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● *Original Contribution*

ACCELERATED CLEARANCE OF ULTRASOUND CONTRAST AGENTS CONTAINING POLYETHYLENE GLYCOL IS ASSOCIATED WITH THE GENERATION OF ANTI-POLYETHYLENE GLYCOL ANTIBODIES

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Abstract—Emerging evidence suggests that the immune system can recognize polyethylene glycol (PEG), leading to the accelerated blood clearance (ABC) of PEGylated particles. Our aim here was to study the generation of anti-PEG immunity and changes in PEGylated microbubble pharmacokinetics during repeated contrast-enhanced ultrasound imaging in rats. We administered homemade PEGylated microbubbles multiple times over a 28-d period and observed dramatically accelerated clearance (4.2 × reduction in half-life), which was associated with robust anti-PEG IgM and anti-PEG IgG antibody production. Dosing animals with free PEG as a competition agent before homemade PEGylated microbubble administration significantly prolonged microbubble circulation, suggesting that ABC was largely driven by circulating anti-PEG antibodies. Experiments with U.S. Food and Drug Administration-approved Definity microbubbles similarly resulted in ABC and the generation of anti-PEG antibodies. Experiments repeated with non-PEGylated Optison microbubbles revealed a slight shift in clearance, indicating that immunologic factors beyond anti-PEG immunity may play a role in ABC, especially of non-PEGylated agents. (E-mail: padayton@email.unc.edu) **© 2018 Published by Elsevier Inc. on behalf of World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Microbubbles, Contrast-enhanced ultrasound, Accelerated blood clearance, Polyethylene glycol, Anti-PEG antibodies.**

INTRODUCTION

Polyethylene glycol (PEG) is used ubiquitously in biomedical research to enhance the *in vivo* stability and circulatory persistence of various particles. The high conformational flexibility and hydrophilicity of PEG make interactions with blood proteins energetically unfavorable (Salmaso and Caliceti 2013). Therefore, PEGylation creates a steric shield surrounding the particle, reducing opsonization and subsequent clearance by the reticuloendothelial system (Butcher et al. 2016). This approach has been used to endow therapeutic proteins (Hirotsu et al. 2017; Liu et al. 2011; Nie et al. 2017), drug-carrying nanoparticles (Haynes and Huang 2016; Liu et al. 2017; Salmaso and Caliceti 2013) and imaging contrast agents (Borden et al. 2006; Garg et al. 2013; Malinge et al. 2017; Zhou et al. 2016) with improved pharmacokinetics, and several of these agents have been translated to clinical use (Appis et al. 2015; Marchal et al. 2015).

Many ultrasound contrast agents (microbubbles) are formulated with PEG to stabilize their phospholipid shell (Abou-Saleh et al. 2014; Martin and Dayton 2013). PEG-mediated stabilization is typically achieved through use of PEGylated surfactants (*e.g.,* polyethylene glycol stearate) or PEGylated phospholipids (Abou-Saleh et al. 2014; Borden et al. 2004; Paefgen et al. 2015). In both cases, the PEGylated molecules incorporate into the microbubble monolayer shell and stabilize the particles against coalescence with other microbubbles and interaction with blood plasma proteins (Abou-Saleh et al. 2014; Borden et al. 2004). Inclusion of these PEGylated molecules has been reported to drastically enhance microbubble stability *in vitro*, prolonging formulation lifetime from approximately 13 min (without PEGylated lipids) to 60 min (with 5%

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PEGylated lipids) (Abou-Saleh et al. 2014). Two of the three U.S. Food and Drug Administration (FDA)-approved microbubble formulations contain PEG. Lumason®/SonoVue® contains PEG-4000 as a stabilizer in the suspending medium (Lumason package insert 2014), and Definity® contains PEGylated phospholipid as a shell component (Definity package insert 2011). The third FDA-approved formulation, Optison™, is stabilized by a human albumin-based shell and does not contain PEG (Optison package insert 2012).

Paradoxically, the immune system can generate specific antibodies that bind to PEG, the molecule originally exploited for its resistance to protein absorption and immunologic shielding (Abu Lila et al. 2013; Yang and Lai 2015). In rodents, anti-PEG immunity is characterized by robust but transient production of anti-PEG immunoglobulin M (IgM), which peaks in concentration approximately 1 wk after the initial dose of PEGylated agents (Yang and Lai 2015). Conversely, in humans, anti-PEG IgG antibodies are more prevalent, suggesting evidence for a potential anti-PEG memory response (Yang et al. 2016). Furthermore, recent evidence suggests that up to 72% of the general population (not previously exposed to PEGylated therapeutics) possess detectable pre-existing anti-PEG antibodies (Yang et al. 2016), possibly because of repeated low-level exposure to PEG-containing household goods such as foods, toothpastes and skin care products.

The pharmacokinetics of PEGylated particles are altered in the presence of anti-PEG antibodies. This so-called "accelerated blood clearance (ABC) phenomenon" has been associated with >10-fold reductions in particle half-lives in pre-clinical species (Dams et al. 2000; Kaminskas et al. 2011; Suzuki et al. 2012). The ABC phenomenon has been observed in a variety of animals and for PEGylated agents ranging from proteins to liposomes (Sroda et al. 2005; Xu et al. 2015; Zhang et al. 2012). Additionally, the presence of anti-PEG antibodies in humans has been correlated with increased incidence of adverse events and reductions in the therapeutic efficacy of PEGylated proteins in clinical trials, including pegloticase (indicated for severe treatment-refractory gout) and pegaspargase (part of multidrug therapy used to treat acute lymphoblastic leukemia) (Armstrong et al. 2007; Ganson et al. 2006; Hershfield et al. 2014).

We and other researchers have observed accelerated microbubble clearance in the later stages of studies that involve repeat contrast imaging over several days (Zhang et al. 2015). Furthermore, this effect has been associated with reduced target binding in ultrasound molecular imaging studies (Zhang et al. 2015). This effect complicates interpretation of molecular imaging results in serial imaging studies, as changes in target binding are assumed to reflect physiologic changes relating to target expression rather than altered pharmacokinetics of the microbubbles themselves.

The purpose of this study was to evaluate the generation of an anti-PEG immune response after a single or multiple doses of homemade PEGylated ultrasound contrast agents over a 1-mo period and to characterize associated changes in microbubble pharmacokinetics. A secondary aim of this study was to characterize anti-PEG immunity and accelerated clearance after multiple doses of PEGylated Definity microbubbles and non-PEGylated Optison microbubbles.

METHODS

Homemade microbubble fabrication and characterization

To form lipid-shelled microbubbles (referred to as house-MBs), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy(polyethylene-glycol)-2000 (DSPE-mPEG2000) (Avanti Polar Lipids, Alabaster, AL, USA) were combined in a 9:1 molar ratio and dissolved in a phosphate-buffered saline (PBS)-based solution containing 15% (v/v) propylene glycol and 5% (v/v) glycerol for a final lipid concentration of 1.0 mg/mL. Lipid solubilization was achieved using a previously described method (Marshalek et al. 2016). One and one-half milliliter aliquots of the lipid solution were then dispensed into 3.0-mL glass vials. The vial headspace air was replaced with decafluorobutane gas (Fluoromed, Round Rock, TX, USA), and microbubbles were generated by vigorous shaking of the vial using a VialMix (Bristol-Myers-Squibb, New York, NY, USA). House-MB size distributions and concentrations were characterized *via* single-particle optical sizing (AccuSizer 780 AD, Particle Sizing Systems, Port Richey, FL, USA). House-MBs were characterized by a polydisperse size distribution. The average concentration and number-weighted mean diameter of these microbubbles were found to be $(1.0 \pm 0.3) \times 10^{10}$ microbubbles/mL and 1.00 ± 0.02 μm, respectively (N = 3 vials).

Commercially available microbubbles

Definity (Lantheus Medical Imaging, Billerica, MA, USA) and Optison (GE Healthcare, Princeton, NJ, USA) used throughout this study were purchased from the Hospital Pharmacy at the University of North Carolina at Chapel Hill.

Animal preparation

All animal experiments were approved and performed in accordance with the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. First, female Fischer 344 rats were anesthetized *via* inhaled isoflurane (induced at 5% and maintained at 2% isoflurane in oxygen). A 24G catheter was inserted into