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RESEARCH PAPER

A Magnetic Nanoparticle Stabilized Gas Containing Emulsion for Multimodal Imaging and Triggered Drug Release

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ABSTRACT

Purpose To develop a multimodal imaging guided and triggered drug delivery system based on a novel emulsion formulation composed of iron oxide nanoparticles, nanoscopic bubbles, and oil containing drugs.

Methods Iron oxide paramagnetic nanoparticles were synthesized and modified with surface conjugation of polyethylenimide (PEI) or Bovine Serum Albumin (BSA). Both particles were used to disperse and stabilize oil in water emulsions containing coumarin-6 as the model drug. Sulfur hexafluoride was introduced into the oil phase to form nanoscopic bubbles inside the emulsions. The resulted gas containing emulsions were evaluated for their magnetic resonance (MR) and ultrasound (US) imaging properties. The drug release profile triggered by ultrasound was also examined.

Results We have successfully prepared the highly integrated multi-component emulsion system using the surface modified iron oxide nanoparticles to stabilize the interfaces. The resulted structure had distinctive MR and US imaging properties. Upon application of ultrasound waves, the gas containing emulsion would burst and encapsulated drug could be released.

Conclusion The integrated emulsion formulation was multifunctional with paramagnetic, sono-responsive and drug-carrying characteristics, which may have potential applications for disease diagnosis and imaging guided drug release.

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INTRODUCTION

Recent advances in molecular engineering and nanotechnology have prompted the development of multifunctional agents those can be used for both diagnosis and treatment purposes (1). There was even a new term "theranostics" invented by the researchers to emphasize the potential efficiencies and collaborative benefits that may be enabled by these agents (2–5). But it has been quite a challenge to integrate multiple functions into a single system that's reproducible and well characterized. A lot of studies attempted co-encapsulating or chemically conjugating imaging agents and drugs in one structure (6–9). But these entities may compete with each other for space or surface area which could compromise individual function (10). And even more, such simple combination of functional entities may also cause addition of side effects if there was no coordination between the functions.

A more advanced and desirable theranostic approach is to combine diagnostic imaging with imaging directed drug delivery. So the two functions collaborate with each other. The imaging information can be used to direct and promote drug action to the diseased site and spare the normal tissues (11). Tai *et al.* encapsulated magnetic nanoparticles inside thermal sensitive liposomes and proposed to use MR imaging to track liposome distribution and magnetic field to trigger liposome release (12). Park *et al.* used the surface enhanced Raman scattering (SERS) probe gold nanorod (GNR) to visualize tumor as well as to generate the photothermal energy to trigger drug release (13). However, the most popular and practical release-triggering mechanism is believed to be the use of ultrasonic (US) waves (14). US wave can imply a thermogenic effect when focused on tissues, or they act on air/water interface to result in bubble captivation or bubble

burst (15,16). Both effects can be used to initiate drug release (17–20).

In our study, we would also like to take advantage of the US triggered drug release mechanism but at the same time to use MRI to direct and monitor the triggered drug release event. De Smet *et al.* reported the use of thermosensitive liposomes and high intensity focused ultrasound to heat up the tumor to facilitate drug release (21,22). But the liposomes and their distribution in the tumor were not visible under MR and the imaging was only used to monitor the temperature change. We, on the other hand, wanted to be able to observe the drug carrier distribution in order to determine the timing & location of US application. Therefore, we have tried to incorporate distinctive MR signature as well as US responsive property in one emulsion formulation: by using modified magnetic particles as stabilizer at water/oil or water/bubble interfaces.

MATERIALS AND METHODS

Materials

$FeCl_3$, $FeCl_2$, NaOH were all analytical pure, and purchased from Shanghai Chemistry Reagents Company. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), PEI25000 and BSA were purchased from Sigma. SF_6 gas was bought from China Honghua Special Gas Company.

The Preparation of PEI or BSA Conjugated Iron Oxide Nanoparticles

Iron oxide nanoparticles were first synthesized using the co-precipitation method in the presence of poly-lactic acids as described earlier (23). Briefly, $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ in 20 ml degassed water were mixed in the presence of polymerized lactic acid solution at the molar ratio of Fe^{3+} and Fe^{2+} at about 1.8 to 1. NaOH was then added dropwisely until the solution pH reached 11.0 for 30 min at 4°C. The solution was then heated up to 80°C for 60 min, cooled down, and centrifuged at 9,000 rpm twice for 10 min each. Large aggregates in the pellet were removed after centrifugation. The nanoparticles in the supernatant were collected and conjugated with PEI25K or BSA using EDC as the coupling agent. After the conjugation, the solution were dialyzed in a 100,000 MW dialysis bag for 4 h against ddH_2O. The resulted surface modified iron oxide nanoparticles were named MP-PEI and MP-BSA respectively and examined for their size distribution and surface potential using Malvern Zetasizer 3000.

The Preparation and Characterization of O/W Emulsion Stabilized by MP-BSA and MP-PEI Nanoparticles

The final oil in water emulsion was prepared by an inversion method which was found to be more reproducible. First, 100 μl of ddH_2O containing 20 μl of the modified nanoparticles were mixed with 900 μl of soybean oil containing coumarin-6 and dispersed into a w/o emulsion using a probe ultrasound set at 30% intensity. Then, we took 100 μl of this w/o emulsion and mixed it with 1,050 μl of water containing 150 μl of modified nanoparticles. The mixture was again dispersed in a batch sonicator (100 W) for 3–4 min. Microscope examination of the resulted emulsion showed an oil in water structure. The stability of these emulsions were assessed by measuring the settling (separation) time at room temperature after 100× dilution. Finally, for SF_6 containing emulsions, SF_6 was added during the second dispersion phase followed by 2–5 min of sonication. The resulted gas containing emulsion was also diluted up to 500 times to evaluate the emulsion stability. Again, the size distribution of the gas containing emulsions was examined using Malvern zetasizer 3000. Their morphologies were examined using light microscope and transmission electron microscope.

The MR Signal Properties of the Emulsions

The emulsions were diluted to different iron concentrations in ddH_2O and placed in 2 ml Eppendorf tubes. The test tubes were gently rotated to ensure uniformity of the suspension prior to MR measurement. They were then placed vertically in the scanner. T2 weighted images were obtained using a conventional spin-echo sequence (TR 8,000 ms, TE19 ms) using a 3 T MRI scanner from Siemens (TrioTim). The T2 relaxivity (R2) was calculated by least-squares fitting of the 1/relaxation time (s-1) *versus* iron concentration (mM Fe) curve.

The US Imaging of the Gas Containing Emulsions

The emulsions were placed in 2 ml Eppendorf tubes and imaged using a Siemens Acuson Sequioa512 scanner equipped with a 14.0 MHz ultrasound transducer. All images were acquired using the same instrument parameters (Mechanical Index (MI) = 0.63; gain = 8 dB). Series of images were taken at specific time intervals until all the bubble signals were diminished.

Characterization of the Drug Release Profile Triggered by Ultrasound

The gas containing emulsions were diluted and injected into 2,000 MW slide-A-lyzer dialysis cassettes. The cassettes were placed inside a beaker containing 100 ml of PBST solution

(containing 1% tween-20 and 0.2% acetone). The beaker was placed in the center of a bath sonicator and subject to ultrasound waved at different intensities (0, 40%, 70%, 100%, 200% and 300%) for 2 min or after different time (1 min, 2 min, 4 min, 6 min, 8 min, 10 min) under 100% intensity. The amount of released coumarin-6 was measured by fluorescence spectroscopy (excitation wavelength 456 nm, emission wavelength 604 nm) and calculated after the treatment.

MR Imaging of the Emulsion Distribution Inside the Liver in Rats

The distribution of MP and gas containing emulsion *in vivo* after tail vein iv injection was evaluated by MR imaging in three normal adult rats (with a body weight of about 200 g). The animal studies were performed according to the protocol approved by the Internal Review Board of Shanghai Jiaotong University. The rats were anesthetized with Amobarbital (3% in saline solution, 1 ml/kg) and their body temperature was maintained at 37°C. The emulsions stabilized by MP-BSA particles were diluted in 10% sucrose solution to a final iron concentration of 1.8 mM and injected into the tail vein. MR imaging of the liver were taken using a 10 cm circular surface coil in transmit/receive mode. Rats were all imaged in a prone position with their abdomen resting on bottom of the coil. Liver images were acquired on a 3 T MRI scanner from Siemens (TrioTim) using the T2 2D fast low-angle shot (FLASH) sequence with respiratory gating control. The parameters were TR/TE = 6,280 ms/145 ms, flip angle = 350°, FOV = 60 × 60 mm, slice thickness = 2 mm, NEX = 2, in-plane resolution = 0.78 × 0.78 mm^2, and temporal resolution = 21 s.

RESULTS

The Preparation of MP Stabilized "Pickering Emulsion" Formulations Containing SF_6

To prepare a multifunctional theranostic agent that can be used for imaging and imaging triggered drug release, it is desirable to incorporate several functional entities in one simple formulation without compromising their individual properties. So we propose to follow the concept of "pickering emulsion" and use iron oxide nanoparticles as double agents to not only provide the paramagnetic signal but also stabilize the oil/water and air/water interface.

In practice, however, we found that the original iron oxide nanoparticles we synthesized were too hydrophobic to stay at the interface. So we attempted to modify the surface with two different macromolecules, one positively charged (PEI) and the other negatively charged (BSA). Surprisingly, both MP-PEI and MP-BSA nanoparticles were able to generate stabilized emulsions without the help of any surfactant. But the emulsification method we developed was not as straight forward. We had to make a w/o emulsion first and then disperse again in water to convert it into an o/w status. Figure 1a and b depicted the two step process and the general morphology of the resulted emulsion. Based on the preparation mechanism and some preliminary EM investigation, we proposed a possible structure of the emulsion formulation as shown in Fig. 1c.

The biophysical characterizations of the two emulsions were listed in Table I. As expected, the MP-PEI stabilized emulsion droplets had positive zeta potentials >30 mv. The MP-BSA stabilized emulsion droplets had slightly negative zeta potentials. The sizes of these droplets were mostly at sub-micron range. After the introduction of SF_6, there was always a significant increase of droplet sizes, indicating the successful incorporation of air bubbles inside.

Furthermore, we used TEM to examine the detailed morphology of these sub-micron gas containing droplets and listed the representative pictures in Fig. 2. Being different from most organic particles, the MP stabilized droplets were quite strong under the electron beams. More strikingly, the addition of SF_6 made the droplets stood up with distinctive 3D structure features. In detail, we showed series of droplet morphologies before the addition of SF_6, and after the addition of SF_6. The droplets all contained iron oxide nanocrystals shown as black dots under EM. They were mostly located on the surface of droplets. The addition of SF_6 made the structure bigger and fuller, indicating the filling of gas inside.

The MR and US Imaging Properties of SF_6 Containing Emulsions

The emulsions were diluted using ddH_2O to different iron concentrations. Their T2 weighted images were taken using a Siemens 3 T MRI system. T2 relaxivities for both MP-PEI and MP-BSA emulsions were calculated based on these images (Fig. 3). The MP-PEI stabilized emulsion had a T2 relaxivity r2 = 109 Fe $mM^{-1}s^{-1}$, which is higher than the T2 relaxivity of MP-BSA stabilized emulsion (r2 = 28.2 Fe $mM^{-1}s^{-1}$).

The ultrasound images of the two emulsion formulations were shown in Fig. 4. The imaging mode was CPS MSK and transducer was set at 14.0 MHz. At the beginning of imaging experiments, both emulsions had strong echogenic signals as shown in Fig. 4a and c. But after a few minutes of continuous ultrasound treatment, the signal intensity gradually diminished, indicating the gas bubbles inside the droplets burst and disappeared. For the MP-PEI stabilized emulsion, such a process took about 10 min (Fig. 4b). But for the MP-BSA emulsion, most of the bubbles were gone after 3 min (Fig. 4d).

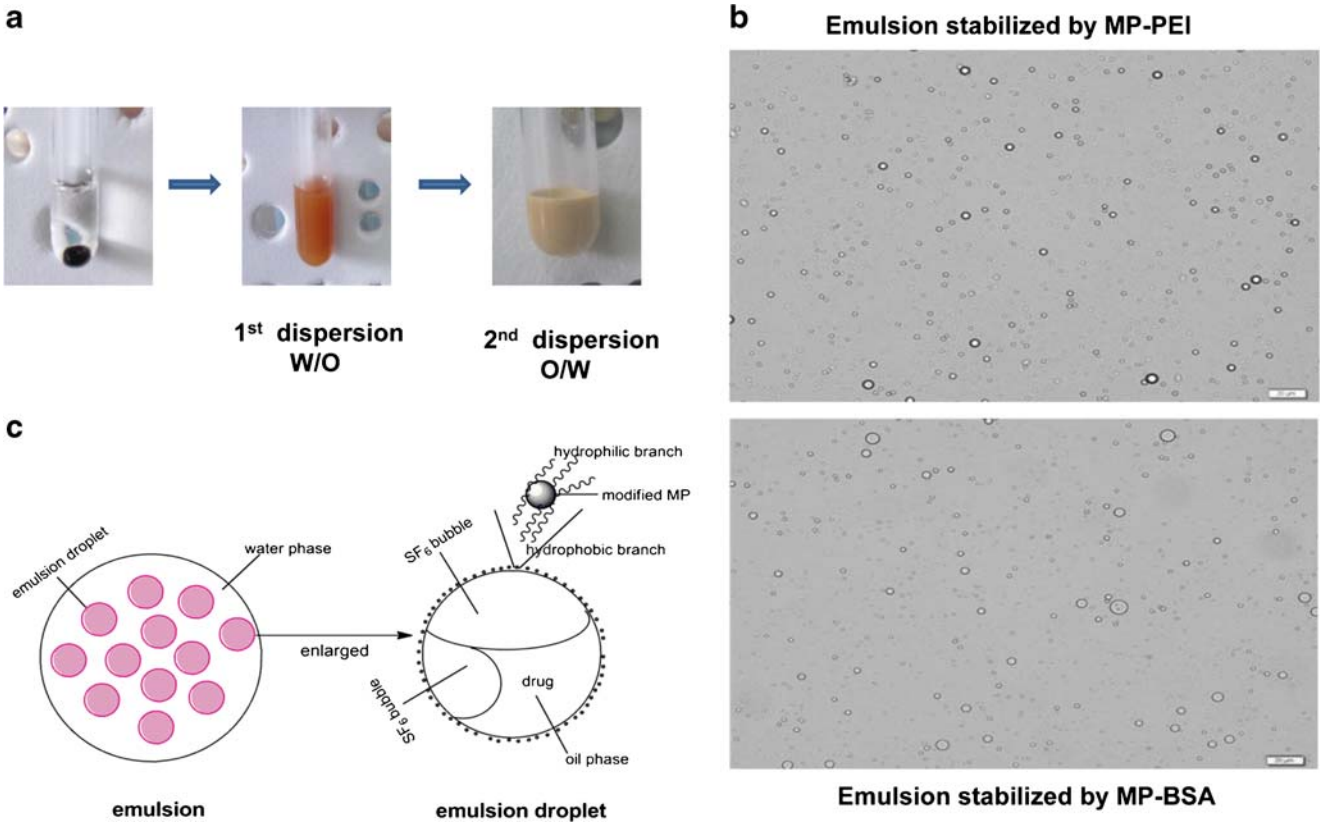

Fig. 1 Preparation of surface modified iron oxide nanoparticle stabilized "pickering emulsion" (*scale bar*: 20 μm). (**a**) The two step process of the emulsions formation. (**b**) The general appearance of the emulsion formulation under optic microscope. (**c**) The schematic drawing of the possible structure of the emulsion droplets.

Drug Release from the MP Stabilized Emulsions Triggered by Ultrasound Wave Application

The model drug Coumarin-6 was encapsulated in the oil phase inside the emulsion droplets. When they were exposed to ultrasound wave, the droplets would burst and the drug was released. Figure 5a plotted the fluorescence intensity of released drugs after treatments of ultrasound wave at different intensities. The data indicated that both emulsions had more drug release after the ultrasound treatments. The fluorescence intensities in the release buffer were proportional to the ultrasound intensity as well as illumination time. In contrary, the control emulsions with no gas added had almost no change of the background fluorescence.

We also detected the triggered drug release of gas-containing emulsions after different illumination time under 100% ultrasound intensity (Fig. 5b). The drugs were shown to release gradually from the MP-PEI emulsion for 6 min, and reached a plateau afterwards. The MP-BSA emulsion, however, released all the drugs within nearly 2 min. Such US responsive properties agreed with what was observed in the ultrasound imaging study very well.

In Vivo MR Imaging Effect of the MP Stabilized Emulsions

In vivo MR imaging studies were carried using rats. The MP stabilized emulsion formulations were injected intravenously and the T2-weighted images of the rat liver at different time

Table I Particle Size and Zeta Potential of the Formed Emulsions

	MP-PEI stabilized emulsion	MP-PEI stabilized emulsion containing SF_6	MP-BSA stabilized emulsion	MP-BSA stabilized emulsion containing SF_6
Particle size	483.7 nm	650.5 nm	802.9 nm	780.5 nm
PDI of the particle size	0.216	0.331	0.238	0.160
Zeta potential	46.3 mv	54.2 mv	−10.1 mv	−7.35 mv

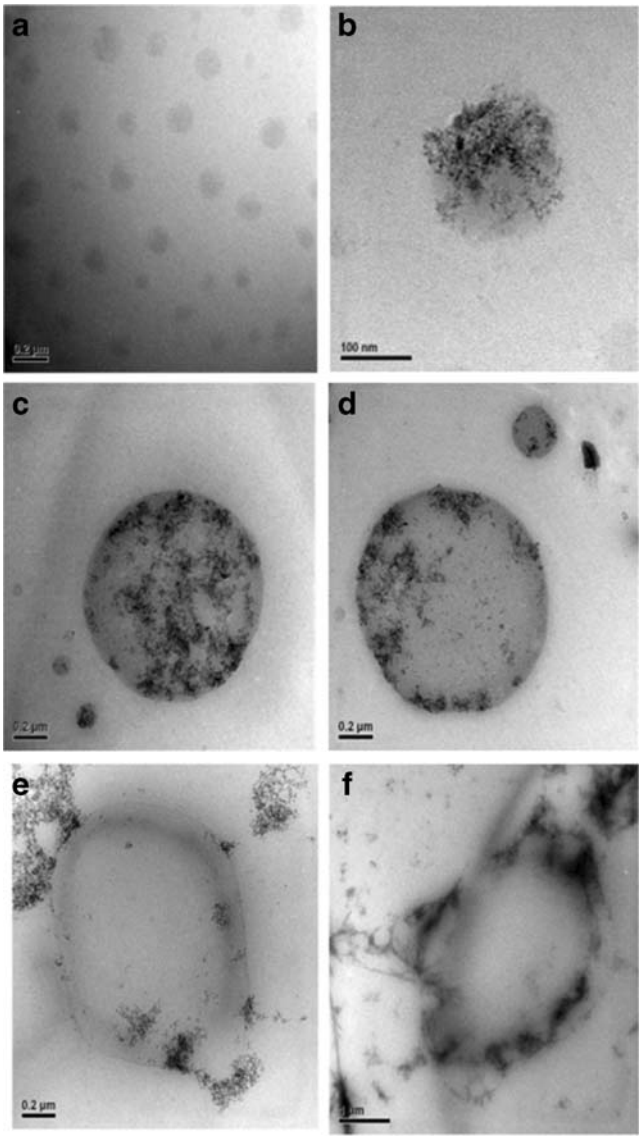

Fig. 2 The TEM micrographs of the MP stabilized emulsion droplets. (**a**) An overview of the MP-PEI stabilized emulsion formulation before the addition of SF_6. (**b**) A single MP-PEI stabilized emulsion droplet before the addition of SF_6. (**c** and **d**) MP-PEI stabilized emulsion droplet containing SF_6. (**e** and **f**) MP-BSA stabilized emulsion droplet containing SF_6.

points were shown in Fig. 6. After the MP-BSA stabilized emulsion injection, the liver tissues became considerably darker soon. We arbitrarily selected three ROIs as outlined by the circles and plotted the averaged signal intensities of the three ROIs at different time points after emulsion injection for each rat (Fig. 6b). All the three rats had significantly lower MR signals immediately after injection, indicating the effect of MPs inside the tissue. Such contrast effect could last for a long time to about 24 h after the injection (data not shown).

For the MP-PEI stabilized emulsion, however, there was significant *in vivo* toxicity, probably due to the toxicity of PEI which was well reported. The imaging study was discontinued after the first case of fatality of rats.

DISCUSSIONS

For the diagnosis of cancer and the monitoring of disease progression, imaging tools including Ultrasound, CT, MRI, and PET are essential. In order to improve imaging quality and reveal more detailed information about the disease, many studies have been tried to develop imaging agents with multiple imaging contrast capabilities to enable multi-modality imaging. But it's considered even more advantageous to combine functions such as diagnostic imaging and imaging directed therapy. Imaging agents such as magnetic nanocapsules and luminescent porous silicon nanoparticles were proposed and tested. But the structures were highly complicated which could limit the types and amounts of payloads they carry for therapeutical purposes. It was also difficult to modulate the drug release profile based on imaging mechanisms.

Recently, several studies have proposed formulations containing nanobubbles for combining ultrasound imaging and ultrasound triggered drug delivery. Different frequencies and intensities of ultrasonic waves may be used for imaging or generating mechanical or thermo effects to trigger drug release. But since both functions of imaging and drug release were based on essentially the same mechanism, it would be difficult to completely separate the two effects. The drug loaded nanoparticles may become unstable during the imaging process and lose the triggered release effect.

In this study, we tried to separate the two modalities and used MR for imaging drug distribution and ultrasound for promoting drug release. MR is considered among all the imaging modalities the least invasive and to have the best spatial resolution. But since the mechanisms of magnetic resonance imaging and ultrasound mechanical force are completely different, we developed a new formulation accommodating both mechanisms based on the concept of Pickering emulsion. Pickering emulsion is an old concept but attracted renewed interests recently (24–27). It was originally described as a method to stabilize microemulsions using solid particles. But nanoparticles were found to have similar effect. There have been many reports of possible applications (28–30). Nanoparticle based pickering emulsions were found to be highly stable with low toxic effects. In order to form pickering emulsions, the solid nanoparticles need to have specific size, shape and surface properties. Especially, the particle surfaces were required to have a fine balance of hydrophilicity and hydrophobicity. Particles such as silicate and metal oxides were used. Some surface modifications may also be required.

We tried to use iron oxide magnetic particles themselves to prepare pickering emulsions but failed. We tried to modify the particle surfaces with commonly used biopolymers.

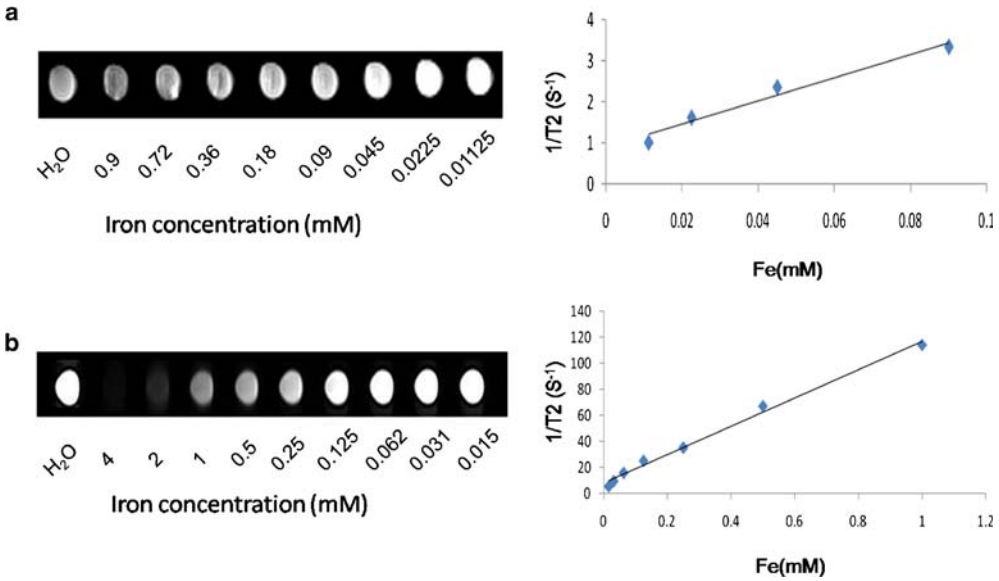

Fig. 3 T2-weighted MRI images (spin-etho sequence: TR = 8,000 ms, TE = 19 ms) of MP stabilized emulsions at a series of iron concentrations and the calculation of T2 relaxivity (1/T2). (**a**) MP-PEI stabilized emulsion; (**b**) MP-BSA stabilized emulsion.

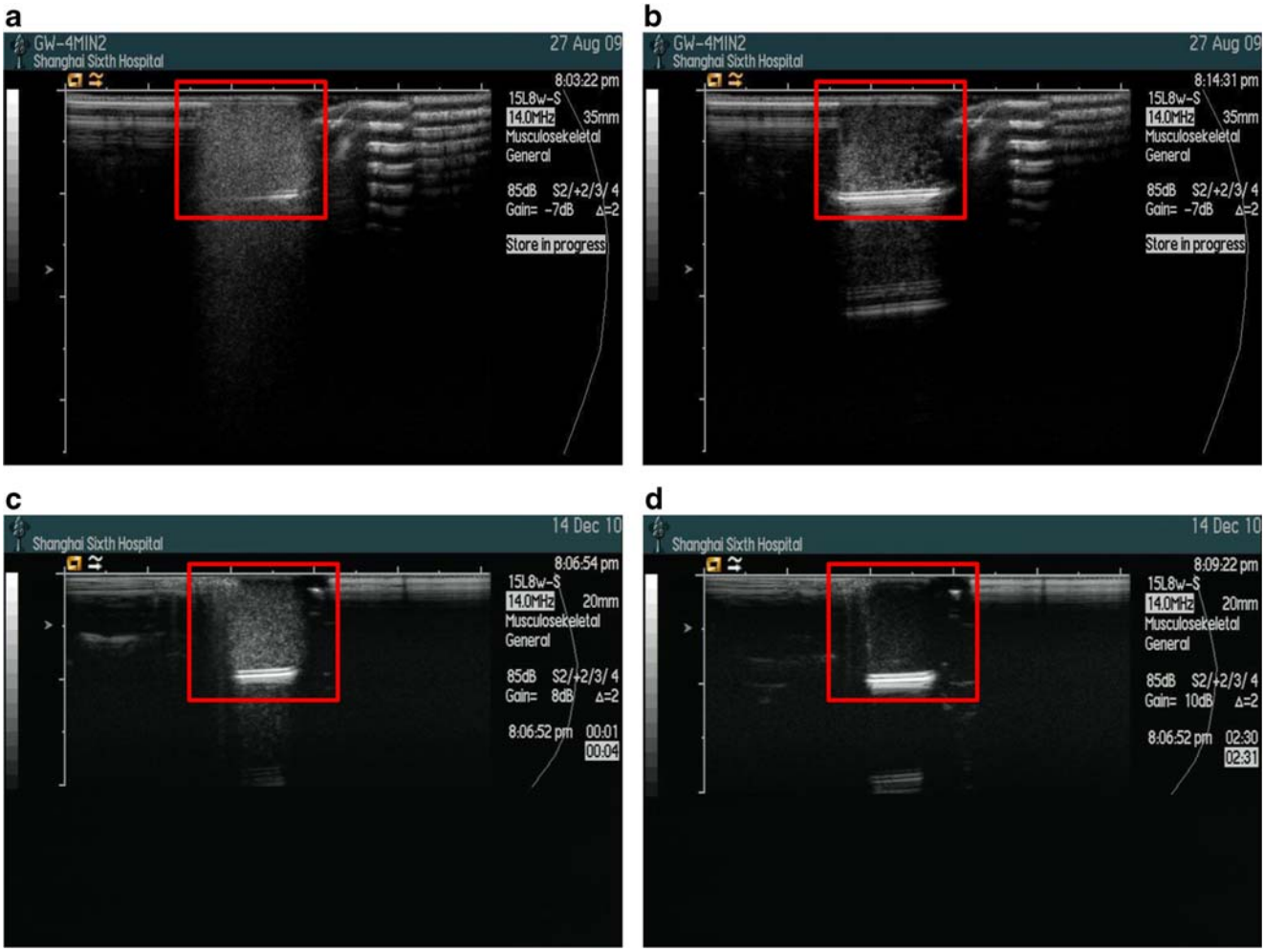

Fig. 4 US imaging of MP stabilized emulsions. (**a**–**b**): US images of MP-PEI stabilized emulsion at the beginning (**a**) and 10 min after continuous US illumination (**b**). (**c**–**d**) US images of MP-BSA stabilized emulsion at the beginning (**c**) and 2.5 min after continuous US illumination (**d**).

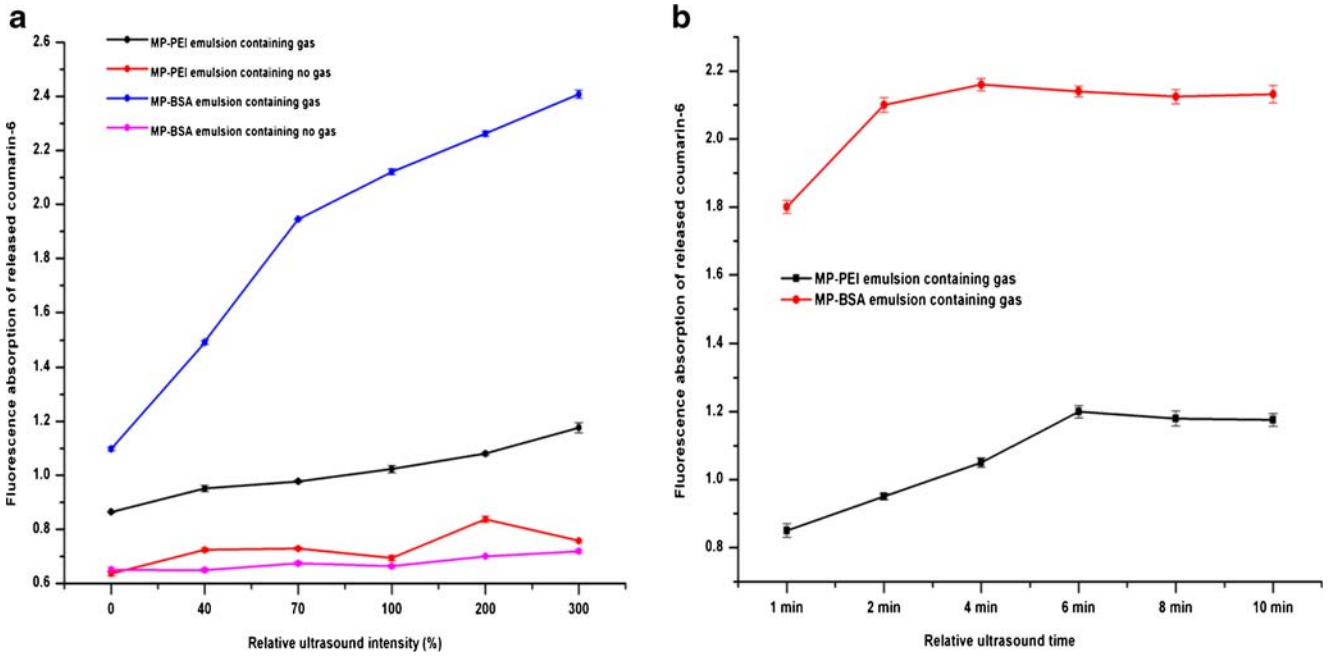

Fig. 5 *In vitro* coumarin-6 release from MP stabilized emulsions with or without encapsulated SF_6. (**a**) The release of coumarin-6 at different ultrasound intensities. (**b**) The release of coumarin-6 after different ultrasound illumination time.

Interestingly, both PEI and BSA modified particles were able to stabilize emulsions and emulsions containing SF_6. The SF_6 containing emulsions are highly stable even after being dried as shown in Fig. 2, which is not possible with other air bubbles

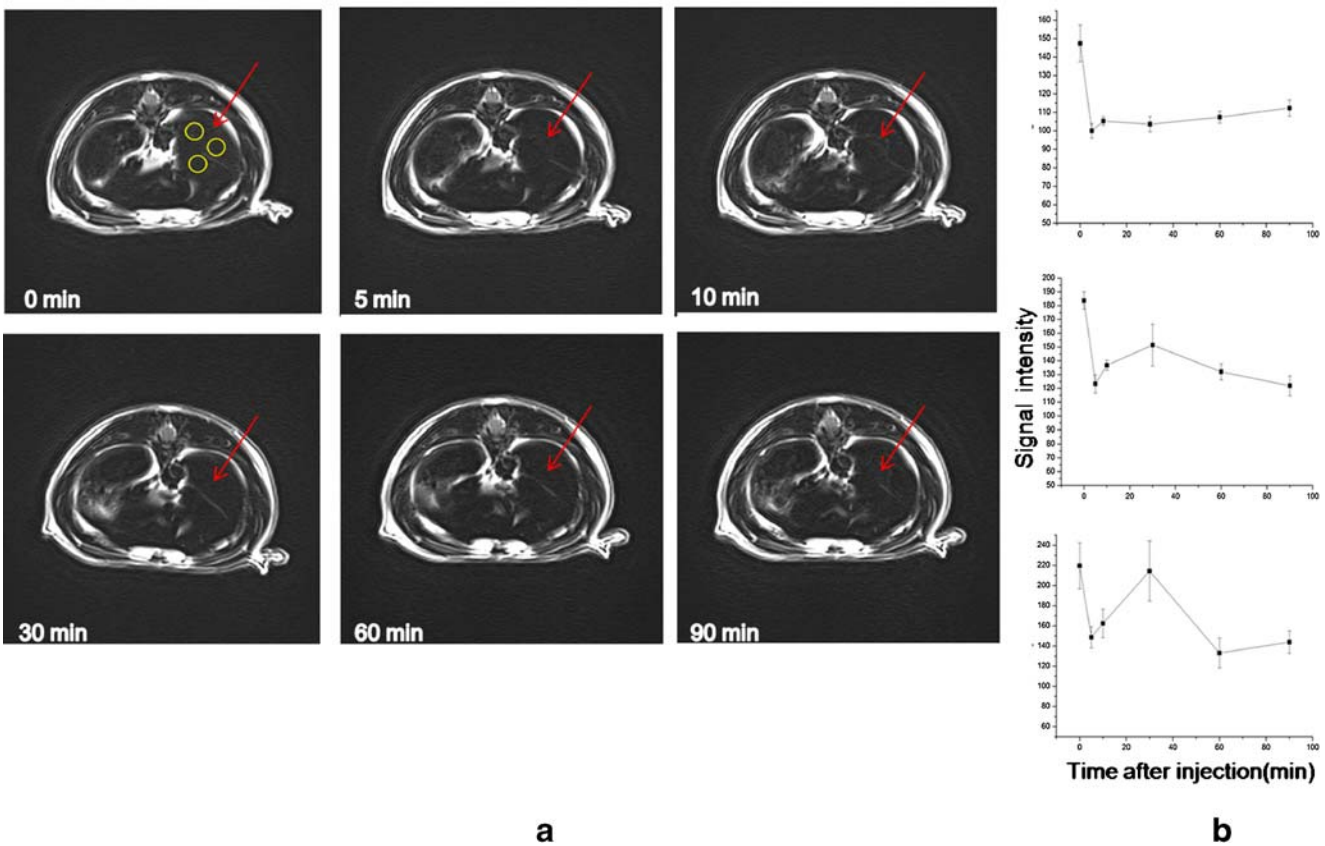

Fig. 6 *In vivo* MR images and the selected area signal intensities in liver tissues of the three rats studied at two adjacent slice locationsafter MP-BSA stabilized emulsion injection. (**a**) The MR images of the rat liver at two adjacent slice locations at different time points after injection for one rat. (**b**) The average signal intensities of three ROIs in the liver images for each rat at different time points after injection. The first image shows the locations of three ROIs.

stabilized by conventional surfactants. The MP-PEI stabilized emulsion was better dispersed and had better stability *in vitro*, because of the highly positive surface charge. Its T2 relaxivity was about 109 Fe $mM^{-1}s^{-1}$, which was also higher than the T2 relaxivity of MP-BSA stabilized emulsion (28.2 Fe $mM^{-1}s^{-1}$). But, because it's too stable, the ultrasound triggered drug release was not as complete as MP-BSA stabilized emulsions. In addition, because of the presence of PEI, it was toxic *in vivo*. Based on these experiences, we plan to test other biopolymers as surface modifier of the MP particles to achieve even better biocompatibility and safety.

To summarize, we developed a novel emulsion formulation containing encapsulated drugs as well as paramagnetic particles and nanobubbles. It has the potential to be used as a theranostic agent to enable MR imaging and US mediated drug release.

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