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Characterization of mechanical properties of hybrid contrast agents by combining atomic force microscopy with acoustic/optic assessments

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ABSTRACT

Multi-parameter fitting algorithms, which are currently used for the characterization of coated-bubbles, inevitably introduce uncertainty into the results. Therefore, a better technique that can accurately determine the microbubbles' mechanical properties is urgently needed. A comprehensive technology combining atomic force microscopy, optical, and acoustic measurements with simulations of coated-bubble dynamics was developed. Using this technique, the mechanical parameters (size distribution, shell thickness, elasticity, and viscosity) of hybrid (ultrasound/magnetic-resonance-imaging) contrast microbubbles and their structure–property relationship were determined. The measurements indicate that when more superparamagnetic iron oxide nanoparticles are embedded in the microbubbles' shells, their mean diameter and effective viscosity increase, and their elastic modulus decreases. This reduces the microbubbles' resonance frequency and thus enhances acoustic scattering and attenuation effects.

1. Introduction

Ultrasound (US)/magnetic resonance imaging (MRI)-guided diagnosis/therapy has been regarded as one of the most promising non-invasive protocols in various strategies. To improve the contrast sensitivity of US and MRI, contrast agents have been used to enhance the acoustic backscattering signals (Postema and Schmitz, 2006; Cosgrove, 2003) or shorten the relaxation times of MRI signals (Michalet et al., 2005; Sandhu et al., 2010). With the increasing demands for continuous clinical improvement of imaging resolution, speed, penetration depth and safety, dual-mode hybrid US/MRI contrast agents have attracted growing interest in medical and scientific communities (Louie, 2010; Jin et al., 2010; Baker, 2010; Sailor and Park, 2012; Huang et al., 2011). Many efforts have been made to integrate superparamagnetic iron oxide nanoparticles (SPIOs) with ultrasound contrast agent (UCA) microbubbles (MBs) (Sailor and Park, 2012; Park et al., 2010; Niu et al., 2013; Chow et al., 2010). However, accurate characterization of the microbubbles' mechanical properties has been recognized to be rather challenging. As reported, the embedding of SPIOs into MB shells can directly change the mechanical properties of hybrid MBs (e.g., MBs' size, shell thickness, elasticity and viscosity), which in turn strongly affects the MBs' dynamic properties (e.g, resonance frequency, sub-harmonic/harmonic responses, acoustic absorption coefficient, and inertial cavitation threshold) and clinical performance (Borden et al., 2005; Hoff et al., 2000; Chitnis et al., 2013; Fouan et al., 2014; Guo et al., 2013). In previous work, the microbubbles' mechanical properties were usually estimated by fitting measured dynamic response signals with coated-bubble dynamic models (Hoff et al., 1996; Hoff et al., 2000; Guo et al., 2013; Goertz et al., 2007). However, since there is usually more than one shell parameter included in the model, multi-parameter fitting algorithms inevitably introduce uncertainty in the fitting results, making it difficult to determine the parameters accurately.

Our previous work has verified that a type of hybrid US/MRI contrast agent MBs can be fabricated by coating SPIOs to albumin-shelled perfluorocarbon MBs (Guo et al., 2014). Here, a comprehensive technology was proposed to characterize the mechanical properties of hybrid MBs with improved accuracy. By combining techniques of atomic force microscopy (AFM), single particle

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optical sensing (SPOS), acoustic attenuation measurement and coated-bubble dynamics simulation, the size distribution, shell thickness, elasticity and viscosity of magnetic MBs were determined one by one without multi-parameter uncertainty being involved in the process. Moreover, systematic studies were performed to investigate the impact of SPIO concentration on the magnetic MBs' mechanical properties and acoustic responses. The results provide a better understanding of the structure–property relationship of hybrid contrast agent MBs, which will benefit advanced design of multifunctional contrast agents by bridging the gap in understanding between their synthesis and application-relevant performance.

2. Materials and methods

2.1. Chemicals and materials

$FeCl_3 \cdot 6H_2O$ (1.0 mol L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.5 mol L^{-1}), $NH_3 \cdot H_2O$ solution, Oleic acid (OA), meso-2,3-Dimercaptosuccinic acid (DMSA), phosphate buffer saline (PBS), ethanol, acetone, sucrose and the bovine serum albumin were bought from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China. The C_3F_8 gas was bought from Nanjing Special Gas Co., LTD, China (purity 99.98%; molecular weight 188.02 g/mole).

2.2. Synthesis of perfluorocarbon-filled-albumin-SPIO microbubbles

A type of hybrid contrast agent was synthesized by loading SPIOs into albumin-shelled perfluorocarbon MBs (abbreviated as SPIO-albumin). The synthesis procedures included two primary steps: (1) synthesis of water-soluble SPIOs via surface double-change reaction between oleic acid and meso-2,3-dimercaptosuccinic acid; and (2) assembly of SPIO-albumin MBs using a sonication method.

In the first step, 100-ml aqueous solution of $FeCl_3 \cdot 6H_2O$ (1.0 mol/L) and $FeSO_4 \cdot 7H_2O$ (0.5 mol/L) was made to serve as a source of iron. Under a nitrogen atmosphere to prevent oxidation, concentrated $NH_3 \cdot H_2O$ was added into the iron-containing solution with a mechanical stirring at 70 °C, until the pH value of the solution reached 11.0. The reaction was continued for 5 min, with the temperature keeping at 70 °C. Then, 10-ml OA was slowly added into the alkaline solution. After a reaction of 30 min with stirring, the temperature was raised to 85 °C for 1 h, and then cooled down to room temperature. Black precipitates of monodisperse hydrophobic Fe_3O_4-OA nanoparticles were collected by using a permanent magnet and carefully washed 4–5 times with deionized water and ethanol. 240-mg obtained Fe_3O_4-OA and 120-mg DMSA were then dissolved in 120-ml mixed solution of acetone and hexane (volume ratio of 1:1). After stirring the mixed solution at 60 °C for 4 h, monodisperse water-soluble SPIO (viz., Fe_3O_4-DMSA) nanoparticles could be obtained via a surface double-exchange reaction. Through a magnetic separation procedure, SPIO nanoparticles were collected, washed 4–5 times with deionized water, and vacuum-dried. For further purification, the dried SPIO nanoparticles were re-suspended in deionized water at a concentration of 4 mg/ml and sonicated for 60 min in an ultrasound bath. After a pH neutralization process, the solution was dialyzed for 3 days to remove excess impurities. The final sample was filtrated through a 0.22-μm membrane and stored at 4 °C. In the second step, SPIO-albumin MBs were synthesized by using a sonicating method similar to that reported by Porter et al. (1995). In brief, 10% bovine serum albumin and 60% sucrose were mixed with a volume ratio of 1:1 in deionized water. A certain amount of SPIO nanoparticles was then added into the mixed solution with a gentle stirring for 30 min. Sitting in a perfluorocarbon (C_3F_8) environment overnight, the mixed solution was sonicated with a commercial ultrasonic processor (Sonics VCX750, Sonic & Materials Inc., Newtown, CT, USA). The ultrasonic processor worked at a frequency of 20 kHz with a maximum power (P_{max}) of 750 W. Ultrasound burst pulses with an on/off ratio 3:1 were applied to the solution for 2 min with an output intensity of 40% P_{max}. During the sonication process, a syringe pump was used to continuously inflate C_3F_8 gas into the solution at a speed of 7.5 ml/min. After sonication, the SPIO-albumin MBs were centrifuged at 800 rpm and then washed twice to eliminate free SPIO nanoparticles. Then, the suspension was stored at 4 °C for at least 4 h to allow the MBs to reach a relatively stable level. Finally the, upper layer of MB suspension was collected for the following assessments.

In order to confirm that SPIOs had been embedded in the albumin-shelled MBs, the diluted microbubble samples were applied to carbon-coated copper grids, fixed with 2.5% glutaraldehyde solution for 2 h at 4 °C, and then washed twice with PBS. Alcohol dehydration was followed in 33%, 50%, 60%, 80%, 90% and 100% ethanol for 20 min respectively. Imaging of microbubble shells was performed by the transmission electron microscopy (TEM, JEM-2100F, JEOL, Japan) at an accelerating voltage of 200 kV.

In order to investigate the structure–property relationship of magnetic MBs, SPIO-albumin MBs were divided into four groups with different SPIO concentrations. Before the sonication process, the surface tension coefficient of the mixed solution in each group was measured by using a BPA-800P bubble tensiometer (Biolin Scientific AB, Stockholm, Sweden). Then, after the formation of SPIO-albumin MBs, Ultraviolet Visible Spectrometry (UV3600, Shimadzu Co., Tokyo, Japan) was used to determine the SPIO concentrations in the groups by measuring the intensity of the light passing through the microbubble suspensions. The wavelength applied in these measurements was 510 nm.

2.3. Size distribution and concentration assessment

A proper size is a key factor for MBs to behave as an effective imaging/therapy agent that can safely pass through the pulmonary and vascular circulation while providing sensitive US and magnetic responses. Here, microbubble suspensions with various SIPO concentrations were diluted 500 times and assayed by Single Particle Optical Sizing (SPOS). Fifteen-ml samples were passed through the optical sensor (LE400-05) of an AccuSizer Model 780 SIS Syringe Injection Sampler (Particle Sizing Systems, Prot Richey, FL, USA) using a syringe pump. This yielded the size distribution and concentration for each of the SPIO-albumin MB samples.

2.4. Thickness measurement using atomic force microscopy

In previous studies, the thin shell material made it difficult to measure the MB shell thickness (d_s) directly. Here, the height image captured by a Multimode 8 AFM with a Nanoscope V controller (Bruker Corporation, Billerica, MA, USA) provided a feasible solution for this problem. Through a vacuum drying process, the gas inside the bubble was driven out to achieve a flat shell surface. AFM scanning was performed in the ScanAsyst-Air mode. The resonant frequency and nominal spring constant of the ScanAsyst-Air cantilever were 70 kHz and 0.4 N/m, respectively. To ensure the minimum force was applied on the MB's surface, the scanning rate was set to 0.5 Hz.

2.5. Assessments of microbubble stiffness based on AFM measurements

AFM has been shown to be capable of evaluating the stiffness of polymer- or phospholipid-shelled MBs (Sboros et al., 2006; Glynos et al., 2009; Buchner Santos et al., 2012; Chen et al., 2013; Cavalieri et al., 2013). Here, the stiffness of SPIO-albumin MBs was interrogated under AFM ScanAsyst-contact mode using tipless cantilevers with a ramp rate of 0.5 Hz. The diluted MBs were dropped on a mica substrate. Prior to measurements, the spring constant was calibrated on the mica plate. The nominal spring constant (k_c) and deflection sensitivity of the cantilever were calibrated to be 0.0228 N/m and 58.91 nm/V, respectively. A compression force (F) was applied to individual MBs through the cantilever. Then, the effective spring stiffness (k_m) of the bubble was assessed by measuring the deflection curve of the cantilever (viz., deflection error vs. z). The deflection error, $d = F/k_c$, represented the deflection distance if the cantilever contacted at hard surface, while z corresponded to the actual cantilever deflection. The effective deformation of the bubble was calculated as $dMB = z - d$. A linear regression fit was applied to the force-deformation (viz., F vs. dMB) curve to obtain k_m. The corresponding bulk modulus was calculated as $K_p = \frac{k_m(1-\nu)}{3(1-2\nu)d_s}$, where ν is Poisson's ratio. At least 3 force-deformation curves (producing no permanent deformation) were acquired for each bubble to insure experimental reproducibility. And for the cases at different SPIO concentration levels, at least 20 microbubbles were measured for each case to get an averaged bulk modulus.

2.6. Estimation of effective shell viscosity based on coated-bubble dynamics simulation

The measurement of acoustic attenuation spectra can give the information on a MB's resonance frequency and attenuation coefficient, which are dependent on the MB's size and visco-elastic properties (Hoff et al., 2000; Guo et al., 2013; Goertz et al., 2007). Thus, as long as the MB's size distribution, shell thickness and elasticity have been characterized based on SPOS and AFM methods, the effective viscosity can be evaluated by applying one-parameter fitting to the measured acoustic attenuation spectrum. Hoff's model (Hoff et al., 1996, 2000) is adopted here to evaluate the effective shell viscosity of SPIO-albumin MBs.

$$\rho_L R\ddot{R} + \frac{3}{2}\rho_L \dot{R}^2 = P_0\left(\frac{R_0}{R}\right)^{3\kappa} - 4\eta_L\frac{\dot{R}}{R} - 3\mu_p\frac{R_0^3\dot{R}}{R^3 R} - 3(K_p - \kappa P_0)\frac{R_0^3}{R^3}\left(1 - \frac{R_0}{R}\right) - P_0 - P_{drive}(t) \quad (1)$$

where R_0 is the ambient bubble radius; κ is the gas polytropic exponent; ρ_L and η_L are liquid density and shear viscosity, respectively; the MB's bulk modulus $K_P = \kappa P_0 + 4G_s\frac{d_s}{R_0}$ and G_s is the MB's shell shear modulus; the MB's bulk viscosity $\mu_P = 4\mu_s\frac{d_s}{R_0}$ and μ_s is the MB's shell shear viscosity; P_0 and P_{drive} are the ambient and acoustic driving pressures, respectively. The MB's extinction cross section is

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