Targeted $T_1$ Magnetic Resonance Imaging Contrast Enhancement with Extraordinarily Small CoFe$_2$O$_4$ Nanoparticles

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ABSTRACT: Extraordinarily small (2.4 nm) cobalt ferrite nanoparticles (ESCIoNs) were synthesized by a one-pot thermal decomposition approach to study their potential as magnetic resonance imaging (MRI) contrast agents. Fine size control was achieved using oleylamine alone, and annular dark-field scanning transmission electron microscopy revealed highly crystalline cubic spinel particles with atomic resolution. Ligand exchange with dimercaptosuccinic acid rendered the particles stable in physiological conditions with a hydrodynamic diameter of 12 nm. The particles displayed superparamagnetic properties and a low $r_2/r_1$ ratio suitable for a $T_1$ contrast agent. The particles were functionalized with bile acid, which improved biocompatibility by significant reduction of reactive oxygen species generation and is a first step toward liver-targeted $T_1$ MRI. Our study demonstrates the potential of ESCIoNs as $T_1$ MRI contrast agents.

KEYWORDS: cobalt ferrite nanoparticles, one-pot synthesis, magnetic resonance imaging, ligand exchange, cytotoxicity, ultrasmall nanoparticles, $T_1$-weighted contrast agent, liver targeting

1. INTRODUCTION

Contrast agents (CA) are useful tools to enhance image contrast in magnetic resonance imaging (MRI). $T_1$ contrast agents, which shorten $T_1$ and generate positive (longitudinal) contrast, are primarily gadolinium (Gd)-based. With seven unpaired electrons, Gd provides effective $T_1$ relaxation of surrounding water protons by dipole interactions. As Gd ions are toxic, chelating agents are used to shield the body from exposure, which accelerate renal clearance and mitigate the toxicity. However, in a recent postmortem study by Mayo Clinic researchers, Gd deposits were found in the brain tissue of patients who had received Gd contrast agent-enhanced MRI scans.¹ The potential for Gd dechelation raised fresh concerns over the safety of Gd-based agents, and in February 2018, the U.K. government suspended the licenses of Omniscan and...
Magnevist, two main commercial Gd-based contrast agents, until further investigation.\(^2\)

The safety concerns for Gd-based agents prompted research into alternative materials, and recently, extremely small-sized iron oxide nanoparticles (<3 nm) emerged as a potential biocompatible approach.\(^1\) Larger superparamagnetic iron oxide nanoparticles (SPIONs) (>5 nm) have proven clinical utility for transverse (\(T_2\)) contrast enhancement through induction of local magnetic field inhomogeneities, which dephase nearby water protons, shortening both \(T_1\) and \(T_2\) relaxation. As SPIONs are uptaken by the reticuloendothelial system and thus predominately accumulate in the liver,\(^3\) they proved suitable for liver-targeted MRI, and two types of SPIONs received FDA approval (Feridex and Resovist).\(^5\) However, Gd-based nonspecific and hepatospecific agents demonstrated better practicality and diagnostic performance for liver cancer and are currently preferred by clinicians.\(^6\) For liver MRI, \(T_1\)-weighted contrast agents are beneficial for detecting metastases and liver lesions.\(^7\) Kim et al. recently showed that \(T_1\)-weighted contrast could be achieved with iron oxide nanoparticles by reducing their size below 3 nm. In a size-dependent study with 1.5, 2.2, 3.0, and 12 nm maghemite particles,\(^8\) they found that the magnetic core of particles smaller than 3 nm was greatly reduced because they were composed mostly of surface atoms, which are magnetically disordered due to spin canting. This led to a decrease in \(T_2\) effect, while the five unpaired electrons provided shortening of \(T_1\). Similar studies that echo this finding have since emerged.\(^9\)-\(^11\)

Iron oxide offers a more biocompatible route than Gd because its degradation products can be stored by ferritin in the body. Although generally considered safe, there are toxicity concerns surrounding iron oxide and, particularly, other metal oxide nanoparticles such as cobalt ferrite, notably due to their potential to generate reactive oxygen species.\(^12\) The toxicity of metal oxide nanoparticles, however, can be effectively mitigated through a biocompatible surface coating, and many different functionalization approaches have emerged.\(^13\) In addition to tailoring the surface of iron oxide nanoparticles, their magnetic properties can be customized by substituting ferrous ions with other metal ions. Substitution with cobalt ions introduces magnetic anisotropy, which has proved useful for biomedical applications such as magnetic hyperthermia.\(^14\) and Co\(^{2+}\) ions were recently shown to follow the same ferritin remediation pathway as iron.\(^15\) Cobalt ferrite (CoFe\(_2\)O\(_4\)) nanoparticles have also recently demonstrated high potential for MRI imaging with >5 nm particles, showing effective shortening of \(T_2\) relaxation time.\(^16\) In comparison to magnetite, bulk CoFe\(_2\)O\(_4\) possesses 90% saturation magnetization (\(M_s\))\(^17\). The same was observed at the nanoscale, where 6 nm Fe\(_3\)O\(_4\) and CoFe\(_2\)O\(_4\) nanoparticles had \(M_s\) values of 55 and 50 emu g\(^{-1}\), respectively.\(^18\) Considering that lower \(M_s\) values reduce \(T_2\) effects and Co\(^{2+}\) cations have three unpaired electrons and Fe\(^{3+}\) has five, CoFe\(_2\)O\(_4\) nanoparticles could be beneficial for \(T_1\)-weighted MRI; however, to the best of our knowledge, it has not been explored before. In addition, CoFe\(_2\)O\(_4\) is more resistant to oxidation,\(^19\) which can affect the magnetic properties,\(^20\) as well as toxicity.\(^12\) In this context, EScIoNs could be promising candidates for \(T_1\)-weighted MRI applications.

Due to their large surface-to-volume ratio, the properties of particles <5 nm are sensitive to slight changes in their size, shape, and composition. Many existing methods have demonstrated fine control over metal oxide particle size and shape at larger scales (>5 nm);\(^21\)-\(^24\) however, control of these features below 5 nm remains a challenge and often requires the use of multiple solvents, surfactants, and reducing agents. Oleylamine, a long-chained alkylamine with a weak affinity for transition metals, can play a triple role of a solvent, surfactant, and reducing agent, mitigating the need for additional reagents. Nevertheless, to date, only a handful of studies have reported the synthesis of particles with oleylamine alone,\(^25\)-\(^27\) and its role in controlling nanoparticle size is still not well understood.

Although synthesis by thermal decomposition offers superior size control in comparison to other methods such as co-precipitation, the particles produced are only dispersible in nonpolar solvents. One strategy to overcome this is to exchange the hydrophobic ligands for a hydrophilic ligand. Dimercaptosuccinic acid (DMSA), a small molecule composed of carboxylate and thiol groups, has a high affinity for transition metal ions and thus is effective at replacing ligands via a simple ligand exchange technique.\(^28\) Additionally, DMSA is nontoxic and provides a chemically versatile surface for further functionalization.\(^29\) Tailoring the surface of nanoparticles offers the potential for more specific therapeutic and diagnostic in vivo application, such as liver-targeted MRI. For liver-targeted applications, in addition to passive targeting by nanoparticle accumulation in the liver, active targeting with a hepatospecific functional group could be beneficial. Bile acids are amphipathic molecules with a hydrophilic chain and functional head group, which are uptaken into the liver via the hepatic pathway.\(^30\) As such, they have been proposed as ideal surface modifiers for liver-targeted in vivo applications. Kramer et al. effectively demonstrated the use of bile acids as “Trojan horses” to deliver drugs specifically into the liver.\(^31\) Similarly, Zhang et al. found that functionalizing chitosan nanoparticles with cholic acid (CA), a primary bile acid, remarkably improved the efficacy of insulin delivery, owing to CA’s liver-targeting properties.\(^32\) Others have functionalized Gd chelates with CA toward liver-targeted MRI.\(^33,34\) To the best of our knowledge, bile acid-functionalized ferrite nanoparticles have not been explored before.

In this work, we report the synthesis, functionalization, and application of novel ESCIoNs for \(T_1\)-weighted MRI. A simple method for preparing ultrasmall MFe\(_2\)O\(_4\) nanoparticles (M = Fe, Co) with subnanometer-size control is introduced. Following ligand exchange with DMSA, we obtained water-dispersible particles with small hydrodynamic diameters (~12 nm) that were stable in physiological conditions. As the same method and conditions were used to produce iron oxide and cobalt ferrite nanoparticles of the same size, we directly compare the effect of cobalt on the particles’ properties, as well as the induction of cytotoxic and oxidative stress effects to HepG2 cells. For the first time, EScIoNs are shown to act as \(T_1\) MRI contrast agents. We further report the functionalization of EScIoNs with a bile acid derivative (cholic acid), which significantly reduced the production of reactive oxygen species (ROS) in labeled cells and is a promising step toward the development of safe liver-targeted MRI contrast agents.

2. EXPERIMENTAL SECTION

2.1. Synthesis of MFe\(_2\)O\(_4\) (M = Fe or Co) Nanoparticles. To synthesize CoFe\(_2\)O\(_4\) nanoparticles, 0.02 mmol iron acetylacetonate (Fe(acac)\(_3\)), 0.01 mmol cobalt acetylacetonate (Co(acac)\(_2\)), and 60 mmol oleylamine (OLA) were placed into a three-neck flask fitted with a magnetic stirrer, reflux cooler, and thermometer. The mixture
was stirred at 400 rpm under argon for 10 min at room temperature before being heated to 225 °C at a heating rate of 14 °C min⁻¹. The solution was kept at this temperature for 10 min before allowing it to cool. The particles were then collected by centrifugation (4600 rpm, 10 min), washed in ethanol several times, and dispersed in cyclohexane. To prepare iron oxide nanoparticles, 0.03 mmol Fe(NO₃)₃·9H₂O, was used with the conditions described above. Samples were diluted 1:1000 (v/v) in ethanol. Zeta potential measurements (section 9 in the Supporting Information) were performed to estimate the particle concentration. The magnetic properties of the nanoparticles in dried form were assessed using a superconducting quantum interference magnetometer (SQUID) (MPMS3, Quantum Design, Inc.).

2.2. Ligand Exchange with Dimercaptosuccinic Acid (DMSA).

The ligand exchange method was adapted from a previously reported protocol.²⁹ Fe₂O₃@OLA particles were evaporated by rotary evaporation and redispersed in toluene at a concentration of 5 mg/mL. Five milliliters of the particle dispersion was added to a glass vial and sonicated for 5 min in an ultrasonic bath. A DMSA solution was prepared by mixing 200 mg of DMSA in 12 mL of dimethyl sulfoxide (DMSO) by sonication for 5 min. The DMSA solution was added to the nanoparticles and sonicated for 15 min and then stirred at room temperature for 48 h at 200 rpm. To terminate the reaction, 20 mL of ethanol was added, and the particles were magnetically separated. The supernatant was discarded, and the particles were washed three times in ethanol with centrifugation at 4600 rpm for 15 min. Following redispersion in water, the pH was adjusted to 10 using NaOH, and the particles were dialyzed in 500 mL of water (SnakeSkin dialysis tubing (3.5 kDa molecular weight cut-off, 22 mm × 35 feet diameter, Thermo Scientific) in chloroform) for 3 h, with the solvent exchanged at the 1.5 h mark.

2.2. Characterization.

The particles were characterized with transmission electron microscopy (TEM) using a JEOL 3000F microscope (300 kV acceleration voltage) and annular dark-field scanning transmission electron microscopy (ADF-STEM) using an aberration-corrected JEOL ARM200F microscope operating at both 80 and 200 kV. Electron energy loss spectroscopy (EELS) was performed to analyze the elemental composition of the particles and the oxidation state of the metals. CrystalMaker was used to visualize the crystal structure. Particle diameters were measured from TEM images using automated nanomaterial measurement software developed in-house. Hydrodynamic diameter measurements were obtained using Nanotrac NPA 250 with an external probe. Samples were diluted to approximate concentrations of 30 μg/mL and irrigated with a HeNe laser (red light, 780 nm), and the intensity fluctuations of the scattered light (detected at a backscattering angle of 180°) were analyzed. Three independent measurement runs of 60 s were collected for each sample at a temperature of 20 °C. The mean hydrodynamic diameter was obtained using cumulant analysis and a size distribution using a regularization scheme by number. Zeta potential (ζ) measurements were performed using a Malvern Zetasizer Nano ZSP equipped with a 633 nm laser. Three independent measurements including several subruns of 10 s per subrun were performed for each sample at a temperature of 25 °C. The ζ potential was calculated from the nanoparticle electrophoretic mobility using Smoluchowski theory. Thermogravimetric analysis (TGA) was employed to determine the amount of ligand on the surface of the nanoparticles using PerkinElmer Pyris Diamond TG/DTA6300. Five milligrams of dried nanoparticles were heated to 900 °C at a heating rate of 10 °C min⁻¹ under nitrogen gas flowing at 80 mL/min. To verify ligand coating, Fourier transform IR spectroscopy (FTIR) was employed using Varian Excalibur FTS 3500 FTIR. Transmission electron microscopy investigations on DMSA- and bile acid-functionalized particles were performed using a JEOL JEM-2100 microscope with a LaB₆ cathode operating at an accelerating voltage of 200 kV. For cryogenic-TEM (Cryo-TEM) investigations, 3 μL of nanoparticle solution at a concentration of ~200 μg/mL was deposited onto a holey carbon TEM grid (type S147-4, Plano, Wetzlar, Germany) and then plunged into liquid ethane using a Gatan CP3 cryoplugger. The frozen sample was transferred under liquid nitrogen to a Gatan Cryo-TEM holder (model 914). TEM investigations were performed at 100 K using the same microscope conditions described above. To obtain elemental concentrations, inductively coupled plasma optical emission spectrometry (ICP-OES) measurements were conducted using Horiba Jobin Yvon Ultima 2 (pressure: 2.15 bar; flow: 0.75 L/min; Ce: λ = 238.892 nm; Fe: λ = 238.206 nm). Samples were diluted 1:1000 (v/v) in water. Subsequent calculations (section 9 in the Supporting Information) were performed to estimate the particle concentration. The magnetic properties of the particles in dried form were assessed using a superconducting quantum interference magnetometer (SQUID) (MPMS3, Quantum Design, Inc.).

2.4. Cell Culture.

Adherent epithelial human hepatocarcinoma cells (HepG2) (German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany)) were cultured in an incubator under humidified atmosphere at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (PAN-Biotech 3302-P291205) and dislodged using Cellstripper (Corning, Wiesbaden, Germany).

2.5. Cytotoxicity Analysis.

To evaluate the toxicity of the nanoparticles toward HepG2 cells, CellTox Green assay was performed. Cells were dislodged using Cellstripper, counted by an automated cell counter (CASY Model TT, OLS-OMNI Life Science, Bremen, Germany), and seeded with a density of 75,000/mL (100 μL per well) in 96-well BRAND plates (plastic bottom, black wells). HepG2 cells were incubated with nanoparticles of different concentrations in RPMI-1640 medium supplemented with 10% FBS for 24 h at 37 °C. Cell death was measured by the CellTox Green assay (positive control is the lysis solution from the kit). Fluorescence measurements were obtained using a Tecan Infinite M200 Pro plate reader (excitation = 490 nm; emission = 525 nm). Fluorescence values were normalized to the negative control (cell medium = 1) and corrected for optical interference using the percentage interference values from Figure S13A,B (Supporting Information). Three independent assays were performed, and for each assay, samples were tested in triplicate. Particle-induced optical interference was assessed by repeating the protocol above in the absence of cells. To test for particle optical interference in the event of a positive result, 50 μL of 1% Triton X-100 was added per well and incubated with cells for 24 h at 37 °C, after which nanoparticles in RPMI-1640 medium supplemented with 10% FBS were added and fluorescence measurements were performed as above. Interference testing is detailed further in section 10 in the Supporting Information.

2.6. Reactive Oxygen Species Measurements.

Nanoparticle-induced oxidative stress was measured by detecting the oxidation of 2′,7′-dichloroﬂuorescin-diacetate (H₂DCF-DA) into the highly fluorescent compound 2′,7′ dichlorofluorescein (DCF) due to the presence of reactive oxygen species. Cells were dislodged using Cellstripper, counted by an automated cell counter (CASY Model TT, OLS-OMNI Life Science, Bremen, Germany), and seeded with a density of 75,000/mL (100 μL per well) in 96-well BRAND plates (plastic bottom, black wells). HepG2 cells were incubated with nanoparticles of different concentrations in RPMI-1640 medium supplemented with 10% FBS for 24 h at 37 °C. Hydrogen peroxide was used as the positive control. Following incubation, 100 μM H₂DCF-DA diluted in DMEM medium (without phenol red) was added to each well and incubated for 30 min at 37 °C. Fluorescence measurements were obtained using a Tecan Infinite M200 Pro plate reader (excitation = 492 nm; emission = 530 nm). Fluorescence values were normalized to the negative control (cell medium = 1) and corrected for optical interference using the percentage interference values from Figure S13A,B (Supporting Information). Three independent assays were performed, and for each assay, samples were tested in triplicate. Interference testing was conducted by incubating particles with H₂DCF in the absence of cells. H₂DCF-DA was acetylated with N-Acetylcysteine (NAC) according to the protocol of Sun et al.³⁸ Nanoparticles or controls (water, PBS, or 10 μM 3-morpholinosydnonimine (SIN-1)) were incubated with 100 μL of H₂DCF for 1 h at 21 °C in the dark. Fluorescence measurements were then performed as above. To assess for particle reduction of the ROS.
signal, an interference test was repeated as above with the addition of 10 μM SIN-1 per well.

2.7. 9.4 T Preclinical MRI Phantom Test. To evaluate the MRI contrast effect of ESCIoNs, a phantom test was performed using a 9.4 T imaging system with 1 T/m imaging gradients and DirectDrive console (Agilent Technologies, USA), and a 39 mm i.d. quadrature-driven Vshaped resonator was tuned to 400 MHz (Rapid Biomedical, Germany). Nanoparticles were linearly diluted in agarose gel (1% TBE blend) and suspended in 1 mL syringes. Following calibration, the MR contrast was achieved by modifying the protocol of Zhou et al.36 Cholic acid was derivatized to the nanoparticles, an amino group was required. The solid (100 mg) was dissolved in anhydrous THF (20 ml) under (8:1 ratio) as the eluents to give a white powder (122 mg, 41% yield). The solid (100 mg) was dissolved in anhydrous THF (20 ml) under

2.10. Conjugation of Amino Cholate to MFe2O4@DMSA Nanoparticles. The DMSA-coated nanoparticles were conjugated to amino cholate via EDC-NHS coupling (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, EDC). Eight milliliters of nanoparticles in H2O (300 μg/mL) were added to a glass vial, and the pH was lowered to 5 using hydrochloric acid. EDC (22 μL) was added and stirred, then 100 mg of sulfo-NHS in 1 mL of H2O was added, and the solution was stirred at room temperature for 20 min. Another 22 μL of EDC was added, and following 20 min of further stirring, 20 mg of amino cholate in 2 mL of H2O and 20 μL of triethylenediamine was added dropwise to the NP solution, and the solution was stirred overnight. The NPs were purified via centrifugation (4600 rpm, 15 min) and washed in H2O. Finally, the bile acid-functionalized NPs were dialyzed against H2O for 2 h with one water change after 1 h and then filtered through a sterile filter membrane (5 μL pore size). Conjugation was qualitatively checked with a ninhydrin test using ninhydrin (72490-10g), ethanol, and acetic acid.

3. RESULTS AND DISCUSSION

3.1. Synthesis of Extraordinarily Small MFe2O4 Nanoparticles. Extraordinarily small iron oxide (ESIoNs) and cobalt ferrite nanoparticles (ESCiNos) were synthesized following thermal decomposition of iron and cobalt acetylacetonate precursors in oleylamine. Figure 1 shows

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Figure 1. (A, B) TEM micrographs of 2.4 nm (A) ESCiNos and (B) ESIoSs (scale bars are 100 nm). (C, D) Corresponding enlarged TEM micrographs of (C) ESCiNos and (D) ESIoSs (scale bars are 20 nm).
Fe(acac)₃ and oleylamine yielded a face-centered cubic (FCC) spinel structure. Particles synthesized with crystalline phases (Figure S6), which may be due to the formation of two distinct orientation of CoFe₂O₄. To inspect the crystal structure in 22-1086) (section 5 in the Supporting Information).

...cobalt ferrite cubic structures (JCPDS cards 01-075-0449 and 22-1086) (section 5 in the Supporting Information).

...is a precursor/OLA molar ratio of 1:50, below which the size distribution was wide due to the formation of aggregates. Aggregate formation is likely due to an insufficient amount of surfactant present to cap the particles and restrict growth.

...reaction time are also known to affect particle size in thermal decomposition synthesis. We varied the heating rate from 12.6 to 24.7 °C min⁻¹ and found that this had no effect on particle size or shape (Figure S4). Similarly, raising the reaction temperature from 225 to 250 °C had no effect (Table S1). Extending the reaction time from 10 min to 1 h also led to no changes (Figure S5). Thus, the precursor/OLA ratio was the main influence on the particle size in our experiments. The decrease in particle size with the increase in oleylamine concentration could be attributed to oleylamine’s role as a reducing agent, lowering the decomposition temperature and permitting nucleation to occur faster with an increasing precursor/OLA ratio. An excess of oleylamine may also be required to ensure full coverage of the particles and prevent Ostwald ripening. Interestingly, changing the molar ratio of Fe/Co acetylacetonate precursors from 2:1 to 1.5:1 led to a wider size distribution for CoFe₂O₄ (Figure S6), which may be due to the formation of two distinct crystalline phases.

ESIoNs and ESCIoNs were investigated with ADF-STEM to resolve their crystal structure. Particles synthesized with Fe(acac)₃ and oleylamine yielded a face-centered cubic (FCC) spinel structure (Figure 2A). Close inspection of the ADF-STEM image reveals a lattice fringe distance of 4.8 Å, which corresponds to the interplanar d-spacing of the (111) plane of magnetite/maghemite. ESCIoNs (2.4 nm) also had an FCC spinel structure (Figure 2B). ADF-STEM revealed a lattice fringe spacing of 2.4 Å, which is in good agreement with the interplanar d-spacing of the (111) plane of CoFe₂O₄. The Fourier transform was indexed to the [013] crystallographic orientation of CoFe₂O₄. To inspect the crystal structure in closer detail, multiframe fast acquisition and subsequent nonrigid alignment and averaging using SmartAlign software were employed on a representative 4.3 nm CoFe₂O₄ particle. Figure 2C,D reveals an FCC spinel structure in the [110] zone axis orientation with atomic resolution and the corresponding crystal model. XRD patterns of 4.2 nm OLA-ESIoNs and 4.8 nm OLA-ESCIoNs also matched well with magnetite and cobalt ferrite cubic structures (JCPDS cards 01-075-0449 and 22-1086) (section 5 in the Supporting Information).

Table 1. ESIoN and ESCIoN Particle Size in Relation to Amount of Precursor when Synthesized with 60 mmol Oleylamine for 10 min at 225 °C

| total amount of precursor (mmol) | TEM ESIoN size (nm) | TEM ESCIoN size (nm) |
|---------------------------------|---------------------|---------------------|
| 1.4                             | 5.7 ± 3.0           | 4.3 ± 0.8           |
| 0.7                             | 4.6 ± 0.6           | 4.0 ± 0.8           |
| 0.3                             | 4.0 ± 0.4           | 3.5 ± 0.7           |
| 0.15                            | 3.8 ± 0.5           | 3.3 ± 0.6           |
| 0.07                            | 3.1 ± 0.5           | 3.0 ± 0.6           |
| 0.03                            | 2.4 ± 0.4           | 2.4 ± 0.3           |

“For the synthesis of ESCIoNs, a ratio of 2:1 Fe/Co precursors was used throughout. Particle size decreases as the precursor(s)/oleylamine ratio decreases.

EEL spectroscopy detected the K ionization edge of oxygen and L ionization edge of iron, confirming that the ESIoNs are composed of iron and oxygen (Figure 3A). Quantification using the Hartree–Slater model showed an atomic ratio (O/I) of 0.68 ± 0.07% for iron, which matches best with maghemite. Quantitative analysis of the Fe–L₂,3 ionization edges can also indicate the oxidation state of iron oxide because the (L₂/L₁) intensity ratio varies depending on the oxidation state. The intensity ratio of L₂/L₁ was found to be 4.49, which is in good agreement with the value for maghemite. To further investigate the oxidation state, we examined the shape of the Fe 2p L₁ edge, which differs depending on the oxidation state, with maghemite presenting a shapeless L₁ peak and maghemite displaying a pre-shoulder. The inset of Figure 3A shows the L₁ peak in closer detail; the L₁ edge is smooth without a shoulder, which also suggests that the composition of the particles is Fe₂O₄. Due to their small size, it is possible that the particles are a mixture of magnetite and maghemite or that they oxidize to maghemite over time. Differentiating between the two is notoriously challenging at the nanoscale, and it was not possible to quantitatively distinguish between them. Nonetheless, the black color of the ESIoNs (Figure S1) strongly suggests that the particles are predominantly maghemite. EEL spectroscopy of 2.4 nm ESCIoNs revealed three peaks corresponding to the K ionization edge of oxygen and L ionization edges of iron and cobalt, confirming the presence of cobalt in the particles (Figure 3B). Quantitative analysis of the EELS spectrum using the Hartree–Slater model revealed a relative composition of 6% ± 6% oxygen, 27 ± 3% iron, and 17 ± 2% cobalt, which

Figure 2. (A, B) ADF images and corresponding fast Fourier transform (FFT) of (A) 2.4 nm ESIoN in [110] zone axis orientation and (B) 2.4 nm ESCIoN in [013] zone axis orientation. (C, D) SmartAlign ADF image and corresponding FFT of a 4.3 nm ESCIoN in [110] zone axis orientation. Scale bar = 2 nm. (E) Modeled crystal structure of cobalt ferrite [110].
corresponds well with the composition of cobalt ferrite (CoFe$_2$O$_4$). EELS elemental mapping revealed a homogeneous distribution of cobalt and iron in the particles (Figure 3C).

The magnetic properties of 4.3, 3.5, and 2.4 nm OLA-ESClO$_n$s were assessed. The particles displayed typical superparamagnetic behavior with a loss of hysteresis (Figure 4A). The magnetization saturation ($M_s$) decreased with particle size, as anticipated, with 4.3, 3.5, and 2.4 nm particles having $M_s$ values of 44, 37, and 22 emu/g, respectively, at 300 K. Analogously, the blocking temperature ($T_B$) decreased with particle size from 131 to 101 and 52 K for 4.3, 3.5, and 2.4 nm particles (Figure 4B). The magnetic properties were reassessed following ligand exchange with DMSA, as detailed in the next section.

### 3.2. Ligand Exchange with DMSA: Colloidal Stability.

DMSA ligand exchange is a well-established method for transferring metal oxide nanoparticles from nonpolar solvents to water. Oleylamine-coated particles were initially hydrophobic, owing to the hydrocarbon tail of OLA molecules. Following ligand exchange with DMSA, water-dispersible DMSA-ESiO$_n$s and DMSA-ESClo$_n$s were obtained. The suspensions appeared clear, and no precipitation was visible.

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Figure 3. (A) EELS spectrum of ESiO$_n$s showing the oxygen K and iron L ionization edges and (inset) L edges in the range of 705–730 eV presenting a smooth $L_3$ peak. (B) EELS spectrum of 2.4 nm ESClo$_n$s showing three peaks corresponding to the oxygen K and iron and cobalt L ionization edges. (C) EELS elemental map (red = iron, green = cobalt) showing a homogeneous distribution of cobalt and iron atoms in 3 nm ESClo$_n$s. (D) Corresponding EELS spectrum image (scale bar = 10 nm).

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Figure 4. (A) Magnetization curves of 4.3, 3.5, and 2.4 nm OLA-ESClo$_n$s at 300 K. (B) Corresponding temperature-dependent magnetization (zero-field cooling (ZFC) and field-cooling (FC)) curves. The FC curves were obtained at an applied field of 100 Oe.
Aggregation of ultrasmall iron oxide nanoparticles has been shown to decrease the relaxivity of nearby water protons, resulting in a dominant $T_2$ relaxation rate and increase the $T_2$ values.44 Cell toxicity is also affected by particle stability because aggregation can influence uptake into cells, and particle degradation due to acidic pH can lead to leaching of ions.45 To address the stability and reproducibility, we performed DMSA ligand exchange with a range of ESCIoNs and ESIoNs with sizes of 2–5 nm and measured their $D_h$’s and ζ potentials in solutions from pH 3–10. Hydrodynamic diameters were remeasured following 3–12 months storage at 4 °C. $D_h$’s ranged from 7 to 23 nm and ζ potentials ranged from $-28.0 \pm 0.3$ to $-38.4 \pm 0.9$ mV, indicating that monodisperse and stable dispersions were initially obtained for all samples (Table 2). Following storage for 3–12 months, $D_h$’s increased slightly; the largest increase was from 8 to 46 nm after 12 months.

Interestingly, DMSA-ESCiOns were found to be stable in a wider pH range of 3–10 than DMSA-ESIoNs, which began to flocculate below pH 5. As shown in Figure 6A, the $D_h$ of DMSA-ESCiOns increases slightly from 8 ± 2 nm at pH 7 to 17 ± 4 nm at pH 3, whereas the $D_h$ of DMSA-ESIoNs increases from 7 ± 2 to 79 ± 25 nm. DMSA-ESIoN flocculation may be caused by acid-induced reduction of Fe$^{3+}$ to Fe$^{2+}$, which in turn causes oxidation of DMSA. Alternatively, DMSA may have oxidized from oxygen in the solution. Oxidation of DMSA is a known phenomenon that leads to the formation of interparticle disulfide bridges.46 Fauconnier et al.28 found that addition of an alkalinization step after ligand exchange improved colloidal stability significantly. At pH 9, the free SH groups are deprotonated to thiolate groups, which partially replace carboxylate groups at the surface of the particles. Free COO− groups and κ potentials in solutions from pH 3–10. Hydrodynamic diameters were remeasured following 3–12 months storage at 4 °C. $D_h$’s ranged from 7 to 23 nm and ζ potentials ranged from $-28.0 \pm 0.3$ to $-38.4 \pm 0.9$ mV, indicating that monodisperse and stable dispersions were initially obtained for all samples (Table 2). Following storage for 3–12 months, $D_h$’s increased slightly; the largest increase was from 8 to 46 nm after 12 months.

![Figure 5](https://example.com/fig5.png)

Figure 5. Oleylamine-coated ESCIoNs and ESIoNs are obtained following synthesis with acetylacetone precursors and oleylamine at 225 °C for 10 min. Initially hydrophobic, the particles are rendered hydrophilic by stirring with DMSA for 48–72 h. DMSA preferably binds to the metal oxide core, replacing the oleylamine ligands.

| sample | core size (nm) | $D_h$ (nm) | ζ (pH 7) | pH stability range (ζ ≥ −25 mV) | $D_h$ (no. of months after measurement) |
|--------|---------------|------------|----------|---------------------------------|----------------------------------------|
| ESCIoN1 | 2.4 ± 0.4     | 11 ± 2     | $-31.8 \pm 1.8$ | 3–9 | 25 ± 7 (+12) |
| ESCIoN2 | 2.4 ± 0.4     | 13 ± 3     | $-34.2 \pm 1.4$ | 3–9 | 46 ± 14 (+3) |
| ESCIoN3 | 2.3 ± 0.3     | 16 ± 4     | $-28.0 \pm 0.3$ | 3–9 | 15 ± 5 (+3) |
| ESCIoN4 | 2.3 ± 0.3     | 8 ± 2      | $-42.2 \pm 3.7$ | 3–9 | 46 ± 16 (+12) |
| ESCIoN5 | 4.3 ± 0.6     | 12 ± 3     | $-38.4 \pm 0.9$ | 3–9 | 36 ± 7 (+12) |
| ESCIoN6 | 4.3 ± 0.6     | 23 ± 7     | $-40.7 \pm 0.6$ | 3–9 | 22 ± 4 (+12) |
| ESIoN1  | 2.4 ± 0.5     | 7 ± 2      | $-33.9 \pm 0.7$ | 5–9 | 40 ± 10 (+12) |
| ESIoN2  | 4.0 ± 0.8     | 12 ± 2     | $-43.5 \pm 2.5$ | 5–9 | 23 ± 6 (+3) |
| ESIoN3  | 4.2 ± 0.7     | 12 ± 2     | $-41.7 \pm 1.0$ | 5–9 | 24 ± 5 (+12) |
| ESIoN4  | 4.7 ± 0.7     | 16 ± 4     | $-43.2 \pm 6.5$ | 5–9 | 16 ± 3 (+12) |

The hydrodynamic diameters were measured using dynamic light scattering and repeated 3–12 months later. The pH stability range was determined by zeta potential values that exceeded −25 mV. ESCIoNs were stable in a pH range of 3–10, whereas ESIoNs were stable in solutions from pH 5 to 10. The DMSA ligand exchange method was reproducible for both particle types, and only minor flocculation had occurred in some samples after 12 months.

indicating that DMSA had bound to the nanoparticles. A summary of the synthesis and ligand exchange process is outlined in Figure 5. Hydrodynamic diameters ($D_h$) of 11 ± 2 and 8 ± 2 nm were measured for ESCIoN and ESIoNs, respectively. Their zeta (ζ) potentials were $-3.18 \pm 1.8$ and $-33.9 \pm 0.7$ mV, reflecting the negative charges from carboxylate moieties on the surface.

The stability of particles is critical for MRI because particle aggregation has a significant effect on relaxation rate. Aggregation of ultrasmall iron oxide nanoparticles has been shown to decrease the $T_1$ relaxation rate and increase the $T_2$ relaxation rate of nearby water protons, resulting in a dominant $T_2$ effect.44 Cell toxicity is also affected by particle stability because aggregation can influence uptake into cells, and particle degradation due to acidic pH can lead to leaching of ions.45 To address the stability and reproducibility, we performed DMSA ligand exchange with a range of ESCIoNs and ESIoNs with sizes of 2–5 nm and measured their $D_h$’s and ζ potentials in solutions from pH 3–10. Hydrodynamic diameters were remeasured following 3–12 months storage at 4 °C. $D_h$’s ranged from 7 to 23 nm and ζ potentials ranged from $-28.0 \pm 0.3$ to $-38.4 \pm 0.9$ mV, indicating that monodisperse and stable dispersions were initially obtained for all samples (Table 2). Following storage for 3–12 months, $D_h$’s increased slightly; the largest increase was from 8 to 46 nm after 12 months.
magnetite, more S–H groups may be left free on the surface to stabilize the particles in acidic pH. In addition to an alkalization step, we found that dialyzing particles to remove excess oleylamine prior to ligand exchange helped to reproducibly obtain small hydrodynamic diameters. Without prior dialysis, larger $D_h$'s were obtained ($116 \pm 54$ vs $17 \pm 3$ nm for dialyzed particles) (Figure S10).

To view the particles in their native liquid state, cryo-EM was employed. Figure 6D shows DMSA-ESClO$n$s arranged in a chain-like fashion, possibly due to magnetic interactions. Higher particle concentrations were used for cryo-EM than for hydrodynamic diameter measurements, owing to the difficulty in locating such small-sized nanoparticles in a suitably thin area of ice for optimum imaging. Magnetic nanoparticles in high concentration are known to have weak magnetic dipole interactions, resulting in linear chains. When dilute samples were used for cryo-EM, the micrographs revealed mono-disperse nanoparticles (Figure S11A). The particles retained their original core size post exchange, whereas Ostwald ripening or particle dissolution has been reported by others. The stability of particles in water is important for their general application and storage; however, in vivo, the particles will encounter more complex media containing proteins, lipids, salts, and other large molecules, which may interact with the particle. The formation of a protein–particle complex, named the protein corona, can potentially alter the surface charge and hydrodynamic diameter of the particles, affecting their in vivo distribution. As a first step to assess protein adsorption onto DMSA particles, we measured the $D_h$ of the particles in cell culture medium DMEM supplemented with 10% FBS. Figure 6C shows no significant change in $D_h$ for ESClOns ($8 \pm 2$ nm in pure water to $11 \pm 3$ nm in DMEM with 10% FBS), suggesting that protein adsorption was minimal. ESIOns suffered a larger increase from $7 \pm 2$ to $30 \pm 8$ nm, indicating protein adsorption. Cryo-EM of DMSA-ESlOns in DMEM revealed no additional agglomeration or aggregation in comparison to DMSA-ESlOns in water, suggesting that the increase in $D_h$ was due to protein adsorption (Figure S11B).

To confirm DMSA binding to the particles, TGA and FTIR studies were conducted. Figure 7 shows the weight loss profiles of OLA-ESClO$n$s and DMSA-ESClO$n$s. The amount of ligands present on the surface of the nanoparticles was estimated from the weight loss using eq 1 below

$$N = \frac{\omega_m A}{\omega_f M}$$

Figure 6. (A) Hydrodynamic diameters and (B) zeta potentials of 2.4 nm DMSA-ESClO$n$s and DMSA-ESlOns in pH range 3–9. (C) Comparison of their $D_h$'s in water and cell medium. (D) Cryo-EM micrograph of ESClOns in water.
where \( m_j \) is the total weight (in grams) of the ligands calculated from TGA, \( \rho \) is the density of cobalt ferrite (5.2957 g/cm\(^3\)), \( \nu \) is the volume of the 2.4 nm particle, \( A \) is the Avogadro’s constant, \( \omega \) is the total weight (in grams) of the particle core (calculated from TGA), \( M \) is the molar mass of the ligand (oleylamine (267.5 g/mol) or DMSA (182.22 g/mol)), and \( N \) is the number of ligands per particle. The estimated number of DMSA ligands per particle is 90. Considering the surface area of a 2.4 nm particle is 18.1 nm\(^2\), the ligand density is DMSA ligands per particle is 90. Considering the surface area is the number of ligands per particle. The estimated number of carbonyl absorption bands typically observed at 1700 and 1750 cm\(^{-1}\). Shifting occurred because the ligand is bound to the particles, resulting in disassociation of the carboxyl group. Large splitting between these bands is associated with a monodentate interaction between the carboxylate group of DMSA and the iron oxide nanoparticle.29

The magnetic properties of 2.4 nm DMSA-ESIoNs and DMSA-ESCIoNs were measured after ligand exchange with DMSA, as the surface ligand is known to influence their magnetic behavior.51 Indeed, we found that, following ligand exchange of OLA for DMSA, the \( M_s \) and \( T_B \) of ESCIoNs and ESIoNs decreased (Figure S12). Figure 9 shows the magnetization curves of 300 K, with both DMSA-ESIoNs and DMSA-ESCIoNs displaying typical superparamagnetic behavior evidenced by a loss of hysteresis. The magnetization saturation of 2.4 nm DMSA-ESIoNs and DMSA-ESCIoNs was 9.6 and 4.8 emu/g, respectively. The blocking temperature (\( T_B \)) for 2.4 nm DMSA-ESCIoNs was 102 K, which is markedly higher than that of DMSA-ESIoNs at 12 K (Figure 9B). This rise in \( T_B \) is characteristic for CoFe\(_2\)O\(_4\) due to an increase in anisotropy energy courtesy of Co\(^{3+}\) ions. Their magnetic moments (\( m \)) calculated from eq 2 were 38.8 B for DMSA-ESIoNs and 17.5 \( \mu \)B for DMSA-ESCIoNs

\[
m = \frac{M \rho V}{\mu B}
\]

where \( M \) is the mass magnetization, \( \rho \) is the material density, \( V \) is the particle volume, and \( \mu_B \) is the Bohr magneton value.8 The very small magnetic moment of 2.4 nm particles results from a high proportion of the particle being composed of surface atoms and thus subject to spin canting effects.52

### 3.3. Cytotoxicity and Oxidative Stress of ESCIoNs and ESIoNs.

For biomedical applications, it is paramount that nanoparticles are screened for potential cytotoxic effects. The cytotoxicity of DMSA-ESIoNs and DMSA-ESCIoNs toward HepG2 cells was evaluated. HepG2 cells were chosen for the cytotoxicity study because metal oxide particles that are not renally cleared typically accumulate in the liver following in
Figure 10. Comparison of cytotoxicity and oxidative stress measurements of DMSA-EStoNs and DMSA-EStoNs toward HepG2 cells. Values were normalized to the negative control (cell medium = 1). (A) Relative cell death (fluorescence) in relation to particle concentration. No cell death was observed for any particle type. The positive control is the lysis solution from the kit. (B) Relative ROS production (fluorescence) induced by the particles. Both DMSA-EStoNs and DMSA-EStoNs produced ROS; however, DMSA-EStoNs produced significantly higher amounts than DMSA-EStoNs. The positive control is hydrogen peroxide. Results are shown as mean ± standard deviation from three independent experiments.

Figure 11. 9.4 T MRI phantom test of 2.4 nm DMSA-EStoNs embedded in 1% agarose gel. (A) Plot of $r_1$ relaxation rate and (B) $r_2$ relaxation rate against DMSA-EStoNs particle concentration. (C) MRI scout image showing the increase in $T_1$ (positive) contrast with increasing particle concentration. The particles have a less pronounced effect on the $T_2$ (negative) contrast, decreasing slightly with increasing concentration.
100 g/mL. Metal oxide-induced production of ROS is linked to the Fenton reaction, whereby transition metal ions catalyze hydrogen peroxide decomposition with generation of hydroxyl radicals. The increase in ROS production for DMSA-ESCIoNs may be due to the higher catalytic ability of Co$^{2+}$ ions over Fe$^{2+}$. It may also be linked to the production of different ROS species; iron is known to produce ·OH radicals, whereas cobalt produces ·HO$_2$ radicals. Other studies have reported ROS production by Fe$_3$O$_4$ and CoFe$_2$O$_4$ nanoparticles. Conversely, others have reported no cytotoxic effects or ROS production for CoFe$_2$O$_4$ nanoparticles against HepG2 cells. This disparity highlights the importance of testing each new particle system against cells, as surface coating, preparation parameters, and other slight changes in particle characteristics can have an effect. Frequently, metal oxide nanoparticles are tested only for cytotoxic effects and not for oxidative stress, despite their known capability for producing ROS. In section 3.5 of this paper, we demonstrate a route to minimize ROS production via functionalization with a bile acid derivative.

### 3.4. MRI Phantom Tests of 2.4 nm DMSA-ESCIoNs

Iron oxide nanoparticles have been extensively explored for MRI contrast enhancement applications; however, despite promise in other biomedical applications such as magnetic hyperthermia and drug delivery, cobalt ferrite particles remain relatively unexplored as MRI contrast agents. A few reports exist detailing the potential of cobalt ferrite nanoparticles >3 nm as $T_2$ contrast agents, owing to their large magnetic moment. Ghasemian et al. measured the relaxivity of DMSA-coated 16 nm cobalt-zinc ferrite nanoparticles and found their suitability for $T_2$ contrast enhancement with an $r_2/r_1$ ratio of 50 (measured at 1.5 T). Nidhin et al. reported an $r_2/r_1$ ratio of 6.9 for 7.2 nm cobalt ferrite nanoparticles. However, cobalt ferrite nanoparticles smaller than 3 nm have not been tested for MRI applications before.

To assess the potential of 2.4 nm DMSA-ESCIoNs as MRI contrast agents, we designed a phantom test using linearly decreasing particle concentrations and a 9.4 T MRI scanner. Figure 11 shows $T_1$ and $T_2$ phantom images of DMSA-ESCIoNs embedded in 1% agarose gel. (A) Plot of $r_1$ relaxation rate and (B) $r_2$ relaxation rate against DMSA-ESCIoN particle concentration. (C) MRI scout image showing the increase in $T_1$ (positive) contrast with increasing particle concentration.

### Table 3. Comparison of Relaxation Properties of Other Ultrasmall Ferrite Nanoparticle Systems and Commercially Available Contrast Agents at 3.0 and 9.4 T

| nanoparticle/coating | $D_{TEM}$ (nm) | $D_h$ (nm) | $r_1$ (mM$^{-1}$ s$^{-1}$) | $r_2$ (mM$^{-1}$ s$^{-1}$) | $r_2/r_1$ | $B_0$ (T) | reference |
|----------------------|----------------|-------------|---------------------------|---------------------------|-----------|-----------|-----------|
| CoFe$_2$O$_4$@DMSA   | 2.4            | 11          | 2.11                      | 7.81                      | 3.70      | 3.0       | this work |
| Fe$_3$O$_4$@PO-PEG (ESIoNs) | 2.2            | 15          | 4.77                      | 17.5                      | 3.67      | 3.0       | 8         |
| MnFe$_2$O$_4$/mPEG    | 2.0            | 8           | 8.43                      | 21.02                     | 2.49      | 3.0       | 11        |
| Magnevist (Gd-DTPA)   | n/a            | n/a         | 3.7                       | 5.2                       | 1.41      | 3.0       | 61        |
| Gadovist (Gd-DO3A-butrol) | n/a            | n/a         | 5.0                       | $-7.1$                    | 1.42      | 3.0       | 61        |
| CoFe$_2$O$_4$@DMSA    | 2.4            | 11          | 0.75                      | 48.7                      | 65        | 9.4       | this work |
| γ-Fe$_2$O$_3$/citrate | 2.0            | 4           | 0.25                      | 12.4                      | 49.6      | 9.4       | 62        |
| Resovist              | 5              | 63          | 1.67                      | 374.6                     | 224.3     | 9.4       | 63        |

*a: n/a: not available.
against particle concentration, was 48.7 mM$^{-1}$ s$^{-1}$. The $r_2/r_1$ ratio is an important parameter for determining the effectiveness of a contrast agent toward $r_1$ or $r_2$ relaxation: the higher the $r_2/r_1$ ratio, the more effective the agent is toward $T_2$ contrast. The $r_2/r_1$ ratio we obtained for DMSA-ESClO nanoparticles at 9.4 T was 65. The high ratio is due to the large magnetic field strength of the system used because the $r_2/r_1$ ratio increases with the increase in magnetic field strength ($r_1$ decreases and the $r_2$ increases). Following initial testing at 9.4 T, we conducted a further MRI phantom test using a 3.0 T clinical MRI system. The phantom setup consisted of nanoparticle–agarose suspensions in glass tubes, embedded in a tank of 1% agarose. The estimated $r_1$ and $r_2$ relaxivities were 2.11 and 7.81 mM$^{-1}$ s$^{-1}$, respectively (Figure 12). As anticipated, $r_1$ increased with the decrease in field strength from 9.4 to 3.0 T, whereas $r_2$ decreased. This resulted in an estimated $r_2/r_1$ ratio of 3.70. The $r_2/r_1$ ratio is very similar to that achieved by Kim et al.$^8$ with 2.2 nm maghemite nanoparticles and close to commercially available gadolinium-based contrast agents Magnevist (Gd-DTPA) and Gadovist (Gd-DO3A-butrol) with $r_2/r_1$ ratios of 1.41 and 1.42 at 3.0 T, respectively (Table 3). The low $r_2$ value of DMSA-ESClO nanoparticles was due to their very small magnetic moment, influenced by the addition of Co$^{3+}$ ions and small particle size. This minimized the magnetic induction of local magnetic field inhomogeneities and prevented DMSA-ESClO nanoparticles from having a pronounced effect on $T_2$ relaxation. Particle concentration-dependent $T_2$ relaxation was achieved from the unpaired electrons of Co$^{3+}$ and Fe$^{3+}$ and the stability of the particles provided by the DMSA coating. These results demonstrate the potential of ESClO nanoparticles for $T_1$ contrast enhancement for the first time.

3.5. Functionalization of DMSA-ESClO and DMSA-ESIoN with Bile Acid Ligand. DMSA-ESClO nanoparticles have demonstrated potential as $T_1$ contrast imaging applications; however, their biocompatibility must be improved in order for clinical application to be successful. In addition to improving the biocompatibility of agents, design of target-specific agents to improve image resolution in specific biological regions is desired for early detection of disease. Hepatotropic MRI contrast agents are extremely useful for detecting foci of liver lesions in the diagnosis of metastatic liver disease.$^7$ Contrast agents for detecting tissue abnormalities, however, must be able to cross the hepatocyte membrane and eventually be excreted via bile.$^{64}$ Bile acids, amphipathic molecules composed of a sterol scaffold with hydroxyl groups and a carboxyl-terminated side chain,$^{34}$ are transported to and from the liver via the enterohepatic circulation system and thus are ideal candidates for use as liver transport vehicles. Anelli et al.$^{55}$ tested whether cholic acid, cholyglycine, or cholytaurine linked to Gd-DOTA chelates could encourage hepatospecificity and found that cholic acid was an efficient carrier for uptake by hepatocytes.

With this in mind, we have explored the functionalization of cholic acid to DMSA-ESClO nanoparticles for the first time as a step toward designing new liver-targeting nanoparticle MRI contrast agents. Both iron oxide and cobalt ferrite nanoparticles were synthesized for comparative purposes. To attach cholic acid to the particles, we first converted cholic acid into amino cholate by adaption of an existing protocol.$^{36}$ Chieflly, cholic acid was converted to N-hydroxysuccinimide ester using dicyclohexylcarbodiimide (DCC) as the coupling reagent. The activated ester was then transformed into cholate amide, which was reduced by LiAlH$_4$ to afford amino cholate. Nuclear magnetic resonance (NMR) studies were conducted to confirm the product of each step (see section 11 in the Supporting Information). The amino group of amino cholate was then conjugated to carboxylate groups on the surface of DMSA-ESClO nanoparticles by EDC-NHS coupling. A schematic outlining the process of obtaining BA-ESClO and BA-ESIoN (BA, bile acid–functionalized) is shown in Figure 13.

To confirm conjugation of amino cholate to DMSA nanoparticles, FTIR was conducted. Figure 14 compares the FTIR spectra acquired for DMSA- and BA-functionalized ESClO nanoparticles. Following conjugation with bile acid, the broad peak at 3400 cm$^{-1}$ (in the same region as OH stretching) sharpens due to NH stretching of the secondary amide group. Sharpening of the peaks at 2865 and 2930 cm$^{-1}$ can be attributed to the symmetric and asymmetric CH$_2$ groups of amino cholate,$^{66}$ and the peak at 2970 cm$^{-1}$ belongs to the asymmetric vibration of amino cholate’s CH$_3$ groups. Additionally, the small peak at 1650 cm$^{-1}$ is due to C=O amide stretching. The presence of NH and CH$_3$ stretching

![Figure 13. Schematic outlining the synthesis process for BA-ESClO and BA-ESIoN. First, hydrophobic oleylamine-coated particles are rendered hydrophilic via DMSA ligand exchange. Then, DMSA-coated nanoparticles are conjugated to amino cholate by EDC-NHS coupling.](image-url)
peaks indicates conjugation of amino cholate to the DMSA-coated nanoparticles; further verification with the ninhydrin test can be found in section 12 in the Supporting Information.

Cryo-EM of BA-ESIoNs and BA-ESClONs revealed networks of nanoparticles similar to that observed for DMSA-coated particles (Figure 15A,B). Although the appearance of the particles under cryo-EM was similar to that observed for DMSA-ESIoNs and ESCIoNs, noticeable sedimentation of the particles occurred after several days. To examine their stability, hydrodynamic diameter and zeta potential measurements were conducted in different pH levels. The average $D_h$’s for BA-ESIoNs and BA-ESClONs at pH 7 were 476 ± 128 and 369 ± 74 nm, respectively. The significant increase in $D_h$ indicates that particle aggregation has occurred after functionalization with bile acid. Bile acid is known to form helical aggregates due to its hydrophobic sterol backbone, which aids in transport to the liver. Below pH 5, the $D_h$’s of both BA-ESIoNs and BA-ESClONs increased, indicating that the particles are less stable in acidic conditions (Figure 15C). Zeta potential measurements mirror these results; $\zeta$ values of $-36.5 \pm 1.5$ and $-38.7 \pm 1.0$ mV were measured at pH 9 for Fe$_3$O$_4$@BA and CoFe$_2$O$_4$@BA nanoparticles, respectively (Figure 15D), whereas their $\zeta$ potentials at pH 3 increased to $19.1 \pm 0.6$ and $-4.1 \pm 0.2$ mV.

To quantify the amount of bile acid on the surface of the nanoparticles, TGA measurements were performed. The weight loss profiles for BA-ESIoNs and ESCIoNs were almost identical, with total weight losses of 45% (Figure S20). Considering that the weight loss for the same particles prior to bile acid functionalization was 36.4%, approximately 8.6% of the total weight loss is due to the bile acid ligands. Using eq 1, the number of bile acid ligands per ESCIoN particle was estimated to be 28. The approximate radius of a bile acid molecule is 3.5 Å, and a single layer of DMSA is estimated to be 0.1 nm thick. Thus, assuming a double layer of DMSA on a 2.4 nm core, the total surface area would be 24.6 nm$^2$.

Figure 14. FTIR spectrum of DMSA- and BA-functionalized ESCIoNs and ESIoNs.

Figure 15. (A, B) Cryo-EM micrographs of (A) BA-ESIoNs and (B) BA-ESClONs. (C) pH-dependent hydrodynamic measurements. (D) Zeta potential measurements showing that BA particles were stable in neutral and alkaline pH and that BA-ESIoNs were less stable in acidic conditions than BA-ESClONs.
hydrophilic ursodeoxycholic acid (UDCA) has antioxidant properties, whereas hydrophobic bile acids produce ROS, whereas the hydrophobicity of bile acids is linked to the production of ROS. A common issue of ROS generation is the need to obtain biocompatible ferrite nanoparticles and mitigate their oxidative damage. UDCA was found to be an excellent scavenger of RO \cdot \cdot \cdot$$\cdot$$ \mathrm{OH}$$ radicals generated by FeCl\cdot$$, O_{2}$$, and Fe\cdot$$ ions, preferentially interacting with Fe\cdot$$ ions. Notably, the rate constant for the reaction was 10-fold higher than that of nonlabeled Fe\cdot$$ ions. Considering our findings and the order of bile acid hydrophilicity (UDCA < CA < CDCA < DCA < LCA), we propose that cholic acid shares similar antioxidant properties as UDCA and could be an effective site-specific ROS scavenger for free radicals induced by iron-containing nanoparticles. Further investigation to elucidate the mechanism of ROS scavenging by cholic acid is therefore warranted.

Cytotoxic and oxidative stress effects of BA-ESCIoNs and BA-ESIoNs toward HepG2 cells were assessed using the same protocol and conditions used previously for DMSA-coated particles. DMSA-ESCIoNs and ESIoNs were re-evaluated conjunctively to determine any differences due to the presence of bile acid. Figure 16A shows that, at particle concentrations of 100 \mu g/mL, no cytotoxicity was observed with BA-ESCIoNs or BA-ESIoNs. Figure 16B compares the ROS production for DMSA-ESCIoNs and ESIoNs. The ROS production for CoFe$_2$O$_4$ nanoparticles decreased by approximately 67% when bile acid was conjugated to the nanoparticles. Similarly, the ROS production of Fe\cdot$$$_3$$O$_4$ nanoparticles decreased by approximately 62%.

The reduction in ROS we observed suggests that bile acid acted as an ROS scavenger, mitigating oxidative stress effects. Bile acids have demonstrated antioxidant properties by directly intercepting peroxyl radicals, as observed in a previous study. Contrarily, hydrophobic bile acids can induce ROS production through disruption of the mitochondrial membrane. The hydrophobicity of bile acids is linked to production of ROS, whereas hydrophilic ursodeoxycholic acid (UDCA) has antioxidant properties, which are especially relevant toward Fe\cdot$$$_{3+}$$-induced oxidative damage. UDCA was found to be a scavenger of \cdot$$OH radicals. The hydrophobicity of bile acids is linked to production of ROS, whereas hydrophilic ursodeoxycholic acid (UDCA) has antioxidant properties, which are especially relevant toward Fe\cdot$$$_{3+}$$-induced oxidative damage.

4. CONCLUSIONS

Extraordinarily small iron oxide and cobalt ferrite nanoparticles (ESIoNs and ESCIoNs) were synthesized with subnanometer-size precision using a simple thermal decomposition approach. Particle size was finely tuned by varying the oleylamine-to-precursor ratio, and ADF-STEM revealed their highly crystalline FCC spinel structure. Following ligand exchange with DMSA, the particles were stable in water for up to 12 months. DMSA-ESCIoNs and DMSA-ESIoNs displayed superparamagnetic properties with a weak magnetic moment, owing to their small size and thus predominating effect of the magnetically disordered surface layer. Magnetic anisotropy introduced by Co\cdot$$$_{2+}$$ ions lowered the magnetic saturation for 2.4 nm ESIoNs, which proved effective for a diminishing effect on T$_1$ relaxation in MRI phantom studies. The resulting T$_1$/T$_2$ ratio of 3.70 at 3.0 T shows the potential of 2.4 nm ESIoNs as T$_1$-weighted MRI contrast agents for the first time. DMSA-ESCIoNs and ESIoNs displayed no cytotoxic effects toward HepG2 cells; however, elevated levels of ROS were measured following a 24 h incubation period, particularly for DMSA-ESCIoNs. Following conjugation to amino cholate, a bile acid derivative, the ROS levels were, however, significantly reduced, indicating that amino cholate has the potential to act as an effective ROS scavenger for ferrite nanoparticle-induced free radicals. Bile acid-functionalized ESCIoNs could offer a promising route for biocompatible liver-targeted MRI, and thus, further research into this area is encouraged.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b17162. List of chemicals and further information on the synthesis and ligand exchange of ESIoNs and ESCIoNs supported with TEM, XRD, cryo-EM, and SQUID measurements; calculations used to determine particle
concentrations; cytotoxicity and oxidative stress interference testing; NMR, TGA, and ninhydrin results for the synthesis of the bile acid derivative (PDF)

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Notes
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■ ACKNOWLEDGMENTS
This research was supported by the Engineering and Physical Sciences Research Council (South of England Analytical Electron Microscope grant EP/K040375/1, DTA Fellowships Program (D.P.), and IAA block grants (N.G.)). We are also grateful for the financial support from Santander Academic Travel Awards (D.P.), Scattered European Scholarships (D.P.), the European Commission Seventh Framework Program (FP7-PEOPLE-ITN-2008-238363CONTACT), the Royal Society (N.G.), and the European Research Council (ERC-2009-Stg-240500-DEDIGROWTH, ERC-2015-POC-680559-CONDUCT, ERC-2011-POC-309786-DEVICE, and ERC-2016-POC-75474-OxfordNano) (N.G.). The authors thank Rudolf Karos for the XRD measurements, Dr. Daniel Morbidelli, L. Monitoring Endothelial and Tissue Responses to Cobalt Ferrite Nanoparticles and Hybrid Hydrogels. PLoS ONE 2016, 11, e0168727.

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