Continuous synthesis of extremely small-sized iron oxide nanoparticles used for T_1 -weighted magnetic resonance imaging *via* a fluidic reactor

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ABSTRACT Extremely small-sized iron oxide nanoparticles (ESIONPs) with sizes less than 5 nm have shown great promise as T_1 contrast agents for magnetic resonance imaging (MRI). However, their facile and scalable production with simultaneously endowed biocompatible surface chemistry remains difficult to be realized. In this study, by using the coprecipitation method implemented in a specially designed gas/liquid mixed phase fluidic reactor, polyglucose sorbitol carboxymethylether (PSC) coated ESIONPs were continuously synthesized with controllable particle sizes ranging from 1.8 to 4 nm. Among the differently sized ESIONPs, the 3.7-nm ESIONPs exhibit the best performance as T_1 MRI contrast agent, featuring a high r_1 value of $4.11 \text{ (mmol L}^{-1}\text{)}^{-1} \text{ s}^{-1}$ and low r_2/r_1 ratio of 7.90 under a clinical 3 T MR scanning, as well as the excellent T_1 MRI contrast effect in not only water but also the cellular environment and blood vessel. Furthermore, the ESIONPs possess long-term stability and good dispersity in aqueous dispersions, making them ideal candidates as safe and effective T_1 -weighted MRI contrast agent for real clinical use.

Keywords: ESIONPs, T_1 -contrast agents, MRI, coprecipitation method, gas/liquid mixed phase fluidic reactor

INTRODUCTION

Magnetic resonance imaging (MRI) is a critical medical imaging diagnostic modality for visualizing soft tissues and organs [1], because it is noninvasive, radiation-free and capable of real-time monitoring [2,3]. To improve the contrast of MRI, 40%–50% of MR examinations use contrast agents [4,5]. Gadolinium-based contrast agents are extensively used for T_1 -weighted (T_1 is longitudinal relaxation time) MRI in clinics [1,5,6]. However, they pose risks such as Gd deposition and nephrogenic systemic fibrosis [1,5]. Therefore, high-performance and biocompatible MRI contrast agents are in great need.

Due to their excellent biocompatibility, magnetic iron oxide nanoparticles (IONPs) including Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ have

attracted increasing attention as promising MRI contrast agents [5,7–9]. IONPs larger than 10 nm are generally used as T_2 -weighted (T_2 is transversal relaxation time) MRI contrast agents because of their large transversal relaxivity (r_2) [10]. However, the dark signals of T_2 contrast agents are susceptible to interferences such as internal bleeding, air-tissue boundaries and calcification, causing inaccurate diagnoses [1,7,11]. By contrast, T_1 contrast agents with bright signals are preferred for clinical use. IONPs smaller than 5 nm, also termed as extremely small-sized iron oxide nanoparticles (ESIONPs), have been extensively researched as T_1 -weighted MRI contrast agents in recent years, because their large surface-to-volume ratio increases the interaction between the surface iron ions and nearby water protons and enhances the T_1 relaxation effect. Meanwhile, the ultra-small size of ESIONPs decreases their magnetic moment and suppresses the T_2 relaxation effect [2,3,12,13].

ESIONPs have been synthesized by various methods such as coprecipitation method [6,14–16], high temperature thermal decomposition method [1–3,8,17] and polyol method [4,9,12,13,18–20]. The ESIONPs fabricated *via* the high-temperature thermal decomposition method usually possess good crystallinity and monodispersity, but further surface modification is necessary before their biomedical applications due to their hydrophobicity [1,3]. Although the ESIONPs prepared *via* the polyol method are hydrophilic and have high crystallinity, complete removal of the organic solvent remains a significant challenge [5,9]. The coprecipitation method, as compared with other methods, is easier to implement and thus scalable at a lower cost; unfortunately, ESIONPs prepared *via* coprecipitation generally have non-uniform particle size [6,14–16].

Recently, microfluidic reactors have been reported to realize the homogenous and scalable production of a number of nanostructures due to the precise control of reaction conditions [21–27]. The single-phase fluidic systems of miscible fluids are subjected to diffusion-limited mixing and are poorly suited to the synthesis of rapidly formed nanoparticles due to their high susceptibility to fouling, while the droplet-based fluidic systems of immiscible phases (including liquid/liquid and gas/liquid) can

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overcome these limitations because the reaction is confined in spatially isolated droplets [24,28]. Herein, we introduced a gas/liquid mixed phase fluidic reactor where NH_3 was subtly used to create an alkaline condition required for the coprecipitation reaction, and N_2 was used as both carrier gas and protective gas which avoided the oil and water separation of products synthesized by immiscible liquid/liquid droplet-based fluidic systems. We realized the controllable and continuous synthesis of ESIONPs based on this reactor. Polyglucose sorbitol carboxymethylether (PSC, a clinically approved surface coating agent for IONPs, e.g., Feraheme[®]) was used to protect the ESIONPs [12,14,29,30], which exhibited good magnetic property, good biocompatibility, excellent T_1 contrast effect, and kept good dispersity and long-term stability in aqueous dispersions.

EXPERIMENTAL SECTION

Materials

Ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 99.95%, Acros Organics), ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, >99%, Macklin), hydrochloric acid (HCl, 36%–38%, Sinopharm), poly-L-lysine hydrobromide (PLL, $M_w \geq 30$ kDa, Sigma-Aldrich), and polyglucose sorbitol carboxymethylether (PSC, $M_w \sim 10$ kDa, Chiatai Tianqing Pharmaceutical Group Co., Ltd.) were used as received without further purification. Ultrapure water was used throughout the experiments. The NH_3 and N_2 gases were high purity. The reference $\gamma\text{-Fe}_2\text{O}_3$ NPs were the product RuiCun prepared by Chiatai Tianqing Pharmaceutical Group Co., Ltd. The reference Fe_3O_4 NPs were the product prepared by Nanjing Nanoeast Biotech Co., Ltd. A549 cells and human lung cancer line were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. Magnevist[®] (Bayer Schering Pharma AG).

Synthesis of ESIONPs in the gas/liquid mixed phase fluidic reactor

The precursor solutions containing $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (10.0 mmol L^{-1}), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (5.0 mmol L^{-1}) and PSC (3.0 mg mL^{-1}) were prepared and deoxygenated *via* N_2 bubbling for at least 20 min before reaction. Precursor solutions were placed in 50-mL plastic syringes and pumped into the T-shaped channel at a flow rate of 2 mL min^{-1} *via* a micro-injection pump (Longer). The reaction gases NH_3 and N_2 were premixed *via* gas mass flow meters (Beijing Sevenstar Flow Co., Ltd.) with flow rates of 5.0 and 15.0 sccm (standard cubic centimeter per minute), respectively, before injecting into the T-shaped channel. After mixing at the T-junction, a stable gas-liquid segmented flow was obtained in the poly(tetrafluoroethylene) (PTFE) tube (2.2 mm inside diameter and 10 m length) connected to the outlet of the T-shaped junction. The tube was immersed in a water or alcohol bath to tune the temperature from 1 to 90°C . The residence time can be controlled by adjusting the flow rates of liquid and gas phases (Table S1). The final products were collected in a three-necked flask at room temperature and accompanied by 600 r min^{-1} stirring. The obtained samples were cooled at room temperature and then concentrated by centrifugal ultrafiltration (Millipore, molecular size cutoff of 30 kDa), which proceeded at 25°C with a rotational speed of 5000 r min^{-1} for 6 min. The concentrated samples were dialyzed (M_w cutoff 20 kDa) for two days in ultrapure water that was changed every 6 h to get rid of excess ions. The final products

were stored in 4°C refrigerator.

Characterization

The morphology and particle size of ESIONPs were analyzed by using FEI JEM-2100 transmission electron microscope (TEM) operated at 200 kV. At least 150 nanoparticles were measured by using Nano Measure software to evaluate the particle size of nanoparticles. The powders of samples were prepared using a lyophilizer (Advantage 20EL-85, SP Scientific). Powder X-ray diffraction (XRD) patterns were collected with an X-ray diffractometer (D8 ADVANCE, Bruker). The magnetic properties were measured at room temperature by a vibration sample magnetometer (7407, LakeShore). Fourier transform infrared (FTIR) spectra were obtained with a FTIR spectrometer (Nicolet IS10, ThermoFisher). The composition proportion and thermal properties of nanoparticles were analyzed with a thermogravimetric analyzer (STA449 F5, NETZSCH). The X-ray absorption spectroscopy (XAS) analysis was performed on the soft X-ray spectromicroscopy beamline of BL08U1A at Shanghai Synchrotron Radiation Facility (SSRF). The iron concentrations of nanoparticles were measured with a classical C-A (Fe concentration *vs.* absorbance) calibration curve on an ultraviolet-visible (UV-vis) spectrophotometer (UV-3600, Shimadzu). The pH of solutions was tested with a pH meter (FE20, METTLER TOLEDO). The hydrodynamic sizes of nanoparticles were measured by a particle size analyzer (Zetasizer NanoZS90, Malvern).

Cellular uptake and viability evaluation

To ensure an efficient uptake by A549 cells, the surface of ESIONPs was modified by the cationic ion transfection reagent PLL [31]. The mixing dispersion containing 3.7-nm ESIONPs ($1 \text{ mg}_{\text{Fe}} \text{ mL}^{-1}$) and PLL ($40 \mu\text{g mL}^{-1}$) was sonicated below 25°C for more than 3 h to get the PLL-modified ESIONPs. To observe the cellular uptake of nanoparticles, A549 cells were cultured in 6-well plates and incubated with various concentrations of ESIONPs including 0, 50, 100 and $200 \mu\text{g}_{\text{Fe}} \text{ mL}^{-1}$. After 24 h incubation, the cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde. The prussian blue (PB) staining was conducted to detect the iron in A549 cells after ESIONPs labeling. The fixed cells were stained with a mixture of 1% potassium ferrocyanide and 3% hydrochloric acid (Solarbio, Beijing, China). The PB staining images of A549 cells were acquired with microscopy (CKX53, Olympus).

The viability of A549 cells incubated with PLL-modified ESIONPs was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). For MTT assay, A549 cells were seeded into a 96-well plate and grew overnight in $200 \mu\text{L}$ of media. The cells were then incubated with various concentrations of ESIONPs (0, 50, 100 and $200 \mu\text{g}_{\text{Fe}} \text{ mL}^{-1}$) for 24 h. After that, cells were incubated in media containing 0.1 mg mL^{-1} of MTT for 4 h. And then, the culture media was removed and dimethyl sulphoxide (DMSO) was added to dissolve the precipitated formazan. The cell viability was evaluated by measuring the absorbance of each well at 490 nm with a microplate reader (Multiskan Sky, Thermo Scientific).

In vitro MRI and relaxivity measurements

The MR imaging and relaxivity were tested by using a small animal 7 T MR scanner (PharmaScan, Bruker) and clinical 3 T MR scanner (Verio, Siemens). ESIONPs dispersions were line-

arly diluted in ultrapure water ($C_{\text{Fe}} = 0, 0.03, 0.06, 0.13, 0.25, 0.5, 1.0 \text{ mmol L}^{-1}$) and added to the 5-mL centrifugal tubes. The tubes filled with ESIONPs dispersions were placed into the MRI scanner coil center, the MR imaging and relaxivity were measured with the corresponding sequence. The T_1 MRI measurement parameters of 7 T MR scanner were as follows: repetition time (TR) = 400 ms, echo time (TE) = 7.5 ms, field of view (FOV) = $6.0 \times 6.0 \text{ cm}^2$, matrix = 256×256 , slice thickness = 1.0 mm. The T_2 MRI measurement parameters of 7 T MR scanner were as follows: TR = 5000 ms, TE = 99 ms, FOV = $6.0 \times 6.0 \text{ cm}^2$, matrix = 256×256 , slice thickness = 1.0 mm. The T_1 MRI measurement parameters of 3 T MR scanner were as follows: TR = 8.403 ms, TE = 1.854 ms, FOV = $18 \times 18 \text{ cm}^2$, matrix = 384×384 , slice thickness = 3.0 mm. The T_2 MRI measurement parameters of 3 T MR scanner were as follows: TR = 989.596 ms, TE = 20, 40, 60, 80, 100 ms, FOV = $20 \times 20 \text{ cm}^2$, matrix = 384×384 , slice thickness = 3.0 mm.

The A549 cells labelled by 3.7-nm ESIONPs were collected and fixed in the bottom of 1.5-mL centrifugal tubes *via* agarose. The T_1 weighted MR images of A549 cell pellets under 3 T MR scanner were scanned using the following parameters: TR = 15 ms, TE = 1.68 ms, FOV = $35 \times 35 \text{ cm}^2$, matrix = 768×768 , slice thickness = 3.0 mm. The signal-to-noise ratio (SNR) was measured by the mean signal intensity of cell pellets divided by the standard deviation (SD).

In vivo MRI of mouse

All animals were purchased from the Laboratory Animal Center of Southeast University for the applicant, and the experiments were approved by the Animal Ethics Committee of Southeast University (Number of Experimental Facilities certifications SYXK(Su)2021-0022). The *in vivo* MRI of mouse was tested by using a small animal 7 T MR scanner (PharmaScan, Bruker). A series of dosages including 3.5, 5.5, 7.5 and $9.5 \text{ mg}_{\text{Fe}} \text{ kg}^{-1}$ mice of ESIONPs were injected through the tail vein. The dosage of Magnevist® was 1.82 mL kg^{-1} mice, which was calculated according to the ratio of mouse/human body surface area. The mice were anesthetized by the inhalation of isoflurane with adjustable concentration and the respiration rate was maintained at about 30 beats per min. The MRI of pre-injection and different time points after injection were measured by using a 3D spoiled gradient echo sequence (FLASH): FOV = $40 \text{ mm} \times 40 \text{ mm}$, matrix = 256×256 , number of slices = 128, slice thickness = $312.5 \mu\text{m}$, TR = 10 ms, TE = 2.5 ms. The SNR was measured by the mean signal intensity of a region of interest divided by the SD.

RESULTS AND DISCUSSION

Design and fabrication of the gas/liquid mixed phase fluidic reactor

The preparation of IONPs *via* the coprecipitation method involves a series of hydrolysis and condensation reactions of Fe^{2+} and Fe^{3+} ions under an alkaline condition [32]. Such a process is extremely sensitive to solution pH and usually proceeds through pH-dependent transformations that result in multiple intermediates such as $\beta\text{-FeOOH}$, $\alpha\text{-FeOOH}$, and $\alpha\text{-Fe}_2\text{O}_3$; therefore, rapid establishment of a homogeneous alkaline environment could simplify the reaction pathways [33,34]. In order to improve the mixing of reactants and reduce the concentration gradient of ions in the solution [22,23], a gas/liquid mixed phase fluidic reactor was specially designed as shown in Scheme 1. The gas and liquid phase reactants are separately and simultaneously pumped into the fluidic reactor through PTFE tubes connected to the two inlets of a T-shaped junction. After mixing at the T-junction, a stable gas-liquid segmented flow is obtained in the PTFE tube connected to the outlet of the T-shaped junction. The inner surface roughness of the tube and the pressure exerted by the gas segments could promote the mixing of reactants in the liquid segments, and thus enhance the homogeneity of the reaction [22,35].

Synthesis of ESIONPs with different particle sizes

In a typical experiment, the liquid-phase precursor solution contains ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) at a molar ratio of 1:2 to supply iron ions, and PSC acting as the surface capping agent for IONPs. This precursor solution was deoxygenated *via* N_2 bubbling for at least 20 min before being pumped into the fluidic reactor. The gas-phase precursor was composed of NH_3 and N_2 mixed at a volume ratio of 1:3 and was injected to the reactor after premixing. The NH_3 gas was partially dissolved into the liquid segments to create an alkaline reaction environment (typically pH ~ 9.5), and the color of the liquid segments quickly turned from light yellow to brown after leaving the T-junction, suggesting the hydrolysis of the iron precursors. The reaction temperature was controlled by immersing the outlet PTFE tube (typically 10 m in length) in a water/alcohol bath whose temperature can be tuned from 1 to 90°C . Note that the solution segments flow faster at higher temperatures due to the volume expansion of the gas phase. Hence, when the injection rates of liquid and gas phases were 2 mL min^{-1} and 20 sccm, respectively, the residence time of the reaction solution in the tem-

Scheme 1 Schematic diagram of the gas/liquid mixed phase fluidic reactor for the preparation of ESIONPs.

perature control bath was measured to vary from 160 to 66 s as the temperature increased from 1 to 90°C in the water/alcohol bath. The product was continuously collected in a flask, and can be easily scaled up by assembling multiple parallel tubes [36]. We set a series of reaction temperatures including 1, 25, 40, 60, 80 and 90°C (Table 1). The particle size of the collected EIONPs increases from 1.8 nm at 1°C to 4.0 nm at 90°C as shown in their TEM images in Fig. 1. Other factors including the concentration of iron precursor solution and the residence time were found to have little effect on the particle size. For example, when the iron concentration varied from 7.5 to 30.0 mmol L⁻¹ (Table S1), the variation of particle size was less than 0.1 nm (Fig. S1). When the injection rate of reactants was proportionately reduced by 1/4 and 1/2, their residence time at 80°C was prolonged from ~85 s to ~110 and ~165 s, respectively (Table S2), and the variation of particle size was less than 0.4 nm (Fig. S2).

The EIONPs obtained at different reaction temperatures were further characterized with XRD. As shown in Fig. 2, peaks at 30.2°, 35.6°, 43.3°, 57.3°, and 62.9° for the (220), (311), (400), (511), and (440) crystal faces of Fe₃O₄ (JCPDS No. 19-0629) or

γ-Fe₂O₃ (JCPDS No. 39-1346) become more evident for products synthesized at higher temperatures, further indicating the increased particle size [29]. Note that it is difficult to distinguish the Fe₃O₄ and γ-Fe₂O₃ phases *via* XRD analysis due to their almost identical standard XRD patterns [37]. Therefore, the XAS measurement at the Fe L-edges was carried out and compared with that of pure Fe₃O₄ and γ-Fe₂O₃ to identify the compositions of the ESIONPs. As Fig. 2b shows, their XAS spectra exhibit two peaks at around 710.5 and 712.5 eV at L₃ edge, arising from the octahedral coordinated Fe³⁺. As the particle size increases, the peak intensity at 712.5 eV gradually increases and the pattern profile becomes more similar to that of the reference γ-Fe₂O₃ NPs [37,38], which is different from the standard band for Fe₃O₄ at around 711 eV that originates from the octahedral coordinated Fe²⁺ [38]. This suggests that the dominant phase of the ESIONPs larger than 3.5 nm is γ-Fe₂O₃, in agreement with the fact that the Fe₃O₄ phase is readily oxidized to γ-Fe₂O₃ especially at elevated temperatures [37,39]. Fig. 2c shows the field-dependent magnetization curves of ESIONPs prepared at different temperatures. The saturation magnetization of the ESIONPs enhances with increasing particle size. The ESIONPs

Table 1 Synthesis conditions and characterization results of the ESIONPs prepared with different reaction temperatures (*r*₁ and *r*₂ were measured on a 7 T MR scanner)

Sample nomenclature	Temperature (°C)	Residence time (s)	pH	Particle size (nm)	<i>M</i> _s (emu g _{Fe} ⁻¹)	<i>r</i> ₁ ((mmol L ⁻¹) ⁻¹ s ⁻¹)	<i>r</i> ₂ ((mmol L ⁻¹) ⁻¹ s ⁻¹)	<i>r</i> ₂ / <i>r</i> ₁
ESIONPs-1	1	~160	9.50	1.78 ± 0.34	3.73	0.20	\	\
ESIONPs-2	25	~150	9.48	1.96 ± 0.33	2.49	0.16	\	\
ESIONPs-3	40	~132	9.40	2.11 ± 0.45	4.90	0.25	\	\
ESIONPs-4	60	~114	9.47	3.48 ± 1.04	30.25	0.72	17.69	24.6
ESIONPs-5	80	~85	9.49	3.72 ± 0.66	56.85	1.41	32.17	22.8
ESIONPs-6	90	~66	9.51	4.04 ± 0.64	63.61	1.35	35.56	26.3

Figure 1 TEM images and size distributions (insets) of ESIONPs prepared at different reaction temperatures. (a) 1°C, (b) 25°C, (c) 40°C, (d) 60°C, (e) 80°C, (f) 90°C.

smaller than 3 nm exhibit weak paramagnetic, whereas those with sizes of 3.7 and 4.0 nm are superparamagnetic and have saturation magnetizations of 56.85 and 63.61 emu g_{Fe}⁻¹, respectively, which are relatively high among similar-sized ESIONPs reported previously [1,2,16]. We speculate that the effective heat transfer at the small scale of liquid segments during less than 2 min of heating in the temperature control bath endowed the ESIONPs with high crystallinity and magnetization [21]. Our method is highly efficient compared with the hot injection method which usually needs more than one hour of heating [6,16,40].

Dispersion stability of product

We further explored the crystallinity and dispersion stability of the product. The high-resolution TEM image of the ca. 3.7-nm-sized ESIONPs exhibits distinct lattice fringes. For example, an IONP displays 0.25 and 0.25 nm lattice spacings with 86° intersection angle, attributable to the (311) and (T31) planes of IONP, suggesting its single crystalline nature. The selected-area electron diffraction (SAED) pattern of multiple particles exhibits five diffraction rings corresponding to the (220), (311), (400), (511), and (440) crystal faces of γ-Fe₂O₃ or Fe₃O₄ phase, in agreement with their XRD pattern (Fig. 3a). A good dispersion stability is critical for the storage and application of nanomaterials [9], which can be achieved by coating IONPs with PSC based on our previous studies [14,29,30]. The successful coating of PSC on the surface of ESIONPs was confirmed by their FTIR

absorption spectra (Fig. 3b) and thermogravimetry (TG) analysis (Fig. S3). As shown in Fig. 3b, a broad band at 3362 cm⁻¹ is assigned to the O–H bond from water and PSC, the peaks at 2924, 1591, and 1340 cm⁻¹, respectively, correspond to the stretching vibrations of –CH₂–, –COO⁻, and C–H bonds, two peaks at 1416 and 916 cm⁻¹ are the flexural vibration peaks of O–H bond in carboxyl, and peaks at 1154 and 1014 cm⁻¹ are assigned to the stretching vibration peaks of C–O bond, which can be assigned to PSC [41]. Besides, a stretching vibration peak assigned to the Fe–O bond at 590 cm⁻¹ is also observed [9,41]. The hydrodynamic size of the 3.7-nm ESIONPs is about 18.2 nm, and remained unchanged even after 4 weeks of storage (Fig. 3c), suggesting the good stability of the product dispersion. TEM images and hydrodynamic size distributions of three different batches of ESIONPs synthesized at 80°C were characterized to demonstrate the reproducibility of our synthesis method (Fig. S4). And the percentages of Fe³⁺ ions were tested to be ~97% (Table S3), which further demonstrated the dominant phase of 3.7-nm ESIONPs was γ-Fe₂O₃ in agreement with the analysis results of XAS spectra.

MR phantom test of ESIONPs

To evaluate the feasibility of our ESIONPs as T₁ MRI contrast agent, their MR phantom and relaxation properties were measured by using a small animal 7 T MR scanner. The T₁ MRI contrast effects of ESIONPs smaller than 3.0 nm are poor (Fig. 4a, b, and Figs S5 and S6), and failing to show any linear

Figure 2 (a) XRD patterns, (b) Fe L-edges XAS spectra, and (c) field-dependent magnetization curves of the ESIONPs prepared at different reaction temperatures.

Figure 3 (a) High-resolution TEM image and SAED pattern (inset) of 3.7-nm ESIONPs. (b) FTIR absorption spectra of PSC and 3.7-nm ESIONPs. (c) Hydrodynamic size of 3.7-nm ESIONPs stored for 4 weeks.

Figure 4 (a) T_1 weighted MR images and (b) corresponding plots of $1/T_1$ over Fe concentrations of ESIONPs prepared at different temperatures (measured on a 7 T MR scanner). (c) T_1 and T_2 weighted MR images of 3.7-nm ESIONPs (measured on a 3 T MR scanner). (d) Relaxation properties, (e) plots of $1/T_1$ over Fe concentrations and (f) plots of $1/T_2$ over Fe concentrations for different batches of 3.7-nm ESIONPs (measured on a 3 T MR scanner).

correlations between the T_2 relaxation time and the iron concentration (Table 1) [3]. Differently, the 3.7 and 4.0 nm ESIONPs exhibit higher r_1 values of 1.41 and 1.35 (mmol L⁻¹)⁻¹ s⁻¹ at 7 T, respectively. Since the 3.7-nm ESIONPs have a smaller r_2 value of 32.17 (mmol L⁻¹)⁻¹ s⁻¹ than the 4.0 nm ESIONPs (r_2 = 35.56 (mmol L⁻¹)⁻¹ s⁻¹), they are more appropriate as T_1 MRI contrast agent. Both the r_1 and r_2 values of ESIONPs are smaller than those of the commercial product Feraheme®, whose r_1 value is 3.1 (mmol L⁻¹)⁻¹ s⁻¹ and r_2 value is 68 (mmol L⁻¹)⁻¹ s⁻¹ at 7 T. This probably because the Feraheme® has larger particle size of 7 nm and higher saturation magnetizations of 94 emu g_{Fe}⁻¹ [1]. Under a clinical 3 T MR scanner, the 3.7-nm ESIONPs exhibit a much brighter signal (Fig. 4c), and have a relatively higher r_1 value of 4.11 (mmol L⁻¹)⁻¹ s⁻¹ and a lower r_2/r_1 value of 7.9 (Fig. 4d) compared with the 7 T MR scanning, suggesting their potentials for clinical applications. The highly consistent MRI contrast effect of three batches of 3.7-nm ESIONPs under 3 T (Fig. 4e, f) and 7 T MR scanning suggest the good stability of their MRI contrast performance (Fig. S7).

We further demonstrated the potential use of these ESIONPs as cell labeling and imaging agents. To ensure an efficient uptake by cells, 3.7-nm ESIONPs were firstly modified with transfection reagent PLL [31]. As Fig. S8 shows, the hydrodynamic size of the PLL-modified ESIONPs is 20.3 nm, slightly larger than before

the modification (18.2 nm). A549 cells were incubated with the modified ESIONPs at various concentrations in serum-containing cell culture media, and the uptake of the ESIONPs by these cells was confirmed by the PB staining. As shown in Fig. 5a, the cells incubated with 50, 100 and 200 μg_{Fe} mL⁻¹ of ESIONPs exhibit distinct blue compared with the control group without ESIONPs incubation, confirming the successful labeling of the A549 cells with ESIONPs. The cells remained at least 90.6% of viability when the incubation concentration of ESIONPs was up to 200 μg_{Fe} mL⁻¹ (Fig. 5b). *In vitro* T_1 weighted MR images of these ESIONP-labeled A549 cells were obtained on a 3.0 T MR scanner, demonstrating a significant T_1 signal enhancement in contrast to the non-labeled ones (Fig. 5c). The SNR of the MR images of labeled cells demonstrates the incubation concentration of 100 μg_{Fe} mL⁻¹ is high enough for the cell labelling and imaging. Therefore, the 3.7-nm ESIONPs could also provide excellent T_1 contrast effect in the cellular environment besides in water.

In vivo MRI enhancement effect of ESIONPs was evaluated by the magnetic resonance angiography (MRA) in mice. A series of dosages including 3.5, 5.5, 7.5, and 9.5 mg_{Fe} kg_{mice}⁻¹ of ESIONPs were injected through the tail vein (Fig. S9). When the injection dosage was 5.5 mg_{Fe} kg⁻¹, the hearts and blood vessels of mice were brightened significantly on the T_1 weighted MR images after injection (Fig. 6), and no obvious toxic and side effects were

Figure 5 (a) The intracellular accumulation of 3.7-nm ESIONPs stained by PB in A549 human cells incubated with various concentrations of nanoparticles. (b) Viability evaluation of A549 human cells incubated with various concentrations of 3.7-nm ESIONPs. (c) T_1 weighted MR images and corresponding SNR of A549 cell pellets (circled by dotted white lines) incubated with various concentrations of 3.7-nm ESIONPs (measured on a 3 T MR scanner).

Figure 6 ESIONPs-enhanced ($5.5 \text{ mg}_{\text{Fe}} \text{kg}^{-1}$ mice) *in vivo* MR images of mice acquired at pre-injection, post-injection, 10, 20, 30, 50, 60 min, and 24 h after injection.

shown in mice. As shown in Fig. 6 and Fig. S10, the bright signal decreased with time, but it could be maintained for at least 1 h and the signal vanished completely 24 h later. In comparison, although the commercial Gd-based T_1 MRI contrast agent Magnevist® has better contrast effect, the bright signal became indistinct at 10 min post-injection as shown in Fig. S11. So, the longer blood half-life is a great advantage of ESIONPs as MRA contrast agent over commercial Gd-chelator agent. It is important for the clinical diagnosis of angiocardopathy such as

myocardial infarction, atherosclerotic plaque, and thrombosis [1,2].

CONCLUSIONS

PSC-coated ESIONPs with particle sizes smaller than 5 nm were synthesized *via* the coprecipitation method based on a gas/liquid mixed phase fluidic reactor. The preparation method is simple and can be easily scaled up by assembling multiple parallel tubes. The XRD and XAS characterizations reveal that the dominant

phase of ESIONPs larger than 3.5 nm is γ -Fe₂O₃ and they exhibit good magnetic property. The effective heat transfer at the small scale of the liquid segments in the fluidic reactor tube endowed the ESIONPs with high crystallinity and magnetization. Under a clinical 3 T MR scanning the 3.7-nm ESIONPs exhibit a high r_1 value of 4.11 (mmol L⁻¹)⁻¹ s⁻¹ and low r_2/r_1 ratio of 7.90, and demonstrate excellent T_1 MRI contrast effect both in the water, cellular environment, and blood vessel. Importantly, the ESIONPs exhibit good biocompatibility, and keep good dispersity and long-term stability in aqueous dispersions. These features make them ideal candidates as T_1 -weighted MRI contrast agent for clinical applications.

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Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information Supporting data are available in the online version of the paper.

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采用流体反应器连续制备可用于 T_1 加权磁共振成像的极小尺寸氧化铁纳米颗粒

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摘要 粒径小于5 nm的极小尺寸氧化铁纳米颗粒(ESIONPs)作为 T_1 加权磁共振成像(T_1 -MRI)造影剂展现出巨大的发展前景. 然而, 具有良好生物相容性的ESIONPs的批量生产仍然难以实现. 本研究通过特别设计的气/液混合相流体反应器结合共沉淀合成方法, 实现了聚葡萄糖山梨醇羧甲基醚(PSC)包覆的ESIONPs的连续制备, 且其粒径在1.8–4.0 nm范围内可控. 在不同粒径的ESIONPs中, 粒径为3.7 nm的ESIONPs具有最好的 T_1 -MRI造影效果, 特别是在临床3 T磁共振扫描设备下具有高的 r_1 值($4.11 \text{ (mmol L}^{-1})^{-1} \text{ s}^{-1}$)和低的 r_2/r_1 值(7.90), 并且在水相、细胞和血管环境中均展现出良好的 T_1 -MRI对比效果. 此外, PSC包覆的ESIONPs具有良好的生物安全性, 在水相中具有良好的分散性和长期稳定性, 有望成为临床可用的安全有效的 T_1 -MRI造影剂.