior may run the gamut from the echo-limited regime (where the CPMG echo time is short compared to the time to diffuse past the particle), to the motional-averaging regime in outer-sphere relaxation theory (in the limit of long echo times). Indeed, in additional experimental and theoretical work we have found that the CPMG echo-time dependence of apparent T_2 relaxation induced by various SPIONs is consistent with a model^{61,67} that assumes that the scale of local magnetic inhomogeneities^{63–66,68,69}—i.e., originating from transient SPION clustering—is governed by variations in solution conditions (pH, ionic strength, etc.). Once refined (e.g., with quantitative comparison with dynamic light scattering, DLS), this model may ultimately allow determination of cluster size distributions via MR *in situ*, as well as give rise to another technique for generating contrast or mapping local pH variations.

3.5. *In Vitro* Cellular Studies

Investigations of cellular uptake, tolerability, and MR response of these dendron-SPIONs were performed using HeLa cell cultures. (Before continuing, it is worth noting that because the range of greatest pH sensitivity for these SPIONs lies below the normal pH of media for cells in culture—and since in any case, these cells were not in living tissues where altered metabolism can lead to significantly variant local pH values—these experiments were not intended to evoke a particular pH-dependent MR response). First, within error, SPION presence had negligible effect on cell growth when compared to control cultures (see Supporting Information), consistent with tolerability of the SPIONs by these cells. Furthermore, significant cellular uptake was observed for all three dendron-SPIONs tested,

as indicated by Prussian-blue staining/optical microscopy (e.g. Figs. 6(a–d)). MR studies at 7 T of G1/G3-SPION-loaded cells exhibited weak R_2 effects (not shown), and very high R_2^* values (Fig. 6(e)), which are consistent with high cellular uptake but also significant SPION clustering within the cells (see below). Subsequent elemental analysis of the cell plugs indicated significant Fe loading with both the G1 and G3-SPIONs (corresponding to ~11 pg Fe/cell, as shown in Fig. 6(f)). Higher R_2^* values for HeLa cell plugs incubated with G1-SPIONs vs. G3-SPIONs (~8000 vs. ~5000 s⁻¹), while partially explained by the greater cellular density (and hence slightly higher total Fe% in the sample (Fig. 6(f))), also likely indicate greater intracellular clustering of G1-SPION particles vs. G3-SPIONs. Additionally, while these SPIONs exhibited efficient cellular uptake, future SPIONs would be designed to either suppress or encourage cellular uptake depending on the application (i.e., involving either extracellular or intracellular compartments).

3.6. Effects of pH on MR Behavior of SPIONs with Alternative Surface Functionalities

The melamine-dendron SPIONs exhibit sharply pH-sensitive responses, but in a range below what would be relevant for most biomedical applications. As a step towards developing SPIONs with pH-sensitive responses in physiologically relevant regimes, SPIONs with different polymer-based surface functionalizations were synthesized—including dopamine-linked nitrilotriacetic acid-coated SPIONs (“C-SPIONs”) and polyimidazole (“I-SPIONs”; Chart 1). When first examining these polymer-SPIONs using buffers at physiologically relevant ionic strengths, rather poor relaxivities were obtained, and the SPIONs exhibited low sensitivity to pH (data not shown). However, when the experiments were repeated with the buffer ionic strength reduced to 20 mM, the I-SPIONs showed strong relaxivities and pH sensitivity (Fig. 7(a)): a steep *rise* in R_2^m relaxivity is observed at pH 6, with a corresponding reduction in R_2^{m*}/R_2^m ratio (note that imidazole groups usually exhibit a pK_a value of ~6.5⁹). A similar R_2^m result was obtained at 0.5 T, whereas R_1^m exhibited only a weak pH dependence (data not shown). On the other hand, the C-SPIONs exhibited little useful pH dependence regardless of ionic environment (Fig. 7(b)).

When comparing the I-SPION results with those obtained with the melamine-dendron SPIONs described above, one notices that not only has the point of inflection been moved closer to the desired regime for most physiological applications, qualitatively the trends of R_2^m and the R_2^{m*}/R_2^m ratio are *inverted* (*cf.* Fig. 2—the origin of this difference in behavior is the subject of future studies). The greater sensitivity to ionic strength for the I-SPIONs (manifested by the greatly reduced range in pH-sensing functionality, in addition to the poor results obtained with the C-SPIONs) may reflect a smaller effective surface loading of the functional units compared to the melamine dendron-SPIONs (or, reduced charge-bearing capacity compared to what can be achieved with the dendrons, currently under study). In any case, these results support the possibility of tuning these types of environmentally-dependant MR responses—particularly the pH sensitivities—by rational variation of the surface properties.

4. CONCLUSION

In summary, we have investigated the highly pH-sensitive MR responses induced by a series of surface-functionalized SPIONs. First, optimization of the SPIONs functionalized with three different generations of melamine-dendrons led to agents possessing high molar relaxivities (including R_2^m values exceeding 300 s⁻¹·mM⁻¹) and excellent aqueous stabilities—allowing the sensitivity of their MR responses to the local chemical environment and the effects of dendron-generation number to be studied. Relaxivities were found to vary by an order of magnitude by varying the solution pH at physiologically-relevant ionic strengths, with sharp inflections near the pK_a of the monomer of the surface functionalization (and with magnitude of the effect and inflection position depending on the

dendron generation). Opposing R_2^m and R_2^{m*} behavior may allow such SPIONs to act as concentration-independent pH-sensing contrast agents; on the other hand, relatively high ($\sim 30 \text{ s}^{-1} \cdot \text{mM}^{-1}$) and pH-variable R_1 relaxivities at lower, more clinically-relevant fields suggest the possibility of using such SPIONs as T_1 agents (offering the potential advantage of positive MRI contrast). When combined with TEM and studies of MR responses to variances in salt concentration, the observed SPION pH-sensitivities are consistent with transient, reversible SPION clustering modulated by an interplay between surface charge density and solution ionic strength (an effect we are working to model and further exploit in ongoing efforts). Finally, MR studies using SPIONs with alternative functionalities—particularly the vinylimidazole derivatives—demonstrated that the nature of the SPION MR responses to the environment can be highly sensitive to the surface chemical composition; thus, overall our results support the ability to tune these types of SPION pH sensitivities by rational variation of surface properties. Correspondingly, our current efforts concern the development and characterization of novel SPIONs that exhibit pH-sensitive MR responses in physiologically relevant regimes while operating in biological (extracellular or intracellular) environments. The results of these efforts, along with the ongoing modeling of the MR signatures of the underlying clustering mechanisms⁶¹, will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Weissleder R. *Science*. 2006; 312:1168–1171. [PubMed: 16728630]
2. Vaupel P, Rallinowski F, Okunieff P. *Cancer Res*. 1989; 49:6449–6465. [PubMed: 2684393]
3. Tannock IF, ins DH. *Cancer Res*. 1989; 49:4373–4384. [PubMed: 2545340]
4. Martin GR, Jain RK. *Cancer Res*. 1994; 54:5670–5674. [PubMed: 7923215]
5. Gerweck LE, Seetharaman K. *Cancer Res*. 1996; 56:1194–1198. [PubMed: 8640796]
6. Carmeliet P, Jain RK. *Nature*. 2000; 407:249–257. [PubMed: 11001068]
7. Gillies RJ, Raghunand N, Garcia-Martin ML, Gatenby RA. *IEEE Eng Med Biol Mag*. 2004; 23:57–64. [PubMed: 15565800]
8. Vermathen P, Capizzano AA, Maudsley AA. *Magn Reson Med*. 2000; 43:665–675. [PubMed: 10800031]
9. Zhou J, Payen JF, Wilson DA, Traystman RJ, Zijl PCv. *Nature Med*. 2003; 9:1085–1090. [PubMed: 12872167]
10. Stubbs M, Bhujwala ZM, Tozer GM, Rodrigues LM, Maxwell RJ, Morgan R, Howe FA, Griffiths JR. *NMR Biomed*. 1992; 5:315–359.
11. Gillies RJ, ZZL, Bhujwala ZM. *Am J Physiol*. 1994; 267:C195–C203. [PubMed: 8048479]
12. Ackerman JJH, Soto GE, Spees WM, Zhu Z, Evelhoch JL. *Magn Reson Med*. 1996; 36:674–683. [PubMed: 8916017]
13. Soto E, Zhu Z, Evelhoch JL, Ackerman JJH. *Magn Reson Med*. 1996; 36:698–704. [PubMed: 8916020]

14. Deutsch C, Taylor JS, Wilson DF. Proc Natl Acad Sci USA. 1982; 79:7944–7948. [PubMed: 6961462]
15. Mason RP. Current Medicinal Chem. 1999; 6:481–499.
16. Cui W, Otten PYJ, Kodibagkar V, Mason RP. Proc Intl Soc Magn Reson Med. 2003; 11:623–623.
17. Ward KM, Balaban RS. Magn Reson Med. 2000; 44:799–802. [PubMed: 11064415]
18. Zhang S, Winter P, Wu L, Sherry AD. J Am Chem Soc. 2001; 123:1517–1518. [PubMed: 11456734]
19. Lowe MP, Parker D, Reany O, Aime S, Botta M, Castellano G, Gianolio E, Pagliarin R. J Am Chem Soc. 2001; 123:7601–7609. [PubMed: 11480981]
20. Aime S, Barge A, Delli CD, Fedeli F, Mortillaro A, Nielsen FU, Terreno E. Magn Reson Med. 2002; 47:639–648. [PubMed: 11948724]
21. Liu, G.; Li, Y.; Pagel, MD. 48th Expl. NMR Conf; 2007.
22. Beauregard DA, Parker D, Brindle KM. Proc Intl Soc Magn Reson Med. 1998; 6:53–53.
23. Zhang S, Wu K, Sherry AD. Angew Chem Intl Ed Engl. 1999; 38:3192–3194.
24. Raghunand N, Howison C, Sherry AD, Zhang S, Gillies RJ. Magn Reson Med. 2003; 49:249–257. [PubMed: 12541244]
25. Kalman FK, Woods M, Caravan P, Jurek P, Spiller M, Tircso G, Kiraly R, Brucher E, Sherry AD. Inorg Chem. 2007; 46:5260–5270. [PubMed: 17539632]
26. Gallagher FA, Kettunen MI, Day SE, Hu DE, Ardenkjær-Larsen JH, in 't Zandt R, Jensen PR, Karlsson M, Golman K, Lerche MH, Brindle KM. Nature. 2008; 453:940–944. [PubMed: 18509335]
27. Gianolio E, Maciocco L, Imperio D, Giovenzana GB, Simonelli F, Abbas K, Bisi G, Aime S. Chem Commun. 2011; 47:1539–1541.
28. Gallagher FA, Kettunen MI, Brindle KM. NMR Biomed. 2011; 24:1006–1015. [PubMed: 21812047]
29. Hashim AI, Zhang X, Wojtkowiak JW, Martinez GV, Gillies RJ. NMR Biomed. 2011; 24:582–591. [PubMed: 21387439]
30. Tu C, Louie AY. NMR Biomed. 2012 published online. 10.1002/nbm.2870
31. Weissleder R, Elizondo G, Wittenberg J, Lee A, Josephson L, Brady T. Radiology. 1990; 175:494–498. [PubMed: 2326475]
32. Bulte JWM, Douglas T, Witwer B, Zhang S-C, Strable E, Lewis BK, Zywicke HA, Miller B, van Gelderen P, Moskowitz BM, Duncan ID, Frank JA. Nature Biotechnol. 2001; 19:1141–1147. [PubMed: 11731783]
33. Strable E, Bulte JWM, Moskowitz B, Vivekanandan K, Allen M, Douglas T. Chem Mater. 2001; 13:2201–2209.
34. Kalish H, Arbab AS, Miller BR, Lewis BK, Zywicke HA, Bulte JWM Jr, LHB, Frank JA. Magn Reson Med. 2003; 50:275–282. [PubMed: 12876703]
35. Frank JA, Zywicke H, Jordan EK, Mitchell J, Lewis BK, Miller B, Bryant LH, Bulte JW. Acad Radiol. 2002; 9:S484–487. [PubMed: 12188316]
36. Frank JA, Miller BR, Arbab AS, Zywicke HA, Jordan EK, Lewis BK, Bryant LH, Bulte JWM. Radiology. 2003; 228.
37. Dahnke H, Liu W, Herzka D, Frank JA, Schaeffter T. Magn Reson Med. 2008; 60:595–603. [PubMed: 18727097]
38. Liong M, Shao H, Haun JB, Lee H, Weissleder R. Adv Mater. 2010; 22:5168–5172. [PubMed: 20859943]
39. Atanasijevic T, Shusteff M, Fam P, Jasanoff A. Proc Natl Acad Sci USA. 2006; 103:14707–14712. [PubMed: 17003117]
40. Perez JM, Josephson L, O'Loughlin T, Hogemann D, Weissleder R. Nature Biotech. 2002; 20:816–820.
41. Perez JM, Simeone FJ, Saeki Y, Josephson L, Weissleder R. J Am Chem Soc. 2003; 125:10192–10193. [PubMed: 12926940]

42. Elghanian R, Storhoff J, Mucic RC, Letsinger RL, Mirkin CA. *Science*. 1997; 277:1078–1081. [PubMed: 9262471]
43. Hirt RC, Schmitt RG. *Spectrochim Acta*. 1958; 12:127–138.
44. Chaffee, KE.; Crase, BM.; Duanmu, C.; Saha, I.; Davis, AM.; Huggenvik, J.; Gao, Y.; Goodson, BM. 48th Experimental Nuclear Magnetic Resonance Conference; Daytona Beach, FL. 2007.
45. Saha, I.; Davis, AM.; Zielinski, LJ.; Chaffee, KE.; Crase, BM.; Duanmu, C.; Gao, Y.; Goodson, BM. 48th Experimental Nuclear Magnetic Resonance Conference; Daytona Beach, FL. 2007.
46. Duanmu, C.; Saha, I.; Chaffee, KE.; Crase, BM.; Goodson, BM.; Gao, Y. 234th American Chemical Society National Meeting; Boston, MA. 2007. p. 234
47. Buck, LE.; Chaffee, KE.; Duanmu, C.; Sattenapally, N.; Gibson, L.; Stokes, AL.; Saha, I.; McCrudden, J.; Gao, Y.; Goodson, BM. 49th Experimental Nucl. Magn. Reson. Conference; Pacific Grove, CA. 2008.
48. Duanmu, C.; Sattenapally, N.; Buck, LE.; Chaffee, KE.; Saha, I.; Goodson, BM.; Gao, Y. 236th National American Chemical Society Meeting; Philadelphia, PA. 2008. p. 236
49. Crayton SH, Tsourkas A. *ACS Nano*. 2011; 5:9592–9601. [PubMed: 22035454]
50. Minehara H, Naka K, Tanaka K, Narita A, Chujo Y. *Bioorg & Med Chem*. 2011; 19:2282–2286. [PubMed: 21393003]
51. Lim J, Simanek EE. *Mol Pharm*. 2005; 2:273–277. [PubMed: 16053330]
52. Zhang W, Simanek EE. *Org Lett*. 2000; 2:843–845. [PubMed: 10814438]
53. Duanmu C, Saha I, Zheng Y, Goodson BM, Gao Y. *Chem Mater*. 2006; 18:5973–5981.
54. Park J, An K, Hwang Y, Park J-G, Noh H-J, Kim J-Y, Park J-H, Hwang N-M, Hyeon T. *Nature Mater*. 2004; 3:891–895. [PubMed: 15568032]
55. Venditto V, Regino C, Brechbiel M. *Mol Pharm*. 2005; 2:302–311. [PubMed: 16053333]
56. Kim M, Chen Y, Liu Y, Peng X. *Adv Mater*. 2005; 17:1429–1432.
57. Gao X, Cui Y, Levenson RM, Chung LW, Nie S. *Nature Biotechnol*. 2004; 22:969–976. [PubMed: 15258594]
58. Lee H, Lee E, Kim DK, Jang NK, Jeong YY, Jon S. *J Am Chem Soc*. 2006; 128:7383–7389. [PubMed: 16734494]
59. Kievit FM, Veiseh O, Bhattarai N, Fang C, Gunn JW, Lee D, Ellenbogen RG, Olson JM, Zhang M. *Adv Funct Mater*. 2009; 19:2244–2251. [PubMed: 20160995]
60. LaConte LEW, Nitin N, Zurkiya O, Caruntu D, O'Connor CJ, Hu X, Bau G. *J Magn Reson Imaging*. 2007; 26:1634–1641. [PubMed: 17968941]
61. Saha I, Stokes AM, Chaffee KE, Zielinski LJ, Duanmu C, Woods BM, Gao Y, Goodson BM. to be submitted to *J. Magn. Reson*.
62. Kittel, C. *Introduction to Solid State Physics*. 6. Wiley; New York: 1986.
63. Yablonskiy DA, Haacke EM. *Magn Reson Med*. 1994; 32:749–763. [PubMed: 7869897]
64. Jensen JH, Chandra R. *Magn Reson Med*. 2000; 44:144–156. [PubMed: 10893533]
65. Jensen JH, Chandra R. *Magn Reson Med*. 2000; 43:226–236. [PubMed: 10680686]
66. Brooks RA, Moiny F, Gillis P. *Magn Reson Med*. 2001; 45:1014–1020. [PubMed: 11378879]
67. Zielinski LJ, Sen PN. *J Magn Reson*. 2000; 147:95–103. [PubMed: 11042051]
68. Shapiro MG, Atanasijevic T, Faas H, Westmeyer GG, Jasanoff A. *Magn Reson Imaging*. 2006; 24:449–462. [PubMed: 16677952]
69. Matsumoto Y, Jasanoff A. *Magn Reson Imaging*. 2008; 26:994–998. [PubMed: 18479873]

Figure 1.

(a) Example of TEM of melamine dendron-SPIONs⁵³ (here, G3-SPIONs; bar is 25 nm). (b–d) High-field (7 T) relaxivity plots showing the dependencies of R_2 and R_2^* on SPION loading for G1- (b), G2- (c), and G3-SPIONs (d) as functions of Fe concentration (slopes give relaxivities comprised in Table 1). (e,f) Corresponding relaxivity plots of R_1 (e) and R_2 (f) vs. Fe concentration at low field (0.5 T) for G1 and G3 SPIONs. Uncertainties for the individual data points were generally well within the graph symbols.

Figure 2.

Plots of (a) R_2^m and (b) R_2^{*m}/R_2^m ratio vs. pH at high-field (7 T) of gelatin phantoms (4% w/v, 150 mM acetate or phosphate buffer) loaded with G1- (green squares), G2- (red circles), or G3-SPIONs (blue triangles). Data points encircled by the white oval (at pH~5.8) and yellow oval (at pH~6.6) represent relaxivities obtained from gel samples prepared with DI water (no buffer) or PBS, respectively. **Inset:** Corresponding plots of R_1^m (purple symbols, dotted lines) and R_2^m (blue symbols, solid lines) vs. pH obtained at lower field (0.5 T) for gel samples containing G3-SPIONs. Note that for each data point, values and error bars are derived from a linear fit of individual relaxation measurements performed as a function of SPION concentration under the given conditions. Connecting lines are meant only to guide the eye.

Figure 3.

Plots of (a) R_2 values and (b) R_2^*/R_2 ratios (both measured at 7 T) vs. NaCl concentration in gelatin phantoms (4% w/v) each loaded with a fixed amount (40 $\mu\text{g/mL}$) of G1- (green squares), G2- (red circles), or G3-SPIONs (blue triangles). (b) *Inset*: same data as (b), but showing the entire range of R_2^*/R_2 vs. [NaCl]. Connecting lines are meant only to guide the eye. Uncertainties for the individual data points were generally well within the graph symbols. (c) Cartoon depicting transient, reversible microscale clustering of the dendron-SPIONs governed by the interplay of aggregation tendencies vs. charge repulsion forces (modulated by ionic screening).

Figure 4.

R_2^m relaxivities obtained by fitting R_2 values measured from G3-SPION/gelatin suspensions with 100 mM (S_{100}) and 400 mM (S_{400}) [NaCl], compared to the corresponding R_2^m value obtained from samples where the NaCl concentration was changed from 400 to 100 mM ($S_{400 \text{ to } 100}$), showing the apparent reversibility of the MR response to changes in solution ionic strength.

Figure 5. TEM micrographs taken from G3-SPION samples (created from 20 $\mu\text{g/mL}$ aqueous solutions) at low pH (pH~ 3.5, **(a)**) and higher pH (~8, **(b)**). Bars are 100 nm.

Figure 6.

(a–d) Optical micrographs of HeLa cells (40×) stained with Prussian blue to indicate the presence of iron. (a) Cells cultured without SPIONs ('control'); (b–d) HeLa cells labeled with G2-, G1-, and G3-SPIONs, respectively (24 hr incubation with 25 µg/mL SPIONs). Images in (a) and (b) were taken from a different experiment than those in (c) and (d). (e) R_2^* values for cell 'plug' samples comprising G1- or G3-loaded HeLa cells, compared to that of a control sample ('Only Cells'). (f) Fe loading of the cell plugs studied in (e) following acid digestion, reported in both pg Fe per cell (green, left bars) and Fe% (right blue bars). Note: Fe values for the 'Only Cells' control sample are at the lower detection limits of the measurements (and thus the values for this sample likely represent significant overestimates of the true Fe content).

Figure 7.

(a) Plots of R_2^m (black open squares) and R_2^{*m}/R_2^m ratio (blue closed circles) vs. pH at high-field (7 T) from gelatin phantoms (4% w/v, 20 mM acetate or phosphate buffer) loaded with I-SPIONs. Note the shift in the inflections and the opposite behavior in the trend lines compared to the melamine dendron GX-SPIONs (*cf.* Fig. 2). (b) Corresponding plots of R_2^m and R_2^{*m}/R_2^m ratio (red closed circles) for samples loaded with C-SPIONs. Connecting lines are meant only to guide the eye.

Chart 1.

Cartoons showing structures/morphologies of various surface-functionalized SPIONs utilized in the present work (not to scale). The main figure shows dopamine-linked G1, G2, and G3 melamine dendron-functionalized SPIONs ('GX-SPIONs', where X is the dendron generation number). **Inset:** Corresponding diagrams of dopamine-linked nitrilotriacetic acid functionalized SPIONs ('C-SPIONs') and dopamine-linked poly(1-vinylimidazole) functionalized SPIONs ('I-SPIONs').

Table 1

Aqueous ^1H MR relaxivities for melamine dendron-functionalized SPIONs (G1, G2, and G3) suspended in gelatin phantoms prepared with DI water (no buffer); measurements performed at either 7 T or 0.5 T. Uncertainties are from errors obtained from the linear fits.

Relaxivities	G1	G2	G3
$R_1^{m^*}$	0.93 ± 0.4	0.76 ± 0.3	0.54 ± 0.4
$R_1^{m^{\dagger}}$	33 ± 0.7	--	21 ± 1.0
$R_2^{m^*}$	333 ± 10	312 ± 9	304 ± 5
$R_2^{m^{\dagger}}$	197 ± 7	--	175 ± 6
$R_2^{*m^*}$	412 ± 7	338 ± 9	342 ± 14

All relaxivities listed in units of ($\text{s}^{-1} \times \text{mM}^{-1}$)

† 7 T at 18°C

† 0.5 T at 40°C