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Reduced magnetic coupling in ultra-small iron oxide T₁ MRI contrast agents

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Abstract

Contrast agents for magnetic resonance imaging (MRI) are essential for evidential visualization of soft tissues pathologies. Contrast-enhanced MRI can be carried out with T_1 - and T_2 -weighted sequences that require as contrast agents paramagnetic and superparamagnetic materials, respectively. The T_1 -weighted imaging is frequently preferred over T_2 -, as it induces a bright contrast for sharper image analysis and allows more rapid image acquisition. Commonly used and FDA-approved T_1 contrast agents, however, were shown to be associated with nephrogenic systematic fibrosis due to Gd^{3+} release from the injected complexes. Here, ultra-small iron oxide nanocrystals are produced by scalable flame aerosol technology and investigated as T_1 MRI contrast agents, focusing on structure-function relationships and cytocompatibility. The optimized nanocrystals are shown to be a promising cytocompatible alternative to commercial Gd-complexes as they attain comparable relaxivities with no apparent cytotoxicity at clinically relevant concentrations tested *in vitro* against four different cell types (PC3, HepG2, THP-1 and red blood cells). By using SiO_2 as a spacing material, the contrast enhancement could be finely tuned by decreasing the effective magnetic size of iron oxide resulting in significant T_1 contrast enhancement due to reduced magnetic coupling.

Keywords: *large scale synthesis, nanoparticles, silica-supported, MRI, magnetic interaction*

Introduction

Accurate and early diagnosis of diseases is necessary for a successful therapy and a cost-efficient health-care system. Today magnetic resonance imaging (MRI) is preferred clinically, as it allows for a detailed visualization of soft tissue while avoiding harmful ionizing radiation as applied during computer tomography.¹ The contrast in MRI originates from differences in the relaxation times of hydrogen protons. This relaxation process describes the re-alignment of magnetic moments to an external magnetic field after stimulation through a radio-frequency pulse and is strongly influenced by its surrounding environment. Furthermore, it can be classified into transverse and longitudinal, or T_1 and T_2 relaxation, respectively.

Typically, T_1 -weighted MRI is preferred over T_2 - due to the faster data acquisition using gradient-echo sequences. To further increase the contrast between adjacent tissues, contrast agents can be administered as they alter the relaxation times in their vicinity.² For T_1 -weighted MRI, paramagnetic Gd^{3+} ions embedded in chelating molecules are commonly used.³ Although very efficient in terms of contrast enhancement (high r_1 , low r_2/r_1), these Gd-complexes suffer from two major drawbacks. Most importantly it has been shown that, despite their small size (< 7 nm), a moderate amount (2% for healthy patients) of administered Gd-complexes remains within the body after 24 h and is not cleared out.⁴ While initially biocompatible due to their chelated state, Gd^{3+} ions are harmful when released.^{5,6} The risk for Gd leaching out of the complexes is further increased for patients with renal failure due to increased long-term accumulation of the contrast agents. This can lead to nephrogenic systemic fibrosis (NSF)^{5,7-11} causing the European health administration to suggest the replacement of linear Gd-complexes with safer alternatives. Furthermore it is noteworthy, that contrast agent enhanced MRI can still be considered as a comparably new clinical procedure, which renders NSF a rather unknown disease that is expected to gain even more impact in the near future. Additionally, their small size leads to very short blood half-life due to passive glomerular filtration. Even though this can be beneficial in certain procedures, it also limits their targeting efficiency of specific organs² as well as their applicability for long-term imaging. Also, these complexes cannot be functionalized easily for

specific cell targeting, as it is done readily with inorganic nanoparticles, thus further narrowing control over their bio-distribution.

Surprisingly little research, however, has been done on alternatives, despite the enormous commercial potential. This probably can be explained by the limited availability of non-toxic but strongly paramagnetic materials.² Inorganic iron oxide nanoparticles are the most promising candidates.¹²

While already clinically utilized as superparamagnetic T₂ contrast agents, they exhibit paramagnetic properties for particle sizes below 4 nm, due to a reduced magnetic volume and increased surface defects.¹³ A sufficient understanding of these structures and their magnetic properties, however, is yet to be achieved. Recently, first order reversal curves (FORCs), that give detailed information about magnetic property distributions and interactions, have been investigated intensively.¹⁴

Although, it has been shown that such ultra-small iron oxide nanoparticles (ESIONs) can compete with clinically used Gd-complexes in terms of contrast enhancement, they have not been commercially available so far. This can be explained by the comparably complex and hardly scalable methods for preparation of such small nanoparticles. The cost-efficient and reproducible production of nanomaterials is recognized today as a key requirement for a successful clinical translation.¹⁵ The most promising synthesis of ESIONs described so far, however, only resulted in a 5 g per batch production rate through thermal decomposition of an iron-oleate complex.¹³ Other utilized synthesis procedures include polyol method¹⁶, co-precipitation¹⁷ or reduction-precipitation¹⁸, however, all with limited scalability. Flame aerosol technology, on the other hand, has been shown to be scalable to kg h⁻¹.¹⁹ This technology is attractive for biomedical applications as it is a dry and high temperature process leading to inherent sterility of the product particles. Flame aerosol technology has been extensively studied for synthesis of several iron-based nanomaterials, such as iron oxides of different oxidation states²⁰, morphologies²¹, sizes¹⁴, or compositions²². Also, it has been investigated for synthesis of Pd subnanoclusters and even atoms, proving its ability to produce ultra-small particle systems.²³

In this study, flame aerosol technology is used for the synthesis of ultra-small iron oxide nanoclusters as contrast agents for T₁-weighted MRI. In detail, the effect of a SiO₂ support on the structure and performance of the system is investigated. Especially, the contrast enhancement properties are

extensively compared to competing alternatives. Special emphasis on the cytocompatibility of the most promising system is elucidated.

Experimental

Particle synthesis

Pure FeO_x and FeO_x / SiO₂ were prepared via flame spray pyrolysis, as described elsewhere in detail.²¹ In brief, the liquid precursor solution was fed at 2 ml min⁻¹ through a capillary and dispersed by 7 L min⁻¹ O₂ (all gases PanGas, purity > 99%) into fine droplets. The pressure drop over the capillary was constant at 1.6 bar. The formed spray was ignited and stabilized by a premixed CH₄ / O₂ flame (1.5 L min⁻¹ / 3.2 L min⁻¹). The resulting spray flame was sheathed by 10 L min⁻¹ O₂. Particles were formed in the gas phase and collected further downstream on a glass fiber filter (Whatman GF6, 257 mm diameter) with the aid of a vacuum pump (Busch, Seco SV 1040C).

For the precursor solution, iron(III)acetylacetonate (Acros Organics, purity 99+%) was dissolved at a concentration of 0.1 mol_{Fe} L⁻¹ in a 1:1 volume mixture of ethanol and 2-ethylhexanoic acid (both Sigma-Aldrich, purity >97%) and magnetically stirred for at least 1 h. Hexamethyldisiloxane (HMDSO, Sigma-Aldrich, purity ≥ 99%) was added to the solution to attain the desired SiO₂ wt% in the final nanoparticle composition (wt% SiO₂ = m_{SiO₂} / (m_{SiO₂} + m_{Fe₂O₃}) assuming Fe₂O₃ formation.

Particle characteristics

Scanning transmission electron microscopy images were obtained by an aberration-corrected apparatus (Hitachi HD-2700VD) with a probe corrector (CEOS). Measurements were performed at an acceleration potential of 200 kV (electron gun, cold-field emitter) in the ultra-high resolution mode using z-contrast. The FeO_x size distributions were obtained by manual measurements with ImageJ software.

Hydrodynamic diameter distributions of these flame-made agglomerates were measured by dynamic light scattering on a Zetasizer (Malvern Instruments). For this measurement, particles were dispersed

at a concentration of 0.1 mg mL^{-1} in an aqueous bovine serum albumin (BSA) solution (0.01 wt%) by sonication for 30 min in a water bath (Branson, 40 kHz).

Specific surface areas were measured according to the Brunauer Emmett Teller (BET) method on a Tristar II Plus (Micrometrics) at 77 K. All samples were degassed at 150°C under N_2 for at least 1 h prior to measurement. Primary particle size for pure FeO_x was calculated by assuming spherical morphology and a density of 5.24 g cm^{-3} , that corresponds to Fe_2O_3 . X-ray diffraction patterns were measured on a Bruker AXS D8 Advanced spectrometer (40 kV, 40 mA, Cu $K\alpha$ radiation).

Magnetic hysteresis curves were obtained on a Princeton Measurements Corporation vibrating sample magnetometer (PCM-VSM). Values are given in $\text{Am}^2 \text{ kg}^{-1}$ of FeO_x according to the nominal content of Fe_2O_3 in the particles. First order reversal curves¹⁴ were obtained on an alternating force gradient magnetometer (PCM-AGM). Measurements were made with an averaging time of 200 ms. A total of 125 FORCs were measured for each sample with a measurement interval between 2.0 to 2.3 mT. The FORC diagrams were processed with FORCinel software²⁴ using a smoothing factor of 4 for the pure and 45 wt% SiO_2 , and 8 for the 90 wt% SiO_2 and 95 wt% SiO_2 sample.

Iron oxide reduction measurements were performed on an AutoChem II (Micrometrics). Prior to these measurements all samples (approximately 50 mg) were heated up to 600°C (heating rate $10^\circ\text{C min}^{-1}$) under $5 \text{ mL min}^{-1} \text{ O}_2 / 95 \text{ mL min}^{-1} \text{ He}$. During the actual measurements, the chamber was heated to 800°C (heating rate $10^\circ\text{C min}^{-1}$) under $1.5 \text{ mL min}^{-1} \text{ H}_2 / 28.5 \text{ mL min}^{-1} \text{ Ar}$. Exiting gases were detected by mass spectrometry (MS).

MRI contrast enhancement properties

The MRI measurements were performed on a 3 T whole-body scanner (Magnetom Skyra, Siemens Healthcare, Erlangen, Germany). The signal was received using a 64-channel head coil, while the built-in body transmit coil was used for spin excitation. For quantification of the longitudinal relaxation times (T_1 times), a two-dimensional turbo spin echo sequence with inversion recovery preparation was acquired with increasing inversion times ($\text{TR} = 10000 \text{ ms}$, $\text{TE} = 13 \text{ ms}$, $\text{BW} = 130 \text{ Hz/px}$, turbo factor = 5, voxel size = $0.7 \times 0.7 \times 4.0 \text{ mm}^3$, TI = 25-50-100-200-400-700-1200-2000-

3500-8000 ms, acquisition time 33:40 min:sec). Transverse relaxation times (T_2 times) were determined from the signal acquired using a two-dimensional spin echo sequence for increasing times to echo (TR= 5000 ms, BW = 310 Hz/px, voxel size = 0.9x0.9x4.0 mm³, TE =7-12-20-30-45-65-100-150-250-400 ms, acquisition time 50:20 min:sec.). MR-images were processed offline for pixel-wise quantification of the R_1 and R_2 using routines written in Matlab (MATLAB Release 2012, The MathWorks, Inc., Natick, Massachusetts, United States). For all fitting procedures, non-linear least square fits to the signal intensities were performed based on the Levenberg-Marquardt-Algorithm (Matlab “lsqcurvefit”). The longitudinal relaxation rate, R_1 , was estimated by fitting of the inversion recovery MR-signal (S) to the function:²⁵

$$S(TI) = M_0(1 - 2e^{-TI \cdot R_1}) + N \tag{1}$$

where M_0 represents the equilibrium magnetization and N accounts for the image noise. The R_2 relaxation rate was calculated by fitting the signal measured using the spin echo sequence for increasing TE to the equation:²⁵

$$S(TE_i) = S(0) \cdot e^{-TE_i \cdot R_2} + N, \tag{2}$$

where $S(0)$ is the signal at TE = 0 ms. r_1 and r_2 relaxivities were determined from the slope of the linear fit of the corresponding relaxation rates and particle concentrations through at least 3 points (Figures S4 and S5). Samples for MRI measurements were prepared by first dispersing nanoparticles at a concentration of 0.2 mg mL⁻¹ in an aqueous bovine serum albumin (BSA) solution (0.02 wt%) by sonication for 20 min (Sonics Vibra Cell, 8 kJ, power 90%, pulse on / off 29 s / 1 s). Corresponding amounts of these stock solutions were mixed with water to the desired concentrations and a total volume of 750 μ L and filled into Eppendorf tubes. These suspensions were sonicated (Sonics Vibra Cell, 8 kJ, power 90%, pulse on / off 29 s / 1 s) for 1 min. Immediately afterwards, 750 μ L of hot aqueous agar suspension (1.5 wt%) was added to each tube. These final suspensions were cooled down under the above sonication procedure for 5 min. Final particle concentrations were 0, 0.2, 0.4, 0.6, 0.8, and 1 mg mL⁻¹ in 0.75 wt% Agar.

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3 T1 weighted magnetic resonance images of pork skeletal muscle tissues were obtained with the same
4 two-dimensional turbo spin echo sequence with inversion recovery preparation and inversion times of
5 700, 1000 and 1200 ms, respectively. The FeO_x / 95 wt% SiO₂ particles were coated with BSA as
6 described above at a concentration of 5 mg mL⁻¹ (BSA 5 wt%) and diluted with water to the desired
7 concentrations. Volumes of 250 µL were carefully injected through a needle into the tissues.
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13 *Cytotoxicity*

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16 Measurements with PC3, HepG2 and THP-1 cells were carried out according to the protocol from the
17 manufacturer using the Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay kit. The PC-3 and
18 HepG2 cells were cultured in supplemented growth medium (Gibco™ RPMI-1640, Thermo Fisher
19 Scientific) with 10% fetal bovine serum (FBS, Gibco™, Thermo Fisher Scientific) and 1%
20 penicillin/streptomycin (PenStrep, Gibco, Thermo Fisher Scientific) at 37 °C under humidified
21 atmosphere containing 5% CO₂. For the experiments, PC3 or HepG2 cells were seeded into a 96-well
22 plate at a density of 10 000 cells/well (100 µL/well) and cultured for 24 h. For co-incubation of NP
23 with the cells, sterile nanoparticle suspensions were prepared in unsupplemented RPMI-1640 and
24 autoclaved for 20 min at 121°C. Desired NP concentrations were adjusted with unsupplemented
25 RPMI-1640 and twofold supplemented growth medium at least 45 min prior co-incubation with cells.
26 Growth medium in the flat bottom 96-well plate was replaced with thoroughly vortexed NP
27 suspensions securing sample homogeneity or vehicle controls (100 µL). Supplemented RPMI-1640
28 was used as a negative control and Triton™ X-100 (0.1 % solution) as a positive control, the latter
29 applied for cell lysis. The cells were incubated with the nanoparticles for 24 h at 37 °C under
30 humidified atmosphere containing 5% CO₂. After the incubation, the plates were centrifuged at 500g
31 for 5 min at room temperature sedimenting dispersed nanoparticles. The supernatant (50 µL/well) was
32 transferred in a new flat bottom 96-well plate, mixed with 50 µL of substrate mix from Promega
33 CytoTox 96® Non-Radioactive Cytotoxicity Assay kit and incubated in the dark at room temperature
34 for 15 min. Thereafter, the absorbance was measured at 490 nm using a Mithras LB 943 Multimode
35 Plate Reader.
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The human monocytic cells derived from an acute monocytic leukemia patient (THP-1) were cultured in RPMI-1640 with 10% FBS at 37 °C under humidified atmosphere containing 5% CO₂. For the experiments, monocyte cells were seeded into a 48-well plate at a density of 100 000 cells in 225 µL using 10% FBS containing medium. For co-incubation of NP with the cells, freshly prepared NP suspensions at the desired concentration or vehicle controls (25 µL) were added to the wells resulting in a final particle concentration ranging from 0 up to 1000 µg mL⁻¹. The total volume used in a well was 250 µL. Water was used as a negative control and Triton™ X-100 (0.1 % solution) as a positive control. The cells were incubated with the nanoparticles for 24 h at 37 °C under humidified atmosphere containing 5% CO₂. After the incubation, the cells were centrifuged at 2250g for 15 min at room temperature to remove nanoparticles from the supernatant. The supernatant (50 µL/well) was transferred in a new flat bottom 96-well plate, mixed with 50 µL of substrate mix from Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay kit and incubated in the dark at room temperature for 15 min. Thereafter, the absorbance was measured at 490 nm using a Mithras LB 943 Multimode Plate Reader.

To assess hemolytic properties, firstly, after written informed consent (ethical approval, EKSG PB_2016_00816), whole blood from healthy volunteers was collected in vacutainer tubes containing 0.109 mol L⁻¹ citrate. Immediately after collection, the blood collection tubes were centrifuged at 800g. The plasma and the buffy coat layer were discarded. The remaining red blood cells were re-suspended in PBS and washed three times. The hemoglobin concentration was adjusted to approximately 10 mg mL⁻¹. Triton™ X-100 (1% solution) served as positive and PBS as negative control. 180 µL red blood cells and 20 µL of nanoparticle suspension (FeO_x/95 wt% SiO₂ in PBS, 2.5 µg µL⁻¹) were added to Eppendorf tubes. Additionally, nanoparticles were also incubated with free hemoglobin (fully lysed samples) to account for adsorption of hemoglobin on the nanoparticle surface. After incubation for 3 h at 37 °C, the tubes were centrifuged at first at 800g to remove the red blood cells and then at 6000g for 5 min to remove the nanoparticles. The supernatant (100 µL/well) was transferred in a new flat bottom 96-well plate and the absorbance was measured at 570 nm (hemoglobin) using a Mithras LB 943 Multimode Plate Reader.

Results and Discussion

Iron oxide nanoparticles (FeO_x) are co-produced with varying amounts of SiO_2 (0 – 95 wt%) as a support material by flame spray pyrolysis (FSP). Figure 1a shows a z-contrast TEM image of FeO_x / 95 wt% SiO_2 . In the inset, the light silica matrix is homogeneously covered by isolated FeO_x clusters appearing as small bright spots. Three of these FeO_x clusters are exemplarily circled by a dotted line. Figure 1b shows the number size distribution of the FeO_x clusters having an average diameter of 1.5 nm with a geometric standard deviation $\sigma_g = 1.28$ (Figure S1), which is comparable to earlier studies.^{13,16–18} In comparison, pure FeO_x has an average primary particle diameter, d_{BET} , of 3.9 nm corresponding to a specific surface area of $296 \text{ m}^2 \text{ g}^{-1}$. The specific surface area of FeO_x / 95 wt% SiO_2 is $394 \text{ m}^2 \text{ g}^{-1}$. The small FeO_x size in these particles can be explained by the inhibiting effect of SiO_2 on the gas-phase formation/growth of FeO_x .²¹ These particles are similar to mixed FSP-made $\text{Fe}_2\text{O}_3/\text{SiO}_2$,²¹ but here the SiO_2 content is higher, while the high temperature residence time, Fe concentration of the precursor solution (i.e. 0.1 vs. 0.34 M) and enthalpy of solvents (26.8 vs. 34.3 kJ cm^{-3}) are lower, which leads to the present smaller particles.

Figure 1: (a) Transmission electron microscopy (z-contrast) image of FeO_x / 95 wt% SiO_2 . (b) Number size distribution of the FeO_x clusters determined from (a) and of the hydrodynamic diameter of BSA-coated FeO_x / 95 wt% SiO_2 .

This is also why X-ray diffraction patterns (Figure S2) are dominated here by amorphous SiO_2 . Weak or diffuse peaks suggest a minor, if any, crystalline iron oxide fraction for low SiO_2 contents and pure

FeO_x which however could not be matched with known crystalline patterns. Flame-made Zn_{0.4}Fe_{2.6}O₄ at higher temperatures and concentrations that had $d_{\text{XRD}} = 4.4 \text{ nm}^{22}$ exhibited more distinct diffraction peaks than pure FeO_x here, which suggests a smaller crystal size and fraction here. For larger Fe₂O₃ ($d_{\text{XRD}} = 10 \text{ nm}$) with 46 wt% SiO₂ made at hotter flames and higher Fe concentrations,²¹ the XRD patterns indicated a clear crystalline content. The crystal characterization of iron oxides (α -, γ -Fe₂O₃, Fe₃O₄), especially for small particles, by XRD, however, is not straightforward. For flame-made iron oxides the strongly oxidizing synthesis conditions may lead to the predominant formation of α -/ γ -Fe₂O₃ opposed to Fe₃O₄.²¹ Although, higher temperatures lead to the transformation of γ - to α -Fe₂O₃,²⁶ below a critical crystal size the formation of α -Fe₂O₃ is not favorable. Additionally, the formation of α -Fe₂O₃ is suppressed by the presence of SiO₂.²⁷ The present iron oxide particles, however, are referred to as FeO_x, due to the predominantly amorphous XRD patterns (Figure S2).

To stabilize the FeO_x / SiO₂ nanoparticles in liquid dispersion, bovine serum albumin (BSA) was adsorbed onto their surface.²⁸ Figure 1b shows the hydrodynamic diameter distribution (d_{DLS}) of FeO_x / 95 wt% SiO₂, indicating a stable agglomerate size of approximately 100 nm ($\sigma_g = 1.32$).

Figure 2 shows the magnetic hysteresis curves of as-prepared FeO_x / SiO₂ nanoparticles with various SiO₂ contents up to 1000 mT, which allows the qualitative determination of paramagnetic contents. The inset depicts the corresponding coercivities and magnetizations at 1000 mT. All nanoparticles have a significant paramagnetic content as their hysteresis curves have not yet reached complete saturation at 1000 mT. This may indicate crystal sizes below 5 nm¹³ and/or α -Fe₂O₃.¹⁴ The magnetization at 1000 mT continuously decreases for increasing SiO₂ content, suggesting that SiO₂ decreases the magnetic domain size.²¹ Pure FeO_x (red line) shows a magnetization of 2.6 Am² kg⁻¹ at 1000 mT and coercivity of 9 mT, which is comparable to previously reported flame-made iron oxide nanoparticles.¹⁴

Figure 2: Magnetic hysteresis curves of FeO_x / SiO_2 nanoparticles with various SiO_2 contents, y . Magnetization and coercivity decrease with increasing SiO_2 mass fraction.

The coercivity generally decreases for increasing SiO_2 content without, however, fully reaching zero.

The ferro-/ ferrimagnetic (non-paramagnetic) domains of all samples are therefore not fully in a superparamagnetic state, i.e. some particles are magnetically ordered on the scale of the measurement.

The non-zero coercivity might be explained by the magnetic coupling between primary particles within an aggregate, which is reduced for higher SiO_2 contents. It increases the effective magnetic size (dictating the magnetic properties) above the superparamagnetic/single-domain limit (~ 25 nm for Fe_2O_3)²⁹. Here, the primary particle size, d_{BET} , is, however, distinctively below 25 nm (Table S1).

These changes in coercivity emphasize the difference between particle (d_{BET}) and their effective magnetic size, i.e. caused by agglomeration/aggregation, which has to be considered in such systems.

For FeO_x / 95 wt% SiO_2 (light blue) the coercivity could not be accurately determined, as the magnetic signal was too weak.

More detailed information on magnetic coupling phenomena, that is not accessible by conventional hysteresis curves, can be obtained from first order reversal curves (FORCs, Figure 3). In these diagrams the distribution maximum is of main interest and is located for single-domain ferro- or ferrimagnetic nanoparticles (with vertical symmetry) on the B_{CF} -axis. It moves with decreasing magnetic size closer to the origin that marks the superparamagnetic/single-domain limit. For even smaller and therefore superparamagnetic nanoparticles the maximum shifts up along the B_{UF} -axis.³⁰ Paramagnetic fractions, on the other hand, are not detectable by FORC. Furthermore, distribution minima are commonly not analyzed in detail as they come alongside maxima, that give more detailed information on magnetic properties.

Figure 3 shows FORCs of FeO_x / SiO_2 nanoparticles with various SiO_2 contents. For pure FeO_x (a) and FeO_x / 45 wt% SiO_2 (b) the data indicate predominantly single-domain ferro-/ferrimagnetic properties, as the distribution maxima are located on the B_{CF} -axis. Their primary particle sizes, however, are well below the superparamagnetic/single-domain limit, which would suggest a maxima location on the positive B_{UF} -axis. Interestingly, already for 45 wt% SiO_2 content, the distribution is slightly shifted up in comparison to pure FeO_x . In line with hysteresis curves (Figure 2) that also show reduced coercivities for increasing SiO_2 contents, coercivity distributions move into (along the B_{UF} -axis) the superparamagnetic regime for 90 wt% SiO_2 (c). Note that FeO_x / 95 wt% SiO_2 is barely ferro-/ferrimagnetic and paramagnetism cannot be detected by FORCs. So the measurement (Figure S3) is near the sensitivity level of the instrument and therefore the noise level is high. Altogether, this confirms the above hypothesized reduction in magnetic interaction and therefore also reduced effective magnetic size as SiO_2 acts as a spacing material between magnetic domains. This allows the FeO_x particles to act independently resulting in magnetic properties according to the FeO_x primary particle size.²⁷ It should be noted that the magnetization continuously decreases, as seen in the hysteresis measurements (Figure 2), which again shows the smaller magnetic domains.

Figure 3: First order reversal curves of (a) pure FeO_x and $\text{FeO}_x/\text{SiO}_2$ nanoparticles with (b) 45 wt% and (c) 90 wt% SiO_2 content. Magnetic coupling decreases with increasing SiO_2 mass fraction.

In order to investigate the location of FeO_x with respect to the silica support or matrix, the $\text{FeO}_x/\text{SiO}_2$ nanoparticles were annealed to 800 °C under H_2 to determine the FeO_x reduction temperature. Figure 4 shows the H_2O signal in the effluent stream from the reduction chamber as a function of temperature. For pure FeO_x , peaks at 380 °C and 650 °C correspond to the reduction of Fe_2O_3 to Fe_3O_4 and Fe_3O_4 to Fe, respectively.³¹ The first peak shifts continuously to higher temperatures for increasing SiO_2 content, as indicated by the dashed lines. For 95 wt% SiO_2 content, for example, the Fe_2O_3 to Fe_3O_4 reduction takes place at 500 °C. This could be explained by the interaction of FeO_x with the SiO_2 support. At large SiO_2 contents, most likely the SiO_2 matrix partially incorporates, coats or encapsulates the FeO_x particles, as suggested by TEM (Figure 1). The insets in Figure 4 depict this schematically. Such coatings hinder H_2 diffusion to FeO_x and delay its reduction shifting such peaks to higher temperatures. For pure SiO_2 a weak peak at 670 °C was detected (data not shown).

Figure 4: Iron oxide reduction under H_2 atmosphere. Signal of H_2O in the outflow stream as a function of temperature after reduction of FeO_x / SiO_2 nanoparticles with various SiO_2 contents, y . The interaction between FeO_x and SiO_2 increases for increasing SiO_2 content. Dashed lines mark the reduction peaks for pure FeO_x (380 °C) and $FeO_x / 95$ wt% SiO_2 (500 °C).

Efficient contrast agents for T_1 MRI require high r_1 and low r_2 values as well as low r_2/r_1 ratios.² Figure 5a shows T_1 (triangles, left axis) and T_2 (circles, right axis) relaxivities of the as-prepared flame-made FeO_x / SiO_2 with BSA-coating measured as agar suspensions at room temperature as a function of SiO_2 content, which are determined from the slope of the linear fit between T_1 and T_2 relaxation rates as a function particle concentration (Figures S4 and S5). All FeO_x / SiO_2 particles showed linear relaxation rates except for T_2 at large SiO_2 contents (95 wt%). This indicates the desired weak T_2 contrast enhancement performance of these particles. The r_1 remains insignificantly low until a SiO_2 content of 45 wt% is reached and thereafter increases up to $3\text{ s}^{-1}\text{ mM}_{Fe}^{-1}$. The r_2 for pure FeO_x is $r_2 = 21.6\text{ s}^{-1}\text{ mM}_{Fe}^{-1}$. The addition of SiO_2 up to 85 wt% content decreases r_2 , before slightly increasing it to 13.8 mM_{Fe}^{-1} above that content.

The trend of r_1 can be explained by the reduction of the magnetic domain size described above (Figures 2 & 3). In pure FeO_x the magnetic primary particles are in close contact. This leads to strong magnetic coupling between them, increase in effective magnetic size and low r_1 , although the primary particle size (< 4 nm) by itself would imply a good T_1 -contrast enhancement. The addition of SiO_2 , however, leads to increased spacing between the FeO_x particles and therefore reduced magnetic coupling.³² This results in a smaller effective magnetic size down to primary particle or crystal FeO_x size. As a result, the r_1 increases, since the FeO_x structures can now act independently and according to their size. This effect is further enhanced by the reduction of FeO_x size with increasing SiO_2 content compared to bare FeO_x , as discussed above. The marginally increasing r_2 for larger SiO_2 contents cannot be explained at the present stage, although they seem within the error bar of the measurement, which suggests a constant value regardless of SiO_2 content.

Changes in MRI relaxivities due to magnetic coupling have been reported previously, however, only for larger nanoparticles.³³ It was found that r_1 is affected only slightly by agglomeration of iron oxide nanoparticles, while r_2 increases for increased particle-particle interactions.^{34,35} This is not in agreement with the trends found here, where both relaxivities increase with decreased magnetic coupling.

As FeO_x / 95 wt% SiO_2 showed both the highest r_1 and lowest r_2/r_1 of all prepared particles, they were coated with BSA and compared to a clinically applied Gd-complex (Dotarem, Guerbet, France). Their r_1 , r_2 and r_2/r_1 were determined at 37 °C in aqueous dispersion. It can be seen that especially for r_1 (FeO_x / SiO_2 : $2.3 \text{ s}^{-1} \text{ mM}_{\text{Fe}}^{-1}$; Dotarem: $3.5 \text{ s}^{-1} \text{ mM}_{\text{Fe}}^{-1}$) both systems perform comparably. The larger difference in r_2 (FeO_x / SiO_2 : $12.4 \text{ s}^{-1} \text{ mM}_{\text{Fe}}^{-1}$; Dotarem: $4.9 \text{ s}^{-1} \text{ mM}_{\text{Fe}}^{-1}$) can be explained by the partly remaining super-paramagnetism of the FeO_x particles. Interestingly, both relaxivities of the FeO_x / 95 wt% SiO_2 nanoparticles measured at 37 °C (2.3 & $12.4 \text{ s}^{-1} \text{ mM}_{\text{Fe}}^{-1}$) are reduced in comparison to their measurement at room temperature (3 & $13.8 \text{ s}^{-1} \text{ mM}_{\text{Fe}}^{-1}$).³⁶ This shows the importance of biologically relevant measurement conditions, especially when comparing different studies.

For effective performance comparisons, the r_2/r_1 ratio is commonly analyzed. Kim et al.¹³ reported an r_2/r_1 ratio of 3.67 for 2.2 nm iron oxide nanoparticles. Similarly, Shen et al.³⁷ suggest the use of 1.9 nm

sized Fe_3O_4 with r_2/r_1 of 2.03. Zhou et al.³⁸ investigated Gd-embedded iron oxides (4.8 nm) and reported an r_2/r_1 ratio of 5.24 (at 25 °C). The presently reported value of 5.4 (at 37 °C) is therefore in good agreement to the literature. As outlined, the measurement temperature has a significant impact on the retrieved relaxation rate, but unfortunately a fair comparison of different studies is largely hampered due to the lack of reported measurement conditions.

Figure 5: (a) Relaxivities r_1 (triangles, left axis) and r_2 (circles, right axis) of BSA-coated $\text{FeO}_x / \text{SiO}_2$ measured in agar at room temperature as a function of SiO_2 content. Their values increase with increasing SiO_2 content due to reduced magnetic coupling. Inset depicts comparison of BSA-coated $\text{FeO}_x / 95 \text{ wt\% SiO}_2$ with the clinical contrast agent Dotarem (Guerbet) measured as aqueous dispersions at 37 °C. (b) T_1 weighted magnetic resonances images at different inversion times of two different pork skeletal muscle tissues injected with suspensions of $\text{FeO}_x / 95 \text{ wt\% SiO}_2$ at different concentrations.

As a proof-of-concept the best performing FeO_x / 95 wt% SiO_2 particles were injected into pork skeletal muscle tissues at different concentrations ($0.1 - 5 \text{ mg mL}^{-1}$). Figure 5b shows their T_1 weighted magnetic resonance images at different inversion times. The injected suspensions ($250 \mu\text{L}$) gave the desired bright contrast for all concentrations and inversion times. As expected, 5 mg mL^{-1} resulted in the strongest contrast spot. This simplified clinical scenario verifies the excellent contrast enhancement properties of the prepared particles, even in fatty and therefore hard-to-image muscle tissue.

Assessing the cytotoxicity of the prepared particles, Figure 6 shows the fraction of lysed prostate (PC3, a) and liver (HepG2, b) cancer cells, as well as of human monocytes (THP-1, c) after 24 h incubation with FeO_x / 95 wt% SiO_2 (red), Aerosil 200 (blue) and ZnO (green) nanoparticles at concentrations of 25 to $1000 \mu\text{g mL}^{-1}$ in serum containing media relative to full lysis (Triton X). The FeO_x / 95 wt% SiO_2 caused only minimal cell lysis in all three cell lines up to 500 mg L^{-1} and even led to slight stabilization of PC3 at high concentrations.^{39,40} Most importantly, FeO_x / 95 wt% SiO_2 showed better cytocompatibility than ZnO and Aerosil 200 nanoparticles for almost all conditions. Aerosil 200 is commercial fumed silica which is widely used in several biomedical applications (e.g. drug formulations).⁴¹ The ZnO readily dissolves to release toxic Zn^{2+} ions, which served here as a positive nanoparticle control.³⁹ It caused distinct cell-death for concentrations above 100 mg L^{-1} for all three cell-lines. Interestingly, human THP-1 cells showed to be very susceptible to Aerosil 200 and ZnO exposure.⁴²

Figure 6: Cytotoxic activity of FeO_x / 95 wt% SiO_2 , Aerosil 200 and ZnO at different concentrations against (a) PC3, (b) HepG2 and (c) THP-1 cells. Values account for negative (no particles, orange

field) and positive control (Triton X, 0.1%). Data suggests good cytocompatibility of FeO_x / 95 wt% SiO_2 .

Furthermore, the hemolytic properties of the bare, BSA- and HSA-coated FeO_x / 95 wt% SiO_2 nanoparticles at a dose comparable to the recommended dose of Resovist (Bayer HealthCare Ag, Germany, a formerly approved iron oxide based contrast agent)⁴³, were assessed in a hemolysis assay as a function of surface coating (Figure S6). Most importantly, these nanoparticles do not show any hemolytic properties, irrespective of surface functionalization. All FeO_x / 95 wt% SiO_2 solutions show values in the range of the negative control (H_2O). The Aerosil 200, however, shows strong hemolytic properties comparable to the positive control (Triton X).⁴⁴

Conclusions

Ultra-small iron oxides (FeO_x) separated by SiO_2 were produced by scalable and dry flame aerosol technology. For the first time, the importance of small effective magnetic sizes for efficient T_1 MRI contrast enhancement was shown. Through careful addition of silica, the FeO_x particles could be spatially separated during their preparation and the magnetic coupling between them was reduced. The resulting highly efficient FeO_x / SiO_2 had relaxivities comparable to clinically applied contrast agents. The lack of linear dependence of T_2 relaxation rates with particle concentration at high SiO_2 contents, however, suggest poor T_2 contrast enhancement. The exact composition of the produced iron oxide phase has remained unclear so far. Most importantly, the particles were cytocompatible by testing their *in vitro* toxicity on four different cell types. However, *in vivo* experiments are necessary to address biodistribution, degradation and excretion of these nanoparticles. Due to the presence of SiO_2 , the system can be easily surface-functionalized to introduce molecular targeting entities. As current clinical contrast agents for T_1 MRI are composed of Gd-complexes, which have been linked to nephrogenic systemic fibrosis, the proposed flame-made ultra-small iron oxide nanoparticles offer safe and efficacious access to T_1 contrast agents fulfilling major prerequisites for a future translation into clinics.

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Supporting Information

Figures showing TEM size analysis, x-ray diffraction patterns, first order reversal curves, T1 and T2 relaxation rates, hemolytic properties and specific surface areas.

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