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Leading Opinion

The effect of lipid monolayer in-plane rigidity on *in vivo* microbubble circulation persistence

Sumit Garg, Alex A. Thomas, Mark A. Borden*

Department of Mechanical Engineering, Materials Science and Engineering Program, University of Colorado, 1111 Engineering Drive, Boulder, CO 80309-0427, USA

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ABSTRACT

The goal of this study was to increase *in vivo* microbubble circulation persistence for applications in medical imaging and targeted drug delivery. Our approach was to investigate the effect of lipid monolayer in-plane rigidity to reduce the rate of microbubble dissolution, while holding constant the microbubble size, concentration and surface architecture. We first estimated the impact of acyl chain length of the main diacyl phosphatidylcholine (PC) lipid and inter-lipid distance on the cohesive surface energy and, based on these results, we hypothesized that microbubble stability and *in vivo* ultrasound contrast persistence would increase monotonically with increasing acyl chain length. We therefore measured microbubble *in vitro* stability to dilution with and without ultrasound exposure, as well as *in vivo* ultrasound contrast persistence. All measurements showed a sharp rise in stability between DPPC (C16:0) and DSPC (C18:0), which correlates to the wrinkling transition, signaling the onset of significant surface shear and gas permeation resistance, observed in prior single-bubble dissolution studies. Further evidence for the effect of the wrinkling transition came from an *in vitro* and *in vivo* stability comparison of microbubbles coated with pure DPPC with those of lung surfactant extract. Microbubble stability against dilution without ultrasound and *in vivo* ultrasound contrast persistence showed a monotonic increase with acyl chain length from DSPC to DBPC (C22:0). However, we also observed that stability dropped precipitously for all measurements on further increasing lipid acyl chain length from DBPC to DLiPC (C24:0). This result suggests that hydrophobic mismatch between the main PC lipid and the lipopolymer emulsifier, DSPE-PEG5000, may drive a less stable surface microstructure. Overall, these results support our general hypothesis of the role of in-plane rigidity for increasing the lifetime of microbubble circulation.

1. Introduction

Ultrasound (US) medical imaging is safe, inexpensive, portable, and offers excellent spatial resolution and real-time visualization, but its use for advanced diagnostic imaging is hampered by poor soft tissue contrast, which makes the images difficult to interpret. Tissue contrast can be improved by intravascular injection of microbubble ultrasound contrast agents [1], but their short circulation lifetime somewhat limits their broad use in clinical cardiology and radiology. In this study, we overcome this limitation as we significantly prolonged the circulation lifetime of microbubbles by making changes in the microbubble lipid shell composition.

Lipid-coated microbubbles meet two of the key performance criteria for ultrasound contrast agents: biocompatibility and strong ultrasonic backscatter [2–4]. They are spherical gas cores stabilized by polymer-grafted lipid monolayer shells. Lipids monolayers naturally occur in the human body, e.g. the alveolar lining, where they stabilize the gas/liquid interface by reducing surface tension and yet provide a flexible membrane that can adjust to area changes during the breathing cycle. Due to the gas core and soft shell, lipid-coated microbubbles can volumetrically oscillate under low-intensity US and appear bright on the video screen of the ultrasound scanner [5–7]. They have high ultrasonic backscatter because the intra-lipid interactions are not strong enough to significantly dampen the radial oscillations during acoustic stimulation. Despite these advantages, the major limitation is that the microbubble circulation lifetime in the vasculature is relatively short. For example, a bolus injection of Definity® (Lantheus Medical Imaging, Billerica, MA) typically provides only 3–5 min of ultrasound contrast [8].

* Corresponding author. Tel.: +303 492 7750; fax: +303 492 3498.
E-mail address: mark.borden@colorado.edu (M.A. Borden).

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There are two major mechanisms for microbubble clearance in the vasculature: phagocytosis and dissolution. Phagocytosis occurs through the mononuclear phagocyte system (MPS), and is believed to be mediated by the alternative (innate) complement pathway through fixation of protein fragment C3b (Fig. 1). Evidence for the role of phagocytosis has been reported from positron emission tomography (PET) imaging studies of microbubble pharmacokinetics and biodistribution [9,10], which have shown rapid uptake in the lungs, liver and spleen. To reduce complement activation and macrophage uptake, microbubbles are prepared with neutral surface charge [11], which reduces non-specific protein adsorption. In addition, polyethylene glycol (PEG) brushes are attached to the microbubble surface to provide steric hindrance against protein adsorption and complement fixation [2,12,13].

Despite these advances in engineering of the microbubble surface architecture and chemistry to reduce complement activation and phagocytosis, microbubble lifetime *in vivo* remains relatively short [12–15]. We therefore focus here on design modifications of the lipid shell to delay dissolution. Dissolution of microbubbles occurs as the gas core diffuses into the bloodstream and is eliminated from the body through the lungs [16]. The rate of dissolution has been significantly reduced by use of low-solubility perfluorocarbon gases [17–19]. In theory, the use of higher fluorocarbons can further impede dissolution, but in practice this approach is impossible because higher fluorocarbons are in the liquid state under physiological conditions. Therefore the limit of this approach has been reached, and an alternative strategy is needed. We hypothesize that modification of the microbubble shell can enhance the resistance to dissolution.

Our approach is focused on increasing the lateral cohesive forces between the phospholipid molecules to enhance microbubble shell gas permeation resistance and its stability against collapse [4,20–22]. Lipid packing density is affected by lipid acyl chain saturation and length. Unsaturated lipid has a kink (*cis* isomer) in the acyl chain and does not pack as well as the straight (*trans* isomer) saturated lipid. Clinical formulations of microbubbles already use saturated lipids with neutral headgroups. However, prior *in vivo* work has not given enough attention to the lipid acyl chain length, which can significantly enhance the attractive forces between the lipid molecules.

Research has shown that increasing the acyl chain length can have significant effects on the physiochemical behavior of lipid monolayers, and can prolong the time for dissolution of an individual lipid-coated microbubble in a degassed medium [20]. Longer chain lipids have been shown to have increased bending modulus [23] and degree of acyl chain order [24], and reduced lateral density fluctuations [25] owing to the enhanced van der Waals attraction. In addition, longer acyl chains can increase the lipid membrane thickness [26] and provide a thicker barrier for solute permeation. For monolayers on microbubbles, it has been demonstrated that longer chain lipids increase the surface yield shear and shear viscosity [27,28]. Microbubbles in these studies were seen to hold a free projection formed by micropipette aspiration, indicating a strong monolayer cohesion and in-plane rigidity that overcomes surface tension, which would tend to retract the projection. Interestingly, microbubbles coated with longer acyl chains show discontinuous dissolution, with wrinkling and spontaneous restoration of sphericity as the microbubble shrinks [4]. This observation of a “wrinkling transition”, a hallmark of in-plane rigidity, suggests that longer acyl chains on microbubbles enhance the resistance to gas permeation [29,30] and monolayer collapse [4], thereby slowing the dissolution process for individual microbubbles [4,14,20,29].

Although prior bench-top studies have shown greater microbubble stability for shells comprising longer chain lipids, the biomaterials paradigm has not yet been completed since the effect has not been demonstrated *in vivo*. We hypothesized that increasing the lipid acyl chain length would result in an increase in microbubble circulation persistence. Thus, our hypothesis directly links microbubble composition to biomedical performance. To test this hypothesis, we measured *in vitro* stability with and without ultrasound exposure for diluted microbubble ensembles encapsulated by phosphatidylcholine (PC) lipids with acyl chains ranging from 16 to 24 carbons, as well as the *in vivo* ultrasound contrast persistence in healthy, immune-competent mice following intravenous injection. PEG-lipid content was fixed to isolate the effects of PC acyl

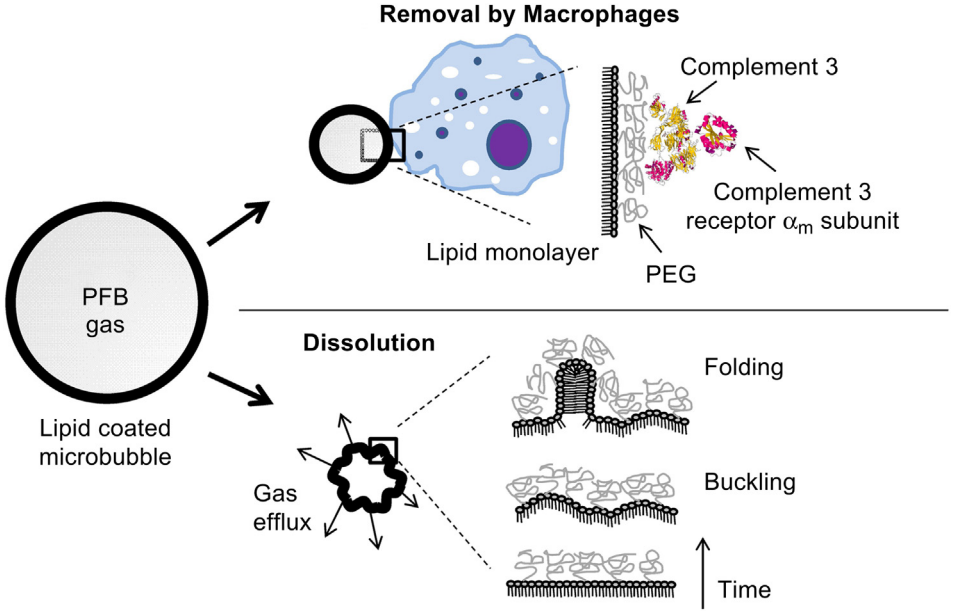

Fig. 1. Schematic representation of the two key mechanisms for removal of lipid-coated microbubbles from *in vivo* circulation. (Top) shows phagocytic removal by macrophages, which is mediated by complement C3b binding to the microbubble surface. (Bottom) shows dissolution into the bloodstream. Rigid lipid monolayers show wrinkling during dissolution owing to enhanced cohesiveness between the lipid constituents.