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Balancing stealth and echogenic properties in an ultrasound contrast agent with drug delivery potential

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ABSTRACT

Contrast agents are currently being modified to combine diagnostic and therapeutic capabilities. For ultrasound (US) imaging with polymeric contrast agents, it is necessary to modify the shell to create "stealth" microbubbles but without these modifications sacrificing the agent's ability to interact with the focused US beam. We hypothesize that addition of the classic immune shielding molecule polyethylene glycol (PEG) to a polylactide (PLA) microbubble shell will affect the acoustic and physical properties of the resulting agents. In an effort to determine the best formulation to achieve a balance between stealth and acoustic activity, we compared two PEGylation techniques; addition of increasing amounts of PEG-PLA copolymer and employing incorporation of a PEG lipid (LipidPEG) into the shell. Loss of acoustic enhancement occurred in a dose-dependent manner for both types of PEGylated agents (loss of signal occurred at >5 wt% PEG-PLA and >1 wt% LipidPEG), while immune activation was also reduced in a dose-dependent manner for the PEG-PLA agents. This study shows that the balance between acoustic behavior and improved immune avoidance was scalable and successful to different degrees with both PEGylation methods, and was best achieved using for PEG-PLA at 5 wt% and for LipidPEG at 1 wt%. Studies are ongoing to evaluate the best method for the targeting and drug delivery capabilities of these agents for applications in cancer treatment. This study represents the basis for understanding the consequences of making modifications to the native polymeric shell.

1. Introduction

Ultrasound contrast agents consist of micron-sized stabilized gas bubbles (MB) small enough to freely transit the vascular system (<6 μm), but large enough to reflect ultrasound (US) and enhance sonographic images [1]. The stabilizing shell can encapsulate and protect a drug, and attached targeting ligands can help direct drug-loaded MB to the desired site [2]. However, the imaging capability of the MB is dependent on complex interactions among the shell, encapsulated gas, and the US beam [3]. A balance between acoustic and clinical requirements is crucial. In cancer applications, MB travel to tumor tissue through the vasculature, where rapid angiogenesis has led to development of leaky capillaries, with pores measuring from 100 to 780 nm in diameter [4]. An additional lack of lymphatic drainage and the presence of tumor-specific vasoactive factors promote accumulation of circulating nanoparticles in the tumor interstitium, termed the "Enhanced Permeability and Retention" (EPR) effect. EPR is being exploited for nanoparticle drug therapy [4–6], but reliance on EPR is limited by slow accumulation (up to 6 h), inconsistent degrees of tumor vessel permeability, and precluded entirely in some tumors that do not exhibit an EPR effect [7,8]. Investigations into methods that do not rely on the EPR effect are ongoing [9–11].

Our ongoing approach to improving delivery beyond reliance on the EPR effect is *in situ* generation of drug-loaded nanoparticles through the interaction of MB and US [12–17]. We have developed hollow, air-filled, poly(lactic acid) (PLA) MB using a double emulsion (w/o/w) process [12,14,18–20]. When insonated at a given frequency, MB will begin to cavitate (contract and expand in response to the fluctuating pressure wave). At elevated pressures (~0.1–2 MPa depending on shell and frequency) [21], they experience inertial cavitation, growing increasingly larger and smaller until they shatter. Inertial cavitation takes place at the resonant

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frequency of the bubble, a function of the bubble size and the material properties of the shell. Our MB are designed such that their resonant frequency is within the diagnostic imaging range (1 MHz to now up to 45 MHz), making them susceptible to inertial cavitation and eventual shattering into nano-sized fragments or shards (n-Sh). In a clinical setting, the n-Sh can pass through a 400 nm sized pore [13,17,22]. The MB are spheres approximately 1–2 μm in diameter, and show high echogenicity (approximately 20 dB enhancement) at low concentrations (i.e. 1.5 μg/50 mL) when evaluated *in vitro* [14,18,20]. The double emulsion (w/o/w) preparation method is versatile for encapsulation of drugs or other species for US-guided delivery, since species can be incorporated into either the organic or aqueous phase [12,15,17,23]. We have reported on encapsulation of doxorubicin (Dox) within the PLA shell, the presence of which has been visualized using confocal microscopy [12,14]. The MB produce n-Sh with an average size of 350 nm measured by dynamic light scattering [13,17,22]. This size would allow passage through leaky pores found in angiogenic tumor vessels (100–780 nm pore diameter) [4], especially if these pores are also enlarged by the incident US beam [24–26]. The radiation and cavitation forces produced by the US beam also work to propel these *in situ* n-Sh toward and through the leaky vascular pores. This produces effective delivery to a targeted area without reliance on the EPR effect [27–29].

Promising *in vivo* studies in a rat hepatocellular carcinoma model demonstrated extravasation potential [4] of these US-generated n-Sh in addition to effective delivery of Dox to tumor tissue, resulting in measureable tumor stasis [13]. However, elevated Dox levels were also observed in the spleen and healthy liver, indicating mononuclear phagocyte system (MPS) involvement [30–34]. Based on these results, we hypothesize that intact MB and/or n-Sh that were not taken up into the tumor were recognized by the immune system while circulating through the blood stream and thus accumulated in the spleen and healthy liver. We wish to develop agents that will be “invisible” to the MPS, not only protecting the spleen and liver, but also increasing the circulation time of MB and n-Sh for possible further accumulation in tumor tissue by subsequent use of the EPR effect after the initial uptake from cavitation-induced n-Sh. However, it is essential that these modifications do not adversely affect the shell properties, which would diminish the echogenicity of the MB and inhibit n-Sh formation. Before proceeding with further drug delivery application studies, we seek to develop “stealth” contrast agents that will shatter into equally “stealth” n-Sh.

Polyethylene glycol (PEG) is one of the most frequently used surface modifiers to shield agents from opsonization and the resulting immune recognition [32,33,35–41]. Using the w/o/w emulsion methods that we developed for the native MB, we chose to compare two surface PEGylation methods to reduce immunogenicity while maintaining excellent echogenic response. The study design was centered on our long-term goals of developing MB with both drug delivery and targeting potential. In one method, PEG-PLA co-polymer (PEG-PLA) was incorporated into the MB shell, and in the second method PEG phospholipids (LipidPEG) were incorporated; however in each case the MB shell remained mostly PLA. The latter method builds on a previous study where we were interested in attaching targeting ligands [42,43]. To date, PEGylated phospholipids have seen wide use with MB composed entirely of phospholipid, but not polymeric MB. Since one of our long-term goals is targeting, we investigated the ability to effectively anchor lipid chains into the polymer shell that also facilitate future conjugation of bioactive molecules for effective targeting. We explore and compare physical characteristics, acoustic consequences, and immunogenic properties of the two forms of PEGylated agents, to determine the method that produces MB that best evade immune recognition while also best retaining the native properties of the polymeric agent. Establishing these baselines will identify either or both agents as candidates for future drug delivery and targeting studies.

2. Materials and methods

2.1. MB preparation

MB were prepared by modifying the water/oil/water (w/o/w) double emulsion process that has been well-established previously in our lab, using camphor and ammonium carbamate as porogens [18]. For PEGylated MB using the co-polymer method, an aliquot of 0.5 g polymer was proportionally comprised of PEG-PLA (100 DL mPEG 5000 6CE, 67 mol% PLA, 33 mol% PEG, 69 kDa, Evonik Biomaterials, Essen, Germany) and PLA (100 DL 7E, 118 kDa, Evonik) by weight, increasing from 1 wt% to 15 wt% PEG-PLA. Of the 500 g total polymer mass, the proportion of PEG to PLA is as follows: 1 wt % PEG-PLA contains 1.60 g PEG to 498.40 g PLA (0.32% PEG to 99.68% PLA), 3.30 g (0.66%) PEG to 496.70 g (99.34%) PLA for 2 wt%, 8.25 g (1.65%) PEG to 491.75 g (98.35%) PLA for 5 wt%, 16.50 g (3.30%) PEG to 483.50 g (96.70%) PLA for 10 wt%, and 24.75 g (4.95%) PEG to 475.25 g (95.05%)PLA for 15 wt% PEG-PLA MB. For PEGylated MB using the LipidPEG method, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[amino(polyethylene glycol)2000] (Avanti Polar Lipids, Alabaster, AL) was used as received and the chloroform was evaporated off under a stream of nitrogen gas before adding the appropriate weight of PLA polymer and methylene chloride to begin the double emulsion process.

2.2. Acoustic characterization of MB

Dose and time response tests were performed in a custom-built acoustic testing system in our lab, using a 5 MHz, 12.7 mm diameter, single element ultrasound transducer (Panametrics, Waltham, MA) spherically focused at a length of 50.8 mm, 6 dB bandwidth of 91%, and a pulse length of 1 μs, as described previously [12,15,18,20]. To determine the cumulative dose response, 20 μL of the MB suspension were added to the sample vessel every 30 s and the acoustic signal was measured at each time point. Time response, or stability over time while circulating in the US beam, was measured by adding 40 μL of the MB suspension to the sample vessel with a fresh 50 mL of warmed PBS and the acoustic signal was measured every minute over a period of 15 min. The test was performed once at a lower mechanical index (0.69 MPa peak negative pressure) to determine the long-term stability within the US beam, and again at a higher mechanical index (1.6 MPa peak negative pressure) to assess the potential for *in situ* n-Sh generation. These tests were performed in triplicate, and the results reported as the average of these readings. Agents were also visualized with a clinical ACUSON S3000 Ultrasound System, HELX Evolution (Siemens Medical Solutions, Mountain View, CA) with a 9L probe operating in nonlinear contrast imaging mode. A concentration of 1.0×10^7 MB in 800 mL PBS was circulated through a tissue mimicking flow phantom (model 524, ATS Laboratories, Bridgeport, CT) with a 6 mm diameter vessel using a peristaltic pump at 350 mL/min. Contrast mode images were taken every 5 min post-injection over a 20 min period.

Resonant frequency of the MB resulting from PEGylation was measured using a pulse-echo setup with a custom-built sample holder, equipped with an acoustically transparent window and an air-backed metallic reflector, holding 250 mL PBS at 37 °C and stirred continuously. This sample holder was submerged in a DI water bath held at 37 °C, and an exchangeable single element, 12.7 mm diameter, unfocused transducer (Panametrics, Waltham,