“Two-in-One” Fabrication of Fe₃O₄/MePEG-PLA Composite Nanocapsules as a Potential Ultrasonic/MRI Dual Contrast Agent

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Supporting Information

ABSTRACT: A new method for the fabrication of Fe₃O₄ nanoparticle-encapsulated polymeric nanocapsules is presented. This method is characterized by combining a double emulsification with the interfacial coprecipitation of iron salts to form Fe₃O₄/polymer composite nanocapsules in a single step. To demonstrate the viability of this approach, methoxy poly(ethylene glycol)-poly(lactide) (MePLEG) was chosen as the shell material for Fe₃O₄/MePLEG nanocapsules. In addition to the versatility offered for fabricating nanocapsules with different shell materials, the method was found to be convenient for adjusting the magnetite content of the nanocapsules from 0 to 43%. In addition to their confirmed T₂-weighted magnetic resonance imaging (MRI) enhancement, the resultant composite nanocapsules display much more obvious acoustic responses than MePLEG nanocapsules in an acoustic investigation. Furthermore, the low toxicity of these composite nanocapsules, as confirmed by our study, combined with their magnetic and acoustic properties ensure that these composite nanocapsules have great potential in acting as ultrasonic/MRI dual contrast agents.

INTRODUCTION

Moving from the macroscale to the nanoscale, superparamagnetic iron oxide (SPIO) nanoparticles demonstrate great potential in various biomedical fields owing to their unique physical, chemical, and thermal properties.1–5 For biomedical applications, a suitable surface coating for SPIO nanoparticles is usually required to avoid recognition by the mononuclear phagocytic system (MPS).1,4,7,8 Macromolecules such as polylactide (PLA)-based copolymers, and polyethylene glycol (PEG) based copolymers are suitable for surface coating because they are known to be biocompatible and versatile, while providing a platform for further biological modification.9–13 Accordingly, coated SPIO composite nanoparticles can be classified as nanospheres or nanocapsules based on whether the macromolecules are absorbed onto the surface of the SPIO nanoparticles or act as a vesicular matrix to encapsulate the SPIO nanoparticles, respectively.

Compared to nanospheres, nanocapsules are more useful for biomedical imaging with ultrasonography due to their capability to further encapsulate gaseous bubbles to act as ultrasonic contrast imaging agents (UCAs).13–17 UCAs enhance ultrasonic contrast by altering the acoustical properties of the tissues, which includes improving, for example, back scattering, nonlinear harmonic, sound attenuation and phase velocity. Considering the T₂-weighted MRI enhancement capability of SPIO nanoparticles, SPIO enveloped composite nanocapsules should be of great potential for acting as ultrasonic/MRI dual contrast imaging agents, which is important for medical imageology because ultrasonic imaging is an ideal complementary diagnostic tool to MRI’s real-time temporal resolution.15

The extensive applications of SPIO based nanoparticles consequentially promoted the development of corresponding synthesis techniques.18–21 As one of the most widely applied SPIO nanoparticles, Fe₃O₄ magnetic nanoparticles with hydrophilic surface modification, are commonly synthesized by coprecipitation in aqueous mediums.21–23 To improve the magnetic properties and size distribution of the resulting magnetic nanoparticles, a facile interfacial coprecipitation to synthesize Fe₃O₄ nanoparticles was developed.24,25 More recently, by combining this interfacial coprecipitation with a double emulsification (W₁/O/W₂) technique Fe₃O₄/PLA magnetic microcapsules with sizes of 2–4 μm were successfully fabricated using a single step method, but the nanosized capsules were still difficult to be prepared by using this method.26 The existing fabrication approaches for Fe₃O₄-based composite
magnetic nanocapsules (MNCs) are usually time-consuming and include at least two steps, that is, the synthesis of Fe₃O₄ nanoparticles and the incorporation of nanoparticles into a polymer nanocapsule by, for example, an emulsion based technique. A new approach that allows for the direct one-step synthesis of MNCs with controllable properties is desirable.

For the fabrication of nanosized capsules by emulsification, the formation and stabilization of the nanoemulsion is critical and requires careful design of the variables, such as the category of shell materials, and emulsification methods. In the present work, MePEG-b-PLA (MePLEG) was chosen to form the building blocks of the polymeric shell of the nanocapsules because of the biocompatibility of both blocks and the efficient protection PEG offers from the MPS. In addition, high-energy ultrasound has been confirmed to be effective with the W₁/O/W₂ emulsification methodology when fabricating MeP-LEG nanocapsules, and thus is introduced here to develop a facile “two-in-one” approach for producing Fe₃O₄/MePLEG MNCs. The “two-in-one” approach realizes the combination of the W₁/O/W₂ emulsification with the interfacial coprecipitation synthesis of Fe₃O₄ nanoparticles, and enables the fabrication of MNCs with a facile one step process. For this approach, both the Fe₃O₄ nanoparticles loading content and the resultant saturation magnetization of the MNCs can be adjusted conveniently by varying the dose of the iron salts. Furthermore, the biocompatibility, acoustic properties, as well as T₂-weighted MRI enhancement of MNCs are investigated. The results show that these magnetic nanocapsules have great potential for acting as ultrasonic/MR dual imaging agents.

**Experimental Section**

**Materials.** Ferric chloride hexahydrate (FeCl₃·6H₂O), ferrous chloride tetrahydrate (FeCl₂·4H₂O), di-n-propylamine and methylene dichloride (DCM) were purchased from Sinopharm Chemical Reagent Co., Ltd. Methoxy poly(ethylene glycol)-poly(lactic acid) (MePEG-PLA, Mₘ = 20 000, MePEG/PLA = 1:9, wt %) was purchased from Daigang Biological Technology Co., Ltd. Poly(vinyl alcohol) (PVA, molecular weight 13 000–23 000 Da, alcoholysis degree 87–89%) was purchased from Sigma-Aldrich, Inc. Sodium periodate (NaIO₄) and hydrated ruthenium dioxide (RuO₂·xH₂O) were purchased from Aladdin chemical reagent Co., Ltd. All of the reagents were used as received.

**Fabrication of NC0, Fe₃O₄/MePEG-PLA Magnetic Nanocapsules (MNCs) and Fe₃O₄ Nanoparticles (MNP).** The Fe₃O₄/MePEG-PLA magnetic nanocapsules were fabricated by a modified “two-in-one” method. Briefly, FeCl₃·6H₂O and FeCl₂·4H₂O were dissolved in 5 mL of deionized water (W₁). 0.5 g of MePEG-PLA and 3 mL of di-n-propylamine were dissolved in 20 mL of DCM (O). A series of Fe³⁺ aqueous solution with concentrations of 0.045 mol/L, 0.15 mol/L, 0.37 mol/L, and 0.57 mol/L were used, and the resultant nanocapsules were named as MNC5, MNC15, MNC30, and MNC40, respectively. The molar ratio of Fe³⁺: Fe²⁺ was fixed to 1:2. The aqueous solution of iron salts was added dropwise into the DCM solution under ultrasonic vibration (300 W, continuous mode) in a nitrogen atmosphere, as shown in Scheme 1. The process of dropping was completed in about 5 min, and ultrasonic oscillation continued for another 5 min to complete the reaction. The obtained W₁/O emulsion was then poured into 40 mL of 1 wt % PVA aqueous solution (W₂). The obtained mixture was ultrasonicated for 1 min (180 W, continuous mode). The second emulsification process was carried out in an ice–water bath. The resultant W₁/O/W₂ double emulsion was poured into 100 mL of 2% (v/v) isopropanol aqueous solution and violently stirred at room temperature for 3 h to evaporate the DCM. The formed nanocapsules were separated by centrifugation (4 °C, 10 000 rpm, 30 min). The precipitate was resuspended in deionized water, the solution was centrifuged and this process was repeated once more. Thereafter, the nanocapsules were purified by magnetic separation (the procedure is described in the Supporting Information). The precipitate was then resuspended in 25 mL of deionized water for an acoustic test. The dry powder sample of the nanocapsules was collected by lyophilization in order to analyze its other characteristics.

For the preparation of NCO in which without Fe₃O₄ encapsulated, the process is similar to that for fabricating MNCs, except that neither iron salts nor di-n-propylamine were added during the fabrication. After the fabrication, The formed nanocapsules were separated by centrifugation (4 °C, 10 000 rpm, 30 min).

The Fe₃O₄ nanoparticles enveloped in MNCs, named MNP5, MNP15, MNP30, and MNP40, were separated from the MNCs by dissolving the composite nanocapsules in chloroform, a good solvent for MePLEG. The samples were collected by a magnetic separation.

A sample of Fe₃O₄ nanoparticles was fabricated by an interfacial coprecipitation progressing to the interface between W₁ and O phase without the addition of MePLEG and is termed as MNP. FeCl₃·6H₂O and FeCl₂·4H₂O were dissolved in 5 mL of deionized water (W₁), and 3 mL of di-n-propylamine were dissolved in 20 mL of DCM (O). The concentration of Fe³⁺ aqueous solution was 0.045 mol/L and the molar ratio of Fe³⁺/Fe²⁺ was fixed to 1:2. The aqueous solution of iron salts was added dropwise into the DCM solution under ultrasonic vibration (300 W, continuous mode) in a nitrogen atmosphere. The process of dropping was completed in about 5 min, and ultrasonic oscillation continued for another 5 min to complete the reaction. Thereafter, the MNP nanoparticles were purified by magnetic separation.

**Size Characterization of Nanocapsules.** The particle size and size distributions (mean diameters and PDI) were determined by photon correlation spectroscopy (PCS) using a particle size analyzer (Zetasizer Nano ZS 90, Malvern Instruments).
Scheme 2. Schematic Diagram of the Device for Sound Attenuation Spectrum Measurement

Transmission Electron Microscopy Observation. Transmission electron microscopy (TEM, 200 kV, JEM-2010, JEOL, Japan) was used to observe the morphology of the nanocapsules. For TEM observation, an aqueous solution of the nanocapsules was sprayed onto a carbon film coated copper grid device, and after evaporating the water, the sample was stained by a 1 wt % ruthenium tetroxide (RuO4) aqueous solution. The RuO4 aqueous solution was prepared freshly according to the literature.30 Briefly, NaIO4 (0.128 g) was dissolved in 10 mL of deionized water at room temperature. RuO4·H2O (0.06 g) was then added to the aqueous solution of NaIO4. RuO4 was synthesized when RuO2·xH2O began to dissolve, and the unreacted RuO2·xH2O settled to the bottom of the bottle.

X-ray Diffraction Analysis. Crystallographic analysis was performed by an X-ray diffraction system (XRD, D/max-2200/PC, Rigaku Corporation, Japan) with Cu radiation to identify the dominant phase of the samples. The 2-theta angle range of the measurements was from 10°–70°. The phase was determined by using standard powder diffraction files from the Joint Committee for Powder Diffraction Studies (JCPDS).

The crystallite size is calculated from the full width at half-maximum (fwhm) of the diffraction peaks of the samples using the Scherrer equation

$$\tau = \frac{k\lambda}{\beta \cos(\theta)}$$

Relative crystallinity was determined from the integral intensity of the diffraction peaks of the samples.

Magnetization Measurements. The magnetization properties of the Fe3O4/MePEG-PLA nanocapsules at 300 K were studied by using a vibrating sample magnetometer (VSM, Model 7407, Lake Shore Cryotronics Inc., USA). Saturation magnetization, coercive force and remnant magnetization were obtained from the hysteresis loops.

In Vitro Acoustic Experiments. Degas water and hybrid nanocapsules (5 mg/mL) were imaged using the ultrasonic imaging system of a GE LOGIQ Book XP Enhanced scanner (GE Medical Systems, USA) where a 4 MHz or an 11 MHz ultrasound transducer acted as a transmitter as well as a receiver. All images were acquired with the same instrument parameters (mechanical index (MI) = 0.5). The schematic diagram of the measurement was shown in Scheme S1 in Supporting Information, that is, SI.

An ultrasound spectroscopy method31 was used to measure the acoustic attenuation spectrum. As shown in Scheme 2, a broadband acoustic pulse was excited and the signals prior to and during insertion of the sample were detected and analyzed by FFT software. The sound attenuation of a sample ($\alpha_s$) can be expressed as

$$\alpha_s = \alpha_w \frac{1}{d} \ln \left( \frac{P_w(\omega)T(\omega)}{P_s(\omega)} \right)$$

where $\alpha_w$ is the sound attenuation in water; $P_w(\omega)$ and $P_s(\omega)$ are the amplitude spectra before and after the insertion of specimen respectively; $T(\omega)$ is the spectrum of the overall transmission coefficient; and $d$ is the thickness of the specimen.

In Vitro MR Imaging Experiments. The relaxivity of magnetic resonance imaging was obtained by a Siemens 3.0 T scanner (Magnemom Trio, Siemens, Munich, Germany) with a wrist coil. Phantom MRI was carried out at various iron concentrations of MNCs from 0 to 0.4 mM in an agarose gel. The spin echo sequence was used. The imaging parameters were: repetition time (TR) 3000 ms; field of view (FOV) 106 × 180 (mm × mm); matrix size 576 × 342 (mm × mm); slice thickness 5.0 mm. Then, the resulting change in the transverse relaxation time ($T_2$) of the nanocapsules suspension was continuously measured by recording the above-mentioned single-slice gradient-echo signal. No phase or frequency encoding was used. According to the monoexponential signal decay as the function of echo time (TE), the transverse relaxation time of well-mixed nanocapsules suspension can be estimated.

Cytotoxicity Assays. To investigate the cytotoxicity of the magnetic nanocapsules, an in vitro experiment was performed using rat liver cell BRL-3A and rat kidney cell NRK cultures. The number of viable cells was determined by the estimation of their mitochondrial reductase activity using the tetrazolium-based colorimetric method (MTT method) and two-color flow cytometry (FCM method).

A MTT assay depends on the cell’s reductive capacity to metabolize the yellow tetrazolium salt into a highly colored formazan product.

BRL-3A cells in the log phase of growth were seeded in RPMI-1640 culture media with 100 U/mL penicillin, 100 U/mL streptomycin, and 10% fetal bovine serum (FBS) at 10 000 cells/mL in an incubator at 37 °C for 12 h. NRK cells in the log phase of growth were seeded in 0.1 mL DMEM/high glucose media containing 100 U/mL penicillin, 100 U/mL streptomycin, and 10% heat-inactivated FBS at 10 000 cells/mL in an incubator at 37 °C for 12 h. The cytotoxicity of the MNCs was evaluated by determining the viability of the cells after coinoculation with different concentrations of MNCs (from 0.050 to 1 mg/mL) with 5% CO2 at 37 °C for 24 h. At the end of the incubation period with the MNCs, cells were incubated with a 200 μL sample of 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT) solution at 37 °C for 4 h. Afterward, 100 μL of DMSO was added in order to dissolve the formazan crystals. The UV absorbance of the solubilized formazan crystals was measured at 492 and 630 nm. Cell viability was expressed as the ratio between the amount of formazan determined for cells treated with the different MNCs suspensions and for control nontreated cells. Each point was performed five times so that the standard deviations were calculated.

For the FCM assay, NRK cells in the log phase of growth were seeded in 0.1 mL DMEM/high glucose media containing 100 U/mL penicillin, 100 U/mL streptomycin, and 10% heat-inactivated FBS. The cells were maintained in at 10 000 cells/mL in an incubator at 37 °C for 12 h. Then cells were coincubated with MNCs in 5% CO2 at 37 °C for 24 h. The concentration of the MNCs was controlled with concentrations of 25, 100, and 400 μg/mL used. Cells were stained with Annexin-V (25 μg/mL) and PI (50 μg/mL) and analyzed with a flow cytometer (Cytomics FC500, Beckman Counter Inc.); 10 000 events were analyzed for each sample.

RESULTS AND DISCUSSION

Fabrication and Composition of Composite Nanocapsules. Interfacial coprecipitation, in which the coprecipitation reaction is confined only to the interface between the water and
the oil phase, has been confirmed to be an effective approach to create hydrophilic magnetite nanoparticles capped with amine groups.24,25 A previous study validated the efficacy of high-energy ultrasound in the fabrication of MePLEG nanocapsules by W1/O/W2 emulsification.32 Therefore, according to the design, the combination of interfacial coprecipitation into W1/O/W2 emulsification would ideally realize a "two-in-one" fabrication of encapsulated iron oxide nanoparticles, or nanocapsules, as described in Scheme 3, and provide a methodology to simplify the fabrication of composite magnetic nanocapsules (MNCs). The coprecipitation was accomplished during the first emulsification and occurred only at the interface between the inner water phase (W1) and the outer MePLEG methylene dichloride solution. To adjust the iron oxide content inside the MNCs so that the magnetic property of resultant MNCs could be controlled the ratio of ferric salt/MePLEG was varied and the resultant MNCs were named NC0, MNC5, MNC15, MNC30, and MNC40, respectively.

The properties of iron oxide nanoparticles relate closely to their composition and crystalline structure.33 To elucidate the composition of the iron oxide nanoparticles inside the MNCs, the nanoparticles named MNP were synthesized by a W1/O emulsification in which pure methylene dichloride without MePLEG was used as oil phase. The diameter of the MNPs was determined by TEM observation as around 10 nm (as shown in the Figure S1 of SI). In addition, as shown in Figure 1, the XRD patterns of MNP and MNC5 confirm their structure as Fe3O4 because the positions and relative intensities of the main peaks match well to those from the JCPDS card (19-0629) for Fe3O4.34 Moreover, compared with that of MNP, the peaks of the XRD pattern for MNC5 are unobvious and broad, which might be because the main composition of the MNC are a MePLEG polymer matrix.

The samples fabricated by this "two-in-one" approach were characterized by various measurements for the purpose of validating the methodology. Shown in Figure 2 are the TGA curves of five samples prepared with different ferric salt/MePLEG ratios. The thermograms of the composite magnetic nanocapsules (MNC5, MNC15, MNC30, and MNC40) exhibit different decomposition stages when compared with nanocapsules without Fe3O4 nanoparticles (NC0), the decomposition stage around 300–400 °C in the thermograms of MNCs may be attributed to the interaction between the polymer and the Fe3O4 nanoparticles which postpones the decomposition of the polymer.35 The residue above 600 °C is ascribed to the inorganic content of the MNCs and thus, the weight percentages of the inorganic iron oxide nanoparticles in the MNCs were calculated from the weight residue of the composite MNCs at 600 °C.

The inorganic content for a series of samples are listed in Table 1, for which the theoretical content was calculated from the iron salt dosed during coprecipitation and is compared with the Fe3O4 content calculated from the residue weight by the TGA curves shown in Figure 2. It is worth noting that during the interfacial coprecipitation and double emulsification, inevitably a small quantity of MePLEG solid nanoparticles are formed without Fe3O4 inside because of the possible escape of inner water...
phase from the oil phase during the second emulsification.\textsuperscript{27} To remove these impurities from the products, the crude products were all purified by magnetic separation as specified in the Experimental Section, and the residue after magnetic separation was confirmed to contain all organic substances which degrade completely before 600 °C (Figure S2 in SI). The Fe\textsubscript{3}O\textsubscript{4} content of the crude products and their TGA results were also examined (Figure S3 in SI). As displayed by Table 1, the Fe\textsubscript{3}O\textsubscript{4} content in the crude products are all lower than the theoretical Fe\textsubscript{3}O\textsubscript{4} content, and the disparity from the theoretical value increases at higher iron salt doses. Considering that both the concentration of di-n-propylamine in methylene dichloride and the W\textsubscript{1}/O interface area were kept constant with increasing doses of iron salt, the existence of a greater disparity at higher iron salt doses is reasonable because of the inefficient reaction between excess iron salts and hydroxyl ions. Additionally, the real Fe\textsubscript{3}O\textsubscript{4} content after magnetic separation calculated from the TGA results were listed in Table 1. After removing the MePLEG solid nanoparticles from the crude products, the Fe\textsubscript{3}O\textsubscript{4} content increased due to the decrease of the total content of MePLEG.

**Morphology and Size Distributions of Composite Nanocapsules.** The morphology of composite MNCs were observed by TEM, as shown in Figure 3a, which is a typical TEM image for the magnification of one capsule from MNCs. As is presented in Figure 3a, the MNCs possess a spherical shape and the Fe\textsubscript{3}O\textsubscript{4} nanoparticles are encapsulated mostly inside the internal portion of the nanocapsules. The light gray halo around the MNCs is speculated to be the PEG corona on the periphery of the nanocapsules and the PVA emulsifier adsorbed on the surface of the nanocapsules, both of which can be stained by RuO\textsubscript{4}.\textsuperscript{36} In the inner water phase results in the increase of the size of the W\textsubscript{1} nanodroplets, and therefore increases the size of the oil droplets during the second emulsification. In addition, the \(D_{h}\) of MNCs from DLS measurements are slightly larger than the 50–200 nm diameters observed from the TEM image, which might be because of the shrinkage of the capsules during the preparation of the TEM samples.\textsuperscript{37–39}

**Magnetic Properties and In Vitro MR Imaging of Composite Nanocapsules.** Shown in Figure 4a are the room temperature hysteresis loops of MNCs fabricated by different doses of iron salts, which displays almost immeasurable coercivity and remanence, suggesting that individual magnetic nanoparticles inside MNCs are of a single domain. Since the saturation magnetization of MNCs increase with increasing Fe\textsubscript{3}O\textsubscript{4} content, this indicates that the magnetic properties of MNCs could be adjusted by controlling the reagent ratio during the first emulsification, a conveniently realized methodology. The inset of Figure 4a demonstrates that MNC5, which possess the lowest saturation magnetization of the MNCs, still displays magnetic responsibility. Additionally, to explore the effects of polymer addition on the magnetic properties of the resultant magnetic nanoparticles inside the MNCs, the room temperature hysteresis loop of MNP nanoparticles was also measured and is displayed in Figure 4a. The saturation magnetization of the MNP synthesized by a W\textsubscript{1}/O interfacial coprecipitation was found to be as high as 78 emu/g, a value higher than that of magnetite nanoparticles synthesized by both aqueous coprecipitation\textsuperscript{21–23} and interfacial coprecipitation proceeding under stirring.\textsuperscript{25} The higher saturation magnetization of MNP can be attributed to the ultrasonic process used here. We suggest that the introduction of ultrasonics in the homogeneous process increased the area of the W\textsubscript{1}/O interface, and thus improved the opportunity for the reaction between the iron salts and the hydroxide ions. With this methodology, an ultrasonic process might play a crucial role in improving the magnetic properties of resultant magnetite nanoparticles.

A previous study disclosed that in aqueous coprecipitation, the addition of a polymer into the medium will affect the crystalline structure and therefore the magnetic properties of the resultant
magnetite nanoparticles. For the purpose of elucidating this point, the saturation magnetization of the MNCs and MNPs (the Fe3O4 nanoparticles inside the MNCs after removing the polymer) are included in Figure 4b. Utilizing the magnetite weight content of the MNCs (WM%) determined by TGA and the saturation magnetization of the MNP (SMMNP), the theoretical saturation magnetization of MNCs (TSMMNCs) were calculated according to eq 3. Notably, the calculation of TSMMNCs by eq 3 is based on the hypothesis that the properties of the magnetite nanoparticles inside the MNCs are the same as that of MNP. From Figure 4b, the saturation magnetization of MNCs (ASMMNCs) is lower than the corresponding TSMMNCs, which indicates that the addition of MePLEG into the oil phase during the W1/O interface coprecipitation affected the crystalline structure, and thus, the magnetic properties of the resultant magnetite nanoparticles.

\[
TS_{\text{MNCs}} = SM_{\text{MNP}} \times (WM\%) 
\]

The XRD measurements were then carried out to investigate the Fe3O4 crystallization, and the resultant XRD patterns of the MNPs are shown in Figure 5. By using the Scherrer equation (eq 1), \( \tau = k\lambda/(\beta \cos \theta) \) the crystallite size of the MNPs were calculated from the fwhm of (311), (440), and (220) reflections. Listed in Table 2 are the crystallite size of the MNPs, the relative crystallinity of the MNPs relative to the MNP, and the saturation magnetization of the MNPs (ASMMNPs) calculated according to eq 4. The ASMMNPs are all lower than the 78 emu/g of ASMMNP suggesting that the crystallinity of the MNPs inside MNCs are not as high as that of MNP because there is a correlation between saturation magnetization and crystallinity. As listed in Table 2, the lower relative crystallinity of MNPs is also verified by the calculation from the XRD patterns.

\[
AS_{\text{MNP}} = AS_{\text{MNC}}/(WM\%) \tag{4} 
\]

Table 2. Crystalline Size of Fe3O4 in Fe3O4/MePEG-PLA Nanocapsules with Different Fe3O4 Contents and Its Corresponding Saturation Magnetization

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (d·m)</th>
<th>Saturation Magnetization (emu/g)</th>
<th>Relative Crystallinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNP5</td>
<td>14.5</td>
<td>25.9</td>
<td>61.4</td>
</tr>
<tr>
<td>MNP15</td>
<td>15.5</td>
<td>44.0</td>
<td>66.1</td>
</tr>
<tr>
<td>MNP30</td>
<td>17.2</td>
<td>33.2</td>
<td>89.5</td>
</tr>
<tr>
<td>MNP40</td>
<td>16.8</td>
<td>36.4</td>
<td>64.3</td>
</tr>
</tbody>
</table>

The saturation magnetization of magnetite nanoparticles were calculated from the saturation magnetization of MNCs as shown in Figure 4b.

To evaluate the T2 enhancing capability, agarose gel with various concentrations of MNCs were investigated by the T2-weighted MRI. As shown in Figure 6, the signal intensity of MRI decreased with the increase of the concentration of MNCs. Figure 6b indicated that the T2 relaxation time decreased as the concentrations of iron increasing and the trend is well fit by a linear line within the analyzed range of iron concentrations, exhibiting the typical properties of Fe3O4 nanoparticles with shortening T2 relaxation time. For example, the T2 relaxation time of MNC5 decreased from 73.3 to 17.7 ms as the concentrations of iron increasing from 0.05 to 0.5 mM. Specifically, the relaxivity, that is, the slope of T2/C01 versus Fe concentration, of MNC5 was calculated to be 107.09 mM/C01 s/C01, which is higher than that of MNP (85.05 mM/C01 s/C01). This may due to the enhanced susceptibility effect of assembled MNP inside the nanocapsules, for the case of MNC, the number of MNP per volume in the inner cavity is much higher than that for the free MNP case.
Acoustic Properties of Composite Nanocapsules. Ultrasonic imaging from sound attenuation is a new technique, which is reported to be effective in helping tissue characterization and diagnosis of early nonoccupancy canceration.\(^4^2\) Sound attenuation may be caused by various acoustical responses, including the scattering and absorbing of ultrasonic waves produced as it crosses an inhomogeneous medium. In addition, on the condition of the incident frequency being consistent with the natural resonance frequency of the ultrasonic contrast imaging agents (UCAs), all of the incident ultrasound energy would be absorbed by the UCAs. As a result, the sound attenuation would reach a peak value and the corresponding video intensity of the ultrasound contrasted image would be enhanced.\(^4^3\) Recent reports concluded that the sound attenuation of soft microbubble UCAs are directly proportional to their concentration, and the resonance frequency is inversely proportional to their particle size.\(^4^4\) However, in the present case, the comparatively harder MePLEG shell, the nanometer sized capsules, as well as the Fe\(_3\)O\(_4\) nanoparticles inside the nanocapsules may result in entirely different acoustical responses from those previously reported.

To investigate the contrast efficiency of MNCs, in vitro ultrasonography was performed with an emission frequency of 4 and 11 MHz, two commonly used frequency in medical diagnostics. As shown in Figure 7, the video intensity is enhanced at both frequencies in the presence of MNCs when compared to that of degassed normal saline and NC0, and the intensity showed a tiny increase with increasing Fe\(_3\)O\(_4\) content. According to the mechanism of 2D imaging, the video intensity is directly proportional to the strength of sound attenuation.\(^4^5\) Therefore the enhancement of the video intensity in in vitro ultrasonography results from the higher sound attenuation provided by MNCs. The enhancement of the acoustic response resulting from the encapsulation of Fe\(_3\)O\(_4\) nanoparticles may provide a new approach to improve the enhancement of UCAs in ultrasound imaging.

For investigating the effect of Fe\(_3\)O\(_4\) nanoparticles on the acoustical properties of MNCs, the MNCs are water-filled to exclude the effect of gas so that the variation of acoustical characteristics of MNCs depends only on the Fe\(_3\)O\(_4\) content. It was reported that the Fe\(_3\)O\(_4\) nanoparticles embedded in the polymeric shell of microcapsules can enhance the contrast of ultrasound signals and that this may be due to the additional acoustic impedance provided by Fe\(_3\)O\(_4\) nanoparticles during ultrasound imaging.\(^1^5,4^6,4^7\) The unique characteristics of the present case are that the Fe\(_3\)O\(_4\) nanoparticles are located in the internal cavity instead of the shell and that the core is filled with water instead of gas. As disclosed by the sound attenuation spectra for NC0 and MNCs shown in the Figure S5 of SI, the sound attenuation is actually enhanced by the presence of Fe\(_3\)O\(_4\) nanoparticles embedded inside the nanocapsules. In addition, with increasing Fe\(_3\)O\(_4\) content, the acoustic attenuation increases accordingly and the resonance frequency shifts to a higher value.

Cytotoxicity of Composite Nanocapsules. For biomedical materials, low cell cytotoxicity is a prerequisite for their application. Although poly(lactic acid)-based polymers are generally biocompatible,\(^4^8\) the cytotoxicity of Fe\(_3\)O\(_4\)/MePEG-PLA composite MNCs nanocapsules were investigated by using various approaches. A classical MTT assay was performed for two metabolic cell cultures, NRK and BRL-3A cells, to evaluate the nanocapsules’ cytotoxicity. The cell viability obtained by the MTT assay was expressed as a fraction of viable cells and normalized to that of cells incubated without MNCs (blank.

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**Figure 6.** (a) T\(_2\)-weighted MR images of MNC5, MNC15, MNC30, MNC40, and MNP. (b) T\(_2\) relaxation rate (1/T\(_2\)) as a function of Fe concentration (mM), TE is 60 ms. (MNC5, ■; MNC15, ●; MNC30, ▲; MNC40, ▼; MNP, ◆).

**Figure 7.** In vitro ultrasonography of degassed normal saline, NC0, MNC5, MNC15, MNC30, and MNC40 with emission frequency of 4 and 11 MHz. The concentration of samples are all 5 mg/mL.
control). The results are shown in Figure 8. For NRK samples, as shown in Figure 8a, the viability of the cells incubated with both NC0 and MNC5 are usually close to 100% and even higher than 100% at higher nanocapsule concentrations, indicating that the NC0 nanocapsules show almost no cytotoxicity, and can even stimulate cell proliferation. As for BRL-3A cells shown in Figure 8b, the results are similar to that for NRK cells, which indicates that Fe3O4/MePEG-PLA nanocapsules impart little cytotoxic effects to metabolic cells.

A flow cytometry fluorescent-activated cell sorting technique (FACS) utilizes a laser beam that differentiates cells based on their size and density to determine the genotoxic potential of MNCs by examining the extent of DNA damage. By using DNA intercalating dyes, the cellular DNA content can be used to determine the proportion of cells undergoing apoptosis.49 The cytotoxicity of MNCs at various concentrations on NRK cells was evaluated by using FACS to detect their apoptosis via a fluorescein annexin-V-FITC/PI double labeling. The rates of apoptosis in the early stage (as defined in the SI) of NRK cells coincubated with 0, 25, 100, and 400 μg/mL MNCs nanocapsules are presented in Figure 9 (the original flow cytometry results for NRK cells are displayed in the SI), the results of which are a direct indicator of the cytotoxicity of MNCs.49 When compared with the control experiment (without coincubation with MNCs), MNCs did not show an effect on the apoptosis of NRK cells, with the rate of apoptosis being lower than 2%. Consistent with MTT results, the results of FACS shows that both NC0 and MNC5 have no obvious cytotoxic effects to NRK cells at the three concentrations examined.

### CONCLUSION
A novel “two-in-one” approach was developed to fabricate Fe3O4/MePEG-PLA nanocapsules with magnetic/ultrasonic dual responses; this approach is characterized by the combination of a double emulsification coupled with an interfacial coprecipitation. Specifically, magnetite nanoparticles were synthesized via the interfacial coprecipitation that proceeded on the W1/O interface during the first emulsification and the resultant magnetite nanoparticles with hydrophilic surfaces thus entered the W1 phase after their formation. The Fe3O4 nanoparticles encapsulated composite nanocapsules were fabricated upon the progress of the second emulsification and the evaporation of oil phase. The sizes, structures, as well as the maghemite contents of the resultant MNCs were investigated by various methods; it is found that the maghemite content of MNCs can be adjusted conveniently from 0% to 43% by controlling the dosage of the iron salts. In addition, the MNCs were confirmed to exhibit magnetic/ultrasonic dual responses and low cytotoxicity. These characteristics enable the MNCs to be used as a potential biomedical material for enhancing the contrast of MR/ultrasonic images.

### ASSOCIATED CONTENT
Supporting Information. Complementary TGA, acoustic attenuation, and FCM results of the composite nanocapsules. This information is available free of charge via the Internet at http://pubs.acs.org

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