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Self-assembled microbubbles as contrast agents for ultrasound/magnetic resonance dual-modality imaging

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ABSTRACT

In this work, superparamagnetic self-assembled microbubbles (SAMBs) consisting of "Poly(acrylic acid)-Iron oxide nanoparticles-Polyamine" sandwich-like shells and tetradecafluorohexane cores were fabricated by a template-free self-assembly approach. The SAMBs exhibit not only magnetic resonance (MR) T_2 imaging functionality, but also ultrasound (US) image contrast, showing great potential as US/MR dual contrast agents. The diameters of the SAMBs can be tuned easily from 450 nm to 1300 nm by changing the precursor ratio, and this size variation directly affects their *in vitro* MRI and US signals. The SAMBs also exhibit *in vivo* contrast enhancement capabilities in rat liver with injection through portal vein, for both MR and US imaging. Additionally, the biodistribution of SAMBs over time suggests normal systemic metabolic activity through the spleen. The results show that the Fe content in rat liver reduces to a level of which Fe cannot be detected in 45 days. The SAMBs exhibit no obvious damage to the primary organs of rat during the metabolic process, indicating their good biocompatibility *in vivo*.

1. Introduction

Iron oxide nanoparticles (IONPs) act as T_2-weighted magnetic resonance imaging (MRI) contrast agents based on their superparamagnetic properties and have great potential in biomedical applications [1,2]. The contrast enhancement provided by IONPs contrast agents is due to the acceleration on the transverse relaxation of the water protons, caused by their interaction with the neighboring contrast agents. This results in a T_2 and T_2^* shortening. Previous studies have revealed that the T_2^* relaxivity, r_2^*, strongly depends on the volume magnetization of the particle and the radius of the particle [3–6]. Hence, embedding IONPs into microcapsules will essentially affect T_2 and T_2^* which are related to the spherical structure of the cluster and to the magnetic field distribution around it. Based on the IONP-conjugated microcapsule structure, microbubbles have been proven to endow the composites with unique sonic properties [7]. For better visualization of specific tissues, microbubbles and perfluorocarbon emulsions of various formulations have been developed and applied clinically as US contrast agents. However, they have suffered from instability due to ultrasonic pressures, broad size distributions, and poor circulating acoustic contrast properties [8,9]. Inorganic nanoparticle/polymer composites and inorganic-material based US contrast agents have recently attracted great attention due to their tunable particle diameters, good compatibility and superior stability over that provided by traditional organic microbubbles under US exposure [10–12]. Meanwhile, IONP-conjugated microbubbles behave as MRI/US dual-modal contrast agents, thus providing more precise diagnostic molecular imaging that combines the susceptibility of US imaging and the exquisite soft tissue contrast provided by the MRI modality [13,14].

Dual-modal contrast agents for T_2-weighted MR and US imaging can be categorized into two general classes, including IONP-conjugated microbubbles bearing lipid shells [13] and polymer shells [7,10,15–18]. IONP-conjugated microbubbles bearing lipid shells have certain limitations that arise not only due to the tedious synthetic procedure *via* the thin film hydration method required for their preparation, but also because of their instability that causes them to be potentially toxic [13]. In comparison with magnetic lipid microbubbles, IONP-conjugated microbubbles bearing biodegradable polymer shells are provided with physical rigidity and may be loaded with drugs or other cargos. The

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biocompatible outer coating provides a biological interface and a potential "scaffold" for targeting species. The only reported method to fabricate IONP-conjugated polymer microbubbles is through the double emulsion method, which builds a platform for dual-mode MRI/US contrast agents [7,10,15–18]. However, only hydrophobic polymers can be selected to form the bubble's shell during the double emulsion process, and the removal of the residual emulsifier is another concern during the synthesis. Hence, it remains a challenge to develop a facile method to fabricate a multifunctional contrast agent for dual MR/US imaging.

The self-assembly approach is a widely used method to fabricate nanoparticle/polymer composite microcapsules with diverse compositions and sizes [19–22]. The self-assembly method can be divided into template-based self-assembly and template-free self-assembly strategies. In particular, the layer-by-layer (LBL) self-assembly method is a well-established template-based method for constructing superparamagnetic IONP-based microcapsules [5]. However, the LBL self-assembly method involves a time-consuming process to form a template with a fixed size and also requires the subsequent removal of the template to generate hollow capsules. Recently, a novel self-assembly method known as the polyamine–salt aggregate (PSA) assembly method was proposed by Wong et al. [23]. This self-assembly route involves the formation of polymer aggregates and the subsequent deposition of particles around these aggregates to form microcapsules. The entire process is based on electrostatic interactions between oppositely charged components. In addition to providing a simplified procedure compared to the LBL method, the PSA approach can also be applied to encapsulate various agents and the shell thickness can be conveniently tuned [3,24–29]. These features provide the PSA assembly strategy with advantages in fabricating nanoparticle-conjugated microbubbles.

In this study, we developed a novel self-assembly approach to fabricate a multifunctional iron oxide nanoparticle-conjugated microbubble as a contrast agent for dual-modality MR/US imaging. The self-assembled microbubble has a unique triple layered shell constructed by "Poly(acrylic acid)-IONPs-Polyamine" composites. The MR and US imaging properties of self-assembled microbubbles were investigated both *in vitro* and *in vivo*. Additionally, metabolic investigations and histological evaluations were performed to characterize the biocompatibility of the microbubbles.

2. Materials and methods

2.1. Materials

Anhydrous ferric chloride, anhydrous ethanol, N,-N-dimethyl formamide (DMF), sodium oleate, n-hexane, acetone, 1,2-dichlorobenzene, toluene, dimethyl sulfoxide (DMSO), citric acid monohydrate, trisodium citrate dehydrate, fluorescein sodium salt and glacial acetic acid (AR) were purchased from Sinopharm Chemical Reagent Co., Ltd. Oleic acid (OA, 90%), 1-octadecene (ODE) (90%), a poly(allylamine) solution (M_w = 65000, 20 wt.% in water), poly(acrylic acid) (PAA, M_w = 1800), tetradecafluorohexane (C_6F_{14}), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 99%, AT) and S-acetylthioglycolic acid *N*-hydroxysuccinimide ester (NHS, 95%, TLC) were purchased from Sigma–Aldrich.

2.2. Preparation of the self-assembled microbubbles (SAMBs)

2.2.1. Preparation of the citrate-capped IONPs

The preparation of the oleic acid-capped IONPs (OA-capped IONPs) was performed by using a previously reported thermal decomposition method [30]. Generally, the iron-oleate complex was prepared by reacting iron chloride ($FeCl_3 \cdot 6H_2O$, 40 mmol) with sodium oleate (120 mmol). Subsequently, the reaction mixture containing the iron-oleate complex, oleic acid and 1-octadecene was heated to 320 °C at a constant heating rate of 3.3 °C min^{-1} and kept at that temperature for 30 min. The synthesized IONPs were then cooled to room temperature, washed with acetone and n-hexane, and separated by centrifugation.

To synthesize water soluble IONPs, ligand exchange reactions [31] were performed to obtain citrate-capped IONPs (CA-capped IONPs). OA-capped IONPs were dispersed into a 50/50 mixture of 1,2-dichlorobenzene and DMF (with a total volume of 15 mL), to which 0.1 g of citric acid was added. The mixture was then stirred at 100 °C for 48 h. The particles were subsequently precipitated by the addition of ethyl ether (40 mL) and recovered by means of a magnet. The particles were redispersed into ethanol and were reprecipitated by means of a magnet three to four times to remove all traces of free citric acid.

2.2.2. Preparation of the self-assembled microcapsules (SAMCs)

2.2.2.1. Preparation of the SAMCs. In a typical preparation, a 2.5 mL poly(allylamine) solution (0.05 mg/mL) was gently mixed with a 7.5 mL trisodium citrate solution ($Na_3Cit \cdot 2H_2O$, 3.47 mg/mL) for 2 min. This solution was then kept still for 2 h. The CA-capped IONPs (0.50 mg/mL, 2.5 mL) were added to the polymer/salt solution to form IONP-deposited spheres. The mixture was then sonicated for 30 min. Subsequently, 1.8 mL of an aqueous poly(acrylic acid) (PAA) solution (5.26 mg/mL) was added to the colloidal dispersion. After 10 min of vibration under ultrasound, the as-formed SAMCs were washed with deionized water and separated by centrifugation. The poly(allylamine) precursor concentration was varied from 0.01 to 0.10 mg/mL to obtain various microcapsules denoted as $SAMC_{0.01}$, $SAMC_{0.05}$ and $SAMC_{0.10}$, as shown in Table 1.

2.2.2.2. Preparation of the fluorescein-labeled polymers and the fluorescent SAMCs. The amino-groups of the poly(allylamine) chains were partially reacted with fluorescein sodium salt to enhance the visibility of these chains and thus determine their location within the products. During the preparation, 1 mL of fluorescein sodium aqueous solution (6.72 mg/mL) was mixed with a 4 mL aqueous EDC solution (25 mg/mL) before the mixture was sonicated for 2 min. Subsequently, a 5 mL aqueous NHS solution (40 mg/mL) was added into the mixture and sonicated for 5 min. A 10 mL aqueous poly(allylamine) solution (1 mg/mL) was then added into the mixture solution and stirred for 12 h. After this reaction, the solution was dialyzed for three days and collected in a brown bottle. All of these steps were performed in the dark.

The poly(acrylic acid) (PAA) chains were partially reacted with Cyanine 5 amine (Cy5) to help observe these chains and thus determine their location within the products. During the preparation, 10 mL of an aqueous PAA solution (10.52 mg/mL) was mixed with a 4 mL aqueous EDC solution (25 mg/mL) before the mixture was sonicated for 2 min. Subsequently, a 5 mL aqueous NHS solution (40 mg/mL) was added into the mixture and sonicated for 5 min. A 1 mL aqueous Cy5 solution (50 μg/mL) was then added into the

Table 1
Self-assembled microcapsules prepared with various concentrations of poly(allylamine) solution.

Sample	[Poly(allylamine)] (mg/mL)	[Cit][a] (mg/mL)	[IONP] (mg/mL)	[PAA] (mg/mL)
$SAMC_{0.01}$	0.01	3.47	0.50	5.26
$SAMC_{0.05}$	0.05	3.47	0.50	5.26
$SAMC_{0.10}$	0.10	3.47	0.50	5.26

[a] Trisodium citrate dehydrate.

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