Published in final edited form as:

Ultrasound Med Biol. 2018 June ; 44(6): 1266–1280. doi:10.1016/j.ultrasmedbio.2018.02.006.

Accelerated clearance of ultrasound contrast agents containing polyethylene glycol is associated with the generation of anti-polyethylene glycol antibodies

Samantha M. Fix^a, A. Gloria Nyankima^b, Morgan D. McSweeney^a, James K. Tsuruta^b, Samuel K. Lai^{a,b,c}, and Paul A. Dayton^{a,b,*}

^aEshelman School of Pharmacy, Division of Pharmacoengineering and Molecular Pharmaceutics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

^bJoint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Chapel Hill, North Carolina, USA

^cDepartment of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Abstract

Emerging evidence suggests that the immune system is able to recognize polyethylene glycol (PEG), leading to the accelerated blood clearance (ABC) of PEGylated particles. Here we aim 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 prior to house-MB administration significantly prolonged microbubble circulation, suggesting that ABC was largely driven by circulating anti-PEG antibodies. Experiments with FDA-approved Definity microbubbles similarly resulted in ABC and the generation of anti-PEG antibodies. Experiments repeated with non-PEGylated Optison microbubbles demonstrated a slight shift in clearance, indicating that immunologic factors beyond anti-PEG immunity may play a role in ABC, especially of non-PEGylated agents.

Keywords

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

*Corresponding author, Address correspondence to: Paul A. Dayton, padayton@email.unc.edu, UNC-NCSU Joint Department of Biomedical Engineering, 152 MacNider Hall, CB7575, Chapel Hill, NC 27599, USA.

flexibility and hydrophilicity of PEG makes 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 the 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 shown to drastically enhance microbubble stability *in vitro*, prolonging formulation lifetime from approximately 13 min (without PEGylated lipids) to 60 min (with 5% PEGylated lipids) (Abou-Saleh, et al. 2014). Two of the three FDA approved microbubble formulations contain PEG. Lumason®/SonoVue® contains PEG-4000 as a stabilizer in the suspending medium (Bracco Diagnostics Inc., 2014) and Definity® contains PEGylated phospholipid as a shell component (Lantheus Medical Imaging, Inc., 2011). The third FDA-approved formulation, Optison™, is stabilized by a human albumin-based shell and does not contain PEG (GE Healthcare Inc., 2012).

Paradoxically, the immune system is able to generate specific antibodies that bind to PEG, the molecule originally exploited for its resistance to protein absorption and immunological 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 IgM, which peaks in concentration approximately one-week following 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 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 due to 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 preclinical 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 physiological changes relating to target expression rather than altered pharmacokinetics of the microbubbles themselves.

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

Materials and 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 780AD, 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 $1.00 \pm 0.02 \mu\text{m}$, 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 24 G catheter was inserted into the tail vein and used to collect blood samples for PEG-specific antibody detection. The catheter was then flushed with a small volume of heparinized saline to prevent clot formation and catheter blockage. Next, the animals' abdominal regions were shaved with an electric clipper and a disposable razor, and ultrasound gel was used to couple the imaging transducer to the animals' skin.

Ultrasound image acquisition

An Acuson Sequoia 512 clinical imaging system equipped with a 14 MHz linear array transducer was used throughout this study (15-L8, Siemens, Mountain View, CA, USA). Anesthetized animals were positioned on their left sides and secured to a heated platform using surgical tape to minimize movement during imaging. The kidney was chosen as our imaging target due to its high vascularity, its proximity to the skin's surface, and because it is not a suspected clearance organ for intact microbubbles (Tartis, et al. 2008, Walday, et al. 1994). The largest transverse plane of right kidney was located via B-mode imaging at 14 MHz and 0.61 mechanical index with an imaging frame rate of 17 Hz. Contrast specific imaging in Cadence™ Contrast Pulse Sequencing (CPS) mode was then initiated with a frequency of 7.0 MHz, mechanical index of 0.18, CPS gain of -10 dB, imaging frame rate of 20 Hz, and dynamic range of 80 dB. Twenty-minute CPS-mode clips were recorded using a capture frame rate of 1.0 Hz. Ten seconds into CPS clip storage, a bolus dose of microbubbles providing approximately 6.7×10^8 microbubbles/kg body weight (see experimental protocols below for more detail) was rapidly administered through the tail vein catheter followed immediately by a 200 μ L flush of sterile saline. Image data were exported from the ultrasound machine in DICOM format and analyzed offline using MATLAB (Mathworks Inc., Natick, MA, USA) and ImageJ software (NIH, Bethesda, MD, USA).

Experiment 1: change in circulatory persistence of PEGylated house-MBs over 28 d

A total of 11 female Fischer 344 rats (average weight 150.5 g) were divided into the experimental groups outlined below and underwent ultrasound imaging (or sham treatment) on days 0, 1, 2, 3, 7, 14, and 28 of this study. For each contrast imaging session, approximately 1×10^8 house-MBs were administered in 90 μ L sterile saline followed by a saline flush.

- 1. Sham (n=2):** These animals underwent ultrasound imaging without contrast administration at each time point. Instead of house-MBs, these animals received a bolus dose of sterile saline. Serum collected from these animals served as a control for anti-PEG antibody expression at each time point.
- 2. Single contrast dose (n=4):** This group was used to study the generation of anti-PEG antibodies following a single dose of PEGylated contrast. These animals were imaged with contrast on day 0. At each subsequent time point, they underwent sham treatment involving catheter insertion, administration of sterile saline, and imaging in the absence of

contrast agents. After the final blood collection on day 28, these animals received follow-up contrast imaging to evaluate the effect of a single dose of contrast on the clearance of house-MBs administered 28 d later.

3. Multiple contrast doses (n=5): These animals received contrast imaging at each time point (days 0, 1, 2, 3, 7, 14, and 28) to study the generation of an anti-PEG antibody response and altered house-MB clearance kinetics during repeat imaging schedules. One animal from this group died on day 14, and data collected up until that point was included in this study. We believe this animal's death was unrelated to the experiment and was likely due to isoflurane sensitivity.

Experiment 2: free PEG competition to recover circulatory persistence of house-MBs

We performed a free PEG competition experiment to explore the causal relationship between anti-PEG antibodies and accelerated clearance of house-MBs. A total of 7 female Fischer 344 rats (average weight 93.7 g) were divided into Control and Free PEG Competition groups. These animals were smaller than those used for experiment 1, and the contrast dose was proportionally reduced to approximately 6.2×10^7 house-MBs administered in 90 μL of sterile saline. All animals received a dose of house-MBs on day 0 and underwent contrast imaging to capture initial microbubble circulation kinetics. On days 1, 2, and 3, all animals received an additional dose of house-MBs to stimulate the accelerated blood clearance effect. On days 7 and 24, Control or Free PEG Competition protocols were performed as detailed below. On day 22, all animals underwent contrast imaging to ensure that the free PEG dose on day 7 did not stimulate further acceleration in MB clearance for animals in the competition group. The timeline of this study is depicted in Figure 1.

1. Control (n=3): On day 7, control animals received an injection of sterile saline equal in volume to that of the free PEG dose administered for the competition group. Approximately 3 hours later, house-MBs were administered and circulation kinetics were captured by contrast ultrasound imaging. This sequence of saline injection followed by contrast imaging was repeated on day 24. Serum was collected on day 0, day 7 (before and ~3hr after saline injection), and day 24 (before and ~3hr after saline injection) to characterize anti-PEG antibody levels.

2. Free PEG Competition (n=4): On day 7, animals in the competition group received a 550 mg per kg body weight (mpk) dose of sterile free PEG in PBS (20 kDa molecular weight, PEG20 kDa) (Sigma-Aldrich, St. Louis, MO, USA). Approximately 3 hours later, house-MBs were administered and contrast ultrasound imaging was performed. This free PEG competition sequence was repeated on day 24 with an increased dose of PEG20 kDa (2,200 mpk). Serum collected on day 0, day 7 (before and ~3hr after PEG injection), and day 24 (before and ~3hr after PEG injection) was used to characterize anti-PEG antibody levels. Serum was collected before and after PEG injection to determine if the free PEG was able to bind a substantial portion of circulating anti-PEG antibodies and therefore decrease detectable antibody concentrations.

Experiment 3: change in circulatory persistence of Definity and Optison with multiple dosing over 30 d

The purpose of this experiment was to study changes in the circulatory persistence of two clinically-approved microbubble formulations - Definity and Optison - when administered multiple times over a 30-d period. Definity is a lipid-based microbubble formulation, which contains mPEG5000-DPPE as a shell component. Conversely, Optison microbubbles are stabilized by a human albumin shell, and the formulation does not contain PEG.

A total of 15 Fischer 344 rats (average weight 146.8 g) were divided into Sham (n=4), Optison (n=6), and Definity (n=5) groups and underwent contrast imaging (or sham treatment) on days 0, 1, 2, 3, 7, 14, and 30 as described for experiment 1 (see procedures for 'Sham' and 'Multiple contrast doses'). Approximately 1×10^8 microbubbles were administered at each time point, according to the stock concentrations reported on the Definity and Optison package inserts (Definity package insert, 2011, Optison package insert, 2012). Serum was collected from all animals at each time point and tested for anti-PEG antibodies.

Quantification of microbubble persistence

B-mode images and CPS videos were imported into MATLAB and converted from DICOM to TIFF and AVI formats, respectively. Each converted file was then opened in ImageJ where a relatively small region of interest (ROI, 60×30 pixel oval) was drawn in the upper half of the kidney. A small ROI was chosen to minimize artifacts due to shadowing in the lower regions of the kidney (Chen, et al. 2012). Mean pixel intensity within the ROI was plotted over time, providing contrast time intensity curves (TICs) for further analysis. After microbubble administration, contrast intensity peaked rapidly and was followed by plateau and washout phases.

From the TICs, three metrics were calculated to quantify the circulatory kinetics of the microbubbles: area under the curve (AUC), elimination rate (k_e), and half-life ($t_{1/2}$). First, baseline intensity (average of the first 5 frames) was subtracted from the TIC and AUC were calculated in GraphPad Prism 6.0h (GraphPad Software, Inc., La Jolla, CA, USA). Additionally, a one-phase exponential decay equation was fit to the TIC washout phase, which was used to calculate the exponential decay constant referred to here as elimination rate (k_e , min^{-1}) and microbubble half-life ($t_{1/2}$, min) using GraphPad. All curve fits were associated with R^2 values between 0.90–0.99. AUC, k_e , and $t_{1/2}$ values were averaged among animals at each time point and are reported here as mean $\pm$ standard deviation.

For experiment 2, image collection on day 24 was erroneously terminated before the 20-min end point. Therefore, AUC values calculated from 0 – 7 min ($\text{AUC}_{0-7\text{min}}$) were compared among groups for this experiment. One-phase exponential decays were fit to the shorted curves on day 24 with strong R^2 values >0.96 and used to calculate $t_{1/2}$ and k_e values.

Detection of PEG-specific antibodies

For all experiments, blood was collected as described in the 'Animal Preparation' section and allowed to clot at room temperature for 15–60 min. Serum was then isolated by centrifugation at 2,000 g for 10 min and stored at –60°C for future use.

Serum concentrations of anti-PEG IgM or anti-PEG IgG antibodies were analyzed separately using their respective enzyme-linked immunosorbent assay (ELISA) kits (Life Diagnostics, Inc., West Chester, PA, USA) performed according to the manufacturer's protocol. For all experiments, serum samples were diluted 100× for the anti-PEG IgG assay. For experiment 1, serum samples were diluted 1000× for the anti-PEG IgM assay. Serum dilution was reduced to 250× for the anti-PEG IgM assay for experiments 2 and 3. Upon completion of the assay, optical density at 450 nm was read using an HTS 7000 Bioassay Plate Reader (PerkinElmer, Waltham, MA, USA) and the absorbance of a blank well was subtracted from all experimental values. All serum samples were tested in duplicate, and standard curves generated in duplicate on each ELISA plate were used to calculate the relative concentration of anti-PEG IgM or anti-PEG IgG in the samples in arbitrary units per milliliter. Please note that since the concentration of IgM and IgG standards were provided in arbitrary units, relative quantities of experimental anti-PEG antibody can only be compared within each isotype.

For experiment 1, samples from the time points showing maximal antibody concentration (day 7 for IgM and day 14 for IgG) were used in competitive ELISAs to confirm antibody specificity for the PEG component of house-MBs. Anti-PEG IgM and anti-PEG IgG ELISA kits were used as described above except serum samples were diluted with a solution containing the PEGylated lipid component of our microbubbles (DSPE-mPEG2000, 0.9–0.99 mg/mL). The relative quantity of anti-PEG IgM and anti-PEG IgG detected through the competition assay was compared with that observed in the standard ELISA.

Statistical analyses

All statistical analyses were performed in GraphPad. For each experiment, metrics describing microbubble circulatory kinetics (AUC, k_e , $t_{1/2}$) were compared using one-way ANOVA followed by Sidak's multiple comparison testing on significant results. AUC, k_e , and $t_{1/2}$ values describing later time points were compared to the respective initial values calculated on day 0. For experiment 1, kinetic parameters calculated on day 28 were also compared between those animals that had received multiple contrast doses throughout the study and those that had received a single contrast dose on day 0 and follow-up contrast imaging on day 28.

For experiments 1 and 3, mean anti-PEG IgM and anti-PEG IgG levels for each experimental group (i.e. multiple and single dosing for experiment 1, and Definity and Optison for experiment 3) were compared to the respective control mean at each time point using two-way ANOVA with respect to time. Dunnet's multiple comparison testing was then performed on significant results. For experiment 1, the concentrations of IgM and IgG antibodies detected in the competitive ELISA assay were compared to the respective

concentrations detected in the non-competitive ELISA using a paired student's t-test. Throughout this study, p-values < 0.05 were considered statistically significant.

For experiment 3, remarkably high anti-PEG IgG values of 3,380 and 5,320 U/mL were detected in one of four control animals on days 14 and 30, respectively. This is in comparison to the 400 ± 200 and 400 ± 300 U/mL IgG detected in the remaining control animals on days 14 and 30, respectively. Therefore, Grubbs' tests were performed, and it was determined that both IgG measurement values from this animal were significant outliers. As such, these data points were excluded from the analysis. Note: this outlying control animal did not demonstrate unusually high anti-PEG IgM concentrations at any time point.

Results and Discussion

Experiment 1: change in circulatory persistence of house-MBs over 28 d

Microbubbles are unable to escape the vascular space due to their relatively large size. By avoiding the measurement of signal in organs that are involved in active microbubble uptake and elimination (liver and spleen), we assumed that any signal observed in contrast-specific imaging was the result of microbubbles in circulation. Thus, microbubble blood clearance following an intravenous bolus dose was measured via quantification of contrast intensity over time.

Here, we imaged circulating microbubbles for 20 min following bolus administration using the kidney as an imaging target. The microbubbles were found to wash into the kidney vasculature within 5 s of the injection (Figure 2B, 2G, 2L), and the high concentration of microbubbles caused shadowing in some cases (white arrow heads in Figure 2C and 2H).

When microbubbles were administered to naïve animals on day 0, contrast was observable for greater than 10 min post-injection (Figure 2A-E). Additionally, contrast intensity faded uniformly throughout the images. Accelerated blood clearance (ABC) of the microbubbles became apparent on day 2, and by day 3, the kidney appeared devoid of contrast signal by 5-min post-injection (Figure 2F-J). The ABC effect continued throughout the remainder of the study (through day 28). Interestingly, we observe that strong signal intensity in the liver persisted well beyond microbubble signal detected in the kidney and surrounding tissue, as designated by white arrows in Figure 2I and 2N. Such an effect was never observed for the first contrast dose in naïve animals, and this qualitative observation suggests that ABC could be associated with more active liver uptake and clearance of the microbubbles. If the ABC is indeed due to the binding of multiple antibodies per microbubble, we suspect that clearance might be mediated by Fc γ RIIb receptors on liver sinusoidal endothelial cells and by Kupffer cells, as has been reported for other immune complexes (Ganesan, et al. 2012, Jenne and Kubes 2013, Steffan, et al. 1986) and microbubbles (Iijima, et al. 2006, Yanagisawa, et al. 2007). Clearance to the liver and spleen may also be guided by erythrocytes following binding of opsonized microbubbles by complement receptor one (Pascual and Schifferli 1995).

Contrast intensity was quantified in ImageJ and used to generate the time-intensity curves (TICs) presented in Figure 3. Corresponding with our qualitative observations, a leftward shift in the TICs was first observed 2 days after the initial microbubble dose (Figure 3A). This effect continued to become more dramatic throughout the study, reaching a maximum on day 14 that was matched at day 28. This observation is reflected quantitatively in terms of elimination rate (k_e), half-life ($t_{1/2}$) and area under curve (AUC) (Table 1 and Figure 4). k_e rates calculated on days 14 and 28 were approximately 4-times faster than that of the initial dose. Similarly, $t_{1/2}$ and AUC decreased throughout the study, reaching a minimum on day 14, which was approximately matched on day 28. Half-lives and AUCs at these time points were approximately 4- and 6.5-times less than those of the initial dose, respectively.

ABC was also observed on day 28 for animals that had received a single prior dose of microbubbles. However, the effect was not as dramatic as that seen on the 28th day in animals that had received multiple microbubble doses over 28 d (Figure 3B). In the single dose group, k_e was found to increase by 2.3-times, while $t_{1/2}$ and AUC were both found to decrease by approximately 2-times compared to the initial dose. However, k_e was found to be significantly lower and AUC significantly higher in the single dose animals compared to the animals that received multiple dosing throughout the study ($p < 0.01$ for both). These data indicate that cumulative microbubble exposure as well as dosing interval may be important in determining the magnitude of the ABC effect for PEGylated microbubbles.

Experiment 1: generation of a PEG-specific immune response in response to house-MB dosing

Commercially available ELISA kits were used to compare serum anti-PEG IgM and anti-PEG IgG levels between sham-treated control animals and animals that received either multiple house-MB dosing over 28 d or a single house-MB dose on day 0. As expected, we did not see any changes in anti-PEG IgM levels in sham-treated control animals throughout the study. However, we did see a slight increase in anti-PEG IgG on day 3 in sham control animals (Figure 5A). Since such an elevation in anti-PEG IgG levels was not found in sham control animals at any subsequent time point, we consider the increase on day 3 to be insignificant to the interpretation of this study.

Statistically significant elevations in anti-PEG IgG concentrations were detected on days 7 and 14 in animals that had received multiple microbubble doses (Figure 5A). Anti-PEG IgG peaked in these animals on day 14, and decreased considerably by day 28. As expected, the levels of anti-PEG IgG were lower in the animals that had received a single microbubble dose compared to those that received multiple microbubble doses. Although anti-PEG IgG concentration did not reach statistical significance for animals in the single dose group compared to that of the sham controls, we do note that quantifiable levels of antibody were observed on day 7 (Figure 5A).

Serum anti-PEG IgM concentrations peaked 7 d after the initial microbubble dose for both groups of animals that received microbubbles. In animals that received multiple doses, anti-PEG IgM concentrations were also detected on day 14 but diminished substantially by day 28 (Figure 5B). The elevation in anti-PEG IgM concentration on days 7 and 14 in the multiple dosing group showed statistical significance when compared to the IgM

concentration of sham controls at the same time point. Elevation in anti-PEG IgM concentrations did not reach statistical significance at any time point for animals that had received a single microbubble dose; however, detectable levels of antibody were observed on day 7.

To confirm that the detected antibodies were specific for the PEG component of our microbubbles, a competitive ELISA was performed where free mPEG-DSPE was added to compete for antibody binding. Competition was performed on serum samples that showed high antibody expression (i.e. day 7 for anti-PEG IgM and day 14 for anti-PEG IgG). Free mPEG-DSPE competition reduced the detected anti-PEG IgM concentration by 97.4% and 98.7% in the multiple and single dose groups, respectively (Figure 5E-F). Similarly, a 95.3% decrease in apparent anti-PEG IgG concentration was found in the multiple dosing group following free mPEG-DSPE competition (Figure 5C). A lower (64.8%), yet statistically significant, reduction in anti-PEG IgG antibody concentration was found for the single dose group due to the already low antibody levels present without competition (Figure 5D). These data indicate mPEG-DSPE-specificity of the detected IgM and IgG antibodies. Furthermore, we performed additional ELISA assays using plates coated with our microbubble components (DSPC and DSPE-mPEG2000) instead of the PEG-BSA coated plates provided in the ELISA kit. We observed similar trends in serum IgM and IgG levels using the plates coated with microbubble components compared to the kit-provided plates. This confirmed the presence of microbubble-specific antibodies in our serum samples (Supplementary Figure 1).

The time course of initial anti-PEG antibody production coincides with the observed changes in house-MB clearance, both peaking 7–14 d after the initial dose. Furthermore, animals that had received multiple microbubble doses demonstrated a more robust anti-PEG immune response compared to animals receiving a single dose. This corresponds with the more dramatic increase in microbubble clearance for the multiple versus single dose group.

However, we do not see a strong correlation between detected anti-PEG antibody concentration and the rate of microbubble clearance. For example, kinetic parameters describing house-MB clearance are nearly identical on days 14 and 28. Conversely, anti-PEG IgM and anti-PEG IgG levels are considerably greater on day 14 than day 28. We hypothesize that antibody-microbubble binding is not a rate-limiting step in the microbubble clearance pathway and that even on day 28 (when antibody concentrations had declined below the limit of quantification for our ELISA) we have a sufficient molar excess of antibodies to drive maximally accelerated clearance.

Here, we have administered microbubble doses containing approximately 1.0×10^8 house-MBs resulting in a rat blood concentration of $\sim 1.66 \times 10^{-14}$ M. Unfortunately, the lower limits of detection of the commercial anti-PEG IgM and anti-PEG IgG ELISA kits used in this study are unknown. However, we note that ELISA assays for anti-PEG antibodies can have lower limits of detection in the range of 2 – 15 ng/mL (Yang, et al. 2016). This implies that serum antibody concentrations near the detection limits of our assays may correspond to an antibody molar excess of >800 antibodies per microbubble. Thus, antibody concentrations well below our ELISA's probable lower limit of detection would likely be

sufficient to induce the accelerated clearance of this dose of microbubbles as immune complexes.

Experiment 2: free PEG competition to recover circulatory persistence of house-MBs

A competition experiment was performed to explore the mechanistic connection between anti-PEG antibody generation and accelerated clearance of PEGylated house-MBs. We dosed rats with house-MBs on days 0, 1, 2, and 3 to induce anti-PEG antibody production and accelerated microbubble clearance. On day 7, we administered free PEG20 kDa at a dose of 550 mpk in hopes of occupying the circulating anti-PEG antibodies. We subsequently administered house-MBs and characterized their circulatory persistence. This competition protocol did not provide recovery of house-MB dwell time compared to control animals that had received a saline injection prior to the house-MB dose. The initial $t_{1/2}$ on day 0 was found to be 8 ± 3 min, whereas Control and Competition groups on day 7 were characterized by $t_{1/2}$ values of 2.5 ± 0.5 min and 2 ± 0.7 min, respectively. ELISA analysis demonstrated high anti-PEG IgM and anti-PEG IgG concentrations before the PEG20 kDa dose (similar to that found on day 7 in experiment 1 (Figure 5)), which did not decrease after the PEG20kDa injection (Supplemental Figure 2). This indicates that a PEG20 kDa dose of 550 mpk was not sufficient to bind a substantial fraction of the high anti-PEG antibody concentration present on day 7, explaining why free PEG competition did not result in prolonged microbubble dwell time. A competitive ELISA experiment confirmed that free PEG20 kDa is able to compete for antibody-plate binding at a concentration of 0.9 mg/ml for both anti-PEG IgG and anti-PEG IgM (Supplemental Figure 2). This *in vitro* concentration of free PEG would be approximately equal to an *in vivo* free PEG dose of 6,000–15,000 mpk (taking into consideration the 100–250 $\times$ serum dilution used in the ELISAs and assuming a blood volume of 6.5 ml for a 100 g rat). Therefore, PEG20 kDa would theoretically have been a successful competition agent *in vivo* if administered in at a high enough dose.

From experiment 1, we know that anti-PEG antibody concentrations decrease between days 14 – 28 in our model. Therefore, we repeated the competition experiment using a higher dose of free PEG20 kDa (2,200 mpk) at a later time point (day 24) when the serum anti-PEG antibody concentrations were lower. With the revised competition protocol, we did observe a significant prolongation of house-MB dwell time in animals that received free PEG20 kDa compared to control animals that received a saline injection (Figure 6A). Microbubble elimination kinetics (k_e and $t_{1/2}$) after competition matched that of the initial contrast dose. However, the peak intensity of the competition TIC remained slightly lower than that of the initial TIC (day 0). These observations are displayed quantitatively in terms of k_e , $t_{1/2}$, and AUC in Figure 6B-D.

Without competition, k_e was 3.6 $\times$ faster on day 24 than on day 0. With free-PEG competition, elimination rate was reduced to a value that was statistically insignificantly different than that seen on day 0. Similarly, half-life recovered to a value that was insignificantly different from the initial value after free PEG competition. AUC did not reach that of the initial TIC, due to the lower initial peak intensity found for the competition curve. However, competition significantly increased AUC compared to that of the control TIC on

day 24. Collectively, these data support our hypothesis that anti-PEG antibodies are mechanistically involved in the accelerated clearance of our PEGylated house-MBs.

Our data is consistent with the current literature describing the mechanism of the ABC phenomenon for PEGylated liposomes (Ishida, et al. 2006, Ishida and Kiwada 2008, Kawanishi, et al. 2015, Yang and Lai 2015). The binding of IgM or IgG to PEGylated particles is thought to activate the complement system through the classical, antibody-mediated pathway. Such activation leads to particle opsonization by complement proteins, followed by binding/trafficking by erythrocytes (mediated by complement receptor one (Pascual and Schifferli 1995)) or direct accumulation in liver-resident scavenger cells such as liver sinusoidal endothelial cells and Kupffer cells (Yang and Lai 2015). This mechanism is in line with the data presented throughout this manuscript showing (1) anti-PEG antibody detection coinciding with accelerated microbubble clearance, (2) competition with free PEG resulting in recovered microbubble circulation kinetics, and (3) our observation suggesting the possibility of enhanced microbubble accumulation in the liver following clearance from the blood stream (experiment 1).

Experiment 3: change in circulatory persistence of Definity and Optison with multiple dosing over 30 d

FDA-approved Definity microbubbles are stabilized by a PEGylated lipid shell. These microbubbles are similar in composition to our house-MBs, and therefore, we hypothesized that repeat administration of Definity over several weeks would result in anti-PEG antibody production and accelerated clearance. Indeed, we observe a leftward shift in Definity TICs over 30 d (Figure 7A), which corresponded to significant decreases in AUC and $t_{1/2}$ and a significant increase in k_e (Figure 8A-C). A maximum change of $2.1\times$ was found on day 7 for both k_e and $t_{1/2}$. The maximum change in AUC was found on day 14 where AUC was $2.6\times$ lower than the initial value.

The $2.1\text{--}2.6\times$ changes in AUC, $t_{1/2}$, and k_e found for Definity are modest compared to the $4\text{--}6.5\times$ changes found for our house-MBs when administered with the same dosing schedule. We believe that this discrepancy is due to the relatively faster clearance of Definity at baseline compared to our house-MBs. The half-lives of Definity and house-MBs on day 0 were 2.0 ± 0.3 and 3.8 ± 0.3 min, respectively, but both formulations were characterized by the same minimum half-life of 0.9 min when maximally accelerated clearance was exhibited.

Accelerated Definity clearance was associated with the generation of anti-PEG IgM and to a lesser extent anti-PEG IgG (Figure 9). Antibody production followed a similar trend to that observed with house-MB dosing. Highest antibody concentrations were observed on day 7, and antibody levels decreased substantially by the study endpoint.

Optison microbubbles are stabilized by a shell of human albumin and do not contain PEG. Therefore, we were hypothesized that this formulation would not undergo PEG-associated accelerated clearance when administered repeatedly over 30 d. Indeed, Optison administration did not result in the generation of anti-PEG antibodies (Figure 9). However, we did observe a slight shift in TICs towards the end of the 30-d period (Figure 7B). Optison

microbubbles were rapidly cleared at baseline (initial $t_{1/2}$ of 0.5 ± 0.3 min), and $t_{1/2}$ decreased by a modest $1.75\times$ on day 30 (Figure 8E). Similarly, a $1.59\times$ increase in k_e was found on day 30 (Figure 8F). While statistically significant, the experimental relevance of the small changes in Optison $t_{1/2}$ and k_e values warrants further investigation (i.e. Will a decrease in $t_{1/2}$ from 0.5 ± 0.3 min to 0.3 ± 0.1 min change the interpretation of longitudinal contrast enhanced ultrasound studies?). The shift in Optison TICs did not result in a significant change in AUC at any time point (Figure 8D).

The shift in Optison TICs suggests that physical or immunological effects beyond anti-PEG immunity may be involved in the accelerated clearance of microbubbles, especially those that are non-PEGylated. Human serum albumin (the shell material of Optison) is immunogenic in rodents (Britton and Celada 1968, Dahlman-Höglund, et al. 1995), and liposomes decorated with foreign albumin molecules (bovine serum albumin or ovalbumin) have been shown to induce anti-albumin antibody generation and accelerated liposome clearance in rodents (Shek and Heath 1983, Shek, et al. 1986, Tardi, et al. 1997). We hypothesize that the observed shift in Optison TICs during our study is related to an immune response to human albumin in rats or the generation of other opsonizing serum factors in response to the initial microbubble doses. Others have reported that high doses of conventional liposomes (i.e. liposomes not containing PEG or foreign albumin) induce accelerated clearance of subsequently administered PEGylated liposomes (Ishida, et al. 2005). This indicates that serum factors generated in response to an initial dose of particles may opsonize subsequent particles and trigger accelerated clearance by Kupffer cells in the liver (Ishida, et al. 2005). The exact mechanism of accelerated clearance in the case of Optison warrants further investigation. However, these results do suggest that the accelerated blood clearance effect is an important consideration for all microbubble formulations, not only those containing PEG.

Impact for contrast-enhanced ultrasound imaging

Any ultrasound imaging study that involves the repeated administration of PEGylated microbubbles over multiple days is likely to induce the production of anti-PEG antibodies and result in accelerated microbubble clearance. Furthermore, non-PEGylated microbubbles may induce accelerated clearance through other immunological mechanisms. Acceleration of microbubble clearance may become an important consideration for the interpretation of ultrasound imaging studies that involve multiple imaging sessions. For example, several reports have demonstrated the potential of ultrasound time intensity curve (TIC) analysis for monitoring tumor response to chemotherapy in human patients (Lassau, et al. 2010, Schirin-Sokhan, et al. 2012, Ueda, et al. 2016). In such studies, TICs (similar to those in Figure 3) are generated from images of a tumor region periodically throughout the course of treatment. Changes in TIC features are then correlated with therapeutic outcome. In this example, it is critical that changes in the TICs are due to changes in tumor physiology (e.g. decreased vascularization) and not due to fundamental antibody-mediated alterations in the microbubble clearance.

The field of ultrasound molecular imaging (UMI) may also be affected if accelerated clearance is realized for targeted microbubbles. UMI can be used to interrogate disease- or

tissue-specific targets and has applications in diagnosis (Davidson, et al. 2014, Dayton and Ferrara 2002, Machtaler, et al. 2015, McCarty, et al. 2010), evaluation of tumor biomarker expression (Lindsey, et al. 2016, Shelton, et al. 2016, Spivak, et al. 2016, Zhang, et al. 2015), and monitoring treatment response (Palmowski, et al. 2008, Streeter, et al. 2013). Regardless of application, molecular imaging is often repeated on multiple days and changes in microbubble binding are assumed to reflect physiological changes in target/receptor expression. Accelerated microbubble clearance will likely be correlated with reduced microbubble binding, which should be considered when interpreting the results of UMI studies.

It is also important to consider the presence of pre-existing anti-PEG antibodies in human patients. A recent study has found low levels of pre-existing anti-PEG antibodies in over 70% of the general human population (i.e. individuals not previously exposed to PEGylated therapeutics) (Yang, et al. 2016). While reported mean anti-PEG IgG and anti-PEG IgM concentrations were only 52 ng/mL and 22 ng/mL, respectively, these antibody concentrations may be sufficient to influence the circulation kinetics of PEGylated microbubbles. For example, the recommended dose of Definity provides an approximate blood concentration of $\sim 2.6 \times 10^{-15}$ M particles to an 80 kg patient. In the same example patient, the reported average anti-PEG IgM and anti-PEG IgG concentrations would equate to approximate molar excesses of 5,000 $\times$ and 74,000 $\times$, respectively (assuming a plasma volume of 3.4 L, IgM molecular weight of 950 kDa, and IgG molecular weight of 150 kDa). The concentration of anti-PEG antibodies required for inducing clinically significant changes in the pharmacokinetics of PEGylated agents remains undetermined. However, the potential influence of low-level, pre-existing anti-PEG antibodies on contrast-enhanced ultrasound imaging studies warrants consideration and further exploration.

Finally, the safety implications of anti-PEG antibodies (pre-existing or generated in response to an initial microbubble dose) are important to consider with the clinical use of PEGylated ultrasound contrast agents. Previous research regarding PEGylated protein therapies demonstrates that circulating anti-PEG antibodies increase the risk of injection reactions in human patients (Ganson, et al. 2006, Hershfield, et al. 2014). Although the reported incidence of adverse effects following administration of microbubble contrast agents is minute and is often reported as less than other contrast agent types (Abdelmoneim, et al. 2009, Caschera, et al. 2016), clinicians should be aware of this risk when administering PEGylated microbubbles to patients who have suspected hypersensitivity to PEGylated microbubbles or therapeutics. This will require increased awareness of anti-PEG antibodies among relevant physician subsets. A recent study of physicians who prescribe PEGylated therapeutics found that only one quarter of prescribers were aware of anti-PEG antibodies (McSweeney, et al.).

Conclusions

We have demonstrated that the circulatory kinetics of homemade PEGylated microbubbles change dramatically when administered repeatedly over a one-month period in otherwise untreated animals. PEGylated microbubbles appear to be subject to the same ‘accelerated blood clearance (ABC) phenomenon’ observed following the repeated administration of

PEGylated proteins and liposomes. Here we show that the magnitude of accelerated microbubble clearance is related to cumulative microbubble exposure. Microbubble clearance at the study endpoint was faster for those animals that had received 6 prior doses of house-MBs compared to those animals that had only received one prior dose. Furthermore, we detected both anti-PEG IgM and anti-PEG IgG antibodies in all animals that received house-MBs. Once the ABC response had been mounted, dosing animals with free-PEG as a competition agent prior to house-MB administration resulted in recovery of microbubble intravascular dwell time. Therefore, we conclude that the accelerated clearance of PEGylated microbubbles is significantly related to an anti-PEG immune response. Similar trends in accelerated clearance and generation of anti-PEG antibodies were also observed with commercial Definity microbubbles. Interestingly, studies repeated with Optison also illustrated as slight change in clearance rate, which was necessarily independent from PEG-related immunity. We hypothesize that clearance changes in repeatedly administered Optison may have been associated with immunogenicity of the human albumin shell in rats.

In conclusion, it is important to understand the immunogenicity of microbubble shell materials and how this may impact microbubble circulation kinetics during contrast-enhanced ultrasound imaging studies that involve multiple microbubble doses over several days to weeks. Any changes in microbubble circulation driven by anti-PEG or other immune responses should be considered when interpreting the results of longitudinal contrast-enhanced imaging results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Professor David Lawrence for kindly allowing us to use equipment and laboratory space while performing ELISA assays.

Financial support was provided by NSF Graduate Research Fellowship (M.D.M.), NIH NRSA Individual Predoctoral Fellowships (F31) (S.M.F. and A.G.N.), The David and Lucile Packard Foundation (2013–39274, S.K.L.), National Institutes of Health (R21EB017938, S.K.L.; U01CA189281, P.A.D; R01CA195051, P.A.D.), and UNC Research Opportunities Initiative grant in Pharmacoeengineering (S.K.L.).

References

- Abdelmoneim SS, Bernier M, Scott CG, Dhoble A, Ness SA, Hagen ME, Moir S, McCully RB, Pellikka PA, Mulvagh SL. Safety of contrast agent use during stress echocardiography: a 4-year experience from a single-center cohort study of 26,774 patients. *JACC. Cardiovascular imaging* 2009; 2:1048–56. [PubMed: 19761981]
- Abou-Saleh RH, Swain M, Evans SD, Thomson NH. Poly(ethylene glycol) lipid-shelled microbubbles: abundance, stability, and mechanical properties. *Langmuir : the ACS journal of surfaces and colloids* 2014; 30:5557–63. [PubMed: 24758714]
- Abu Lila AS, Kiwada H, Ishida T. The accelerated blood clearance (ABC) phenomenon: clinical challenge and approaches to manage. *Journal of controlled release : official journal of the Controlled Release Society* 2013; 172:38–47. [PubMed: 23933235]
- Appis AW, Tracy MJ, Feinstein SB. Update on the safety and efficacy of commercial ultrasound contrast agents in cardiac applications. *Echo research and practice* 2015; 2:R55–62. [PubMed: 26693339]

- Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, Garratty G. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer* 2007; 110:103–11. [PubMed: 17516438]
- Borden MA, Pu G, Runner GJ, Longo ML. Surface phase behavior and microstructure of lipid/PEG-emulsifier monolayer-coated microbubbles. *Colloids and surfaces. B, Biointerfaces* 2004; 35:209–23. [PubMed: 15261034]
- Borden MA, Sarantos MR, Stieger SM, Simon SI, Ferrara KW, Dayton PA. Ultrasound radiation force modulates ligand availability on targeted contrast agents. *Molecular imaging* 2006; 5:139–47. [PubMed: 16954028]
- Britton S, Celada F. Immunogenicity of human serum albumin: decay in the normal mouse. *Immunology* 1968; 14:503–9. [PubMed: 5653812]
- Butcher NJ, Mortimer GM, Minchin RF. Drug delivery: Unravelling the stealth effect. *Nat Nanotechnol* 2016; 11:310–1. [PubMed: 26878145]
- Caschera L, Lazzara A, Piergallini L, Ricci D, Tusciano B, Vanzulli A. Contrast agents in diagnostic imaging: Present and future. *Pharmacological research* 2016; 110:65–75. [PubMed: 27168225]
- Chen CC, Sirsi SR, Borden MA. EFFECT OF SURFACE ARCHITECTURE ON IN VIVO ULTRASOUND CONTRAST PERSISTENCE OF TARGETED SIZE-SELECTED MICROBUBBLES. *Ultrasound in medicine & biology* 2012; 38:492–503. [PubMed: 22305060]
- Dahlman-Höglund A, Dahlgren U, Ahlstedt S, Hanson LA, Telemo E. Bystander suppression of the immune response to human serum albumin in rats fed ovalbumin. *Immunology* 1995; 86:128–33. [PubMed: 7590873]
- Dams ET, Laverman P, Oyen WJ, Storm G, Scherphof GL, van Der Meer JW, Corstens FH, Boerman OC. Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J Pharmacol Exp Ther* 2000; 292:1071–9. [PubMed: 10688625]
- Davidson BP, Chadderdon SM, Belcik JT, Gupta S, Lindner JR. Ischemic memory imaging in nonhuman primates with echocardiographic molecular imaging of selectin expression. *J Am Soc Echocardiogr* 2014; 27:786–93 e2. [PubMed: 24774222]
- Dayton PA, Ferrara KW. Targeted imaging using ultrasound. *Journal of magnetic resonance imaging : JMRI* 2002; 16:362–77. [PubMed: 12353252]
- DEFINITY®. [package insert]. Lantheus Medical Imaging, Inc. North Billerica, MA. 10, 2011..
- Ganesan LP, Kim J, Wu Y, Mohanty S, Phillips GS, Birmingham DJ, Robinson JM, Anderson CL. FcγRIIb on liver sinusoidal endothelium clears small immune complexes. *Journal of immunology (Baltimore, Md. : 1950)* 2012; 189:4981–8.
- Ganson NJ, Kelly SJ, Scarlett E, Sundry JS, Hershfield MS. Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly(ethylene glycol) (PEG), in a phase I trial of subcutaneous PEGylated urate oxidase. *Arthritis Research & Therapy* 2006; 8:R12–R12. [PubMed: 16356199]
- Garg S, Thomas AA, Borden MA. The effect of lipid monolayer in-plane rigidity on in vivo microbubble circulation persistence. *Biomaterials* 2013; 34:6862–70. [PubMed: 23787108]
- Haynes MT, Huang L. Maximizing the Supported Bilayer Phenomenon: Liposomes Comprised Exclusively of PEGylated Phospholipids for Enhanced Systemic and Lymphatic Delivery. *ACS applied materials & interfaces* 2016; 8:24361–7. [PubMed: 27580273]
- Hershfield MS, Ganson NJ, Kelly SJ, Scarlett EL, Jagers DA, Sundry JS. Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients. *Arthritis Res Ther* 2014; 16:R63. [PubMed: 24602182]
- Hirotsu T, Higashi T, Abu H, II, Misumi S, Wada K, Motoyama K, Arima H. Self-Assembly PEGylation Retaining Activity (SPRA) Technology via a Host-Guest Interaction Surpassing Conventional PEGylation Methods of Proteins. *Molecular pharmaceutics* 2017.
- Iijima H, Moriyasu F, Miyahara T, Yanagisawa K. Ultrasound contrast agent, Levovist microbubbles are phagocytosed by Kupffer cells-In vitro and in vivo studies. *Hepatology research : the official journal of the Japan Society of Hepatology* 2006; 35:235–7. [PubMed: 16831566]
- Ishida T, Harada M, Wang XY, Ichihara M, Irimura K, Kiwada H. Accelerated blood clearance of PEGylated liposomes following preceding liposome injection: effects of lipid dose and PEG

- surface-density and chain length of the first-dose liposomes. *Journal of controlled release : official journal of the Controlled Release Society* 2005; 105:305–17. [PubMed: 15908032]
- Ishida T, Ichihara M, Wang X, Kiwada H. Spleen plays an important role in the induction of accelerated blood clearance of PEGylated liposomes. *Journal of Controlled Release* 2006; 115:243–50. [PubMed: 17011060]
- Ishida T, Kiwada H. Accelerated blood clearance (ABC) phenomenon upon repeated injection of PEGylated liposomes. *International journal of pharmaceutics* 2008; 354:56–62. [PubMed: 18083313]
- Jenne CN, Kubes P. Immune surveillance by the liver. *Nat Immunol* 2013; 14:996–1006. [PubMed: 24048121]
- Kaminskas LM, McLeod VM, Porter CJ, Boyd BJ. Differences in colloidal structure of PEGylated nanomaterials dictate the likelihood of accelerated blood clearance. *Journal of pharmaceutical sciences* 2011; 100:5069–77. [PubMed: 21721002]
- Kawanishi M, Hashimoto Y, Shimizu T, Sagawa I, Ishida T, Kiwada H. Comprehensive analysis of PEGylated liposome-associated proteins relating to the accelerated blood clearance phenomenon by combination with shotgun analysis and conventional methods. *Biotechnology and applied biochemistry* 2015; 62:547–55. [PubMed: 25196743]
- Lassau N, Koscielny S, Albiges L, Chami L, Benatsou B, Chebil M, Roche A, Escudier BJ. Metastatic renal cell carcinoma treated with sunitinib: early evaluation of treatment response using dynamic contrast-enhanced ultrasonography. *Clin Cancer Res* 2010; 16:1216–25. [PubMed: 20145174]
- Lindsey BD, Shelton SE, Foster FS, Dayton PA. Assessment of Molecular Acoustic Angiography for Combined Microvascular and Molecular Imaging in Preclinical Tumor Models. *Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging* 2016.
- Liu D, Xing J, Xiong F, Yang F, Gu N. Preparation and in vivo safety evaluations of antileukemic homoharringtonine-loaded PEGylated liposomes. *Drug development and industrial pharmacy* 2017:1–9.
- Liu Z, Ren Y, Pan L, Xu HM. In vivo anti-tumor activity of polypeptide HM-3 modified by different polyethylene glycols (PEG). *International journal of molecular sciences* 2011; 12:2650–63. [PubMed: 21731464]
- LUMASON®. [package insert]. Bracco Diagnostics Inc., Monroe Twp., NJ. 2014.
- Machtaler S, Knieling F, Luong R, Tian L, Willmann JK. Assessment of Inflammation in an Acute on Chronic Model of Inflammatory Bowel Disease with Ultrasound Molecular Imaging. *Theranostics* 2015; 5:1175–86. [PubMed: 26379784]
- Malinge J, Geraudie B, Savel P, Nataf V, Prignon A, Provost C, Zhang Y, Ou P, Kerrou K, Talbot JN, Siaugue JM, Sollogoub M, Menager C. Liposomes for PET and MR Imaging and for Dual Targeting (Magnetic Field/Glucose Moiety): Synthesis, Properties, and in Vivo Studies. *Molecular pharmaceutics* 2017.
- Marchal S, El Hor A, Millard M, Gillon V, Bezdetnaya L. Anticancer Drug Delivery: An Update on Clinically Applied Nanotherapeutics. *Drugs* 2015; 75:1601–11. [PubMed: 26323338]
- Marshalek JP, Sheeran PS, Ingram P, Dayton PA, Witte RS, Matsunaga TO. Intracellular delivery and ultrasonic activation of folate receptor-targeted phase-change contrast agents in breast cancer cells in vitro. *Journal of Controlled Release* 2016; 243:69–77. [PubMed: 27686582]
- Martin KH, Dayton PA. Current Status and Prospects for Microbubbles in Ultrasound Theranostics. *Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology* 2013; 5:10.1002/wnan.219.
- McCarty OJ, Conley RB, Shentu W, Tormoen GW, Zha D, Xie A, Qi Y, Zhao Y, Carr C, Belcik T, Keene DR, de Groot PG, Lindner JR. Molecular imaging of activated von Willebrand factor to detect high-risk atherosclerotic phenotype. *JACC. Cardiovascular imaging* 2010; 3:947–55. [PubMed: 20846630]
- McSweeney MD, Versfeld ZC, Carpenter DM, Lai SK. Physician Awareness of Immune Responses to Polyethylene Glycol-Drug Conjugates. *Clinical and Translational Science*:n/a-n/a.
- Nie Q, Jia D, Yang H, Feng Y, Fan Q, Shi Q, Wan L, Lu X. Conjugation to 10 kDa Linear PEG Extends Serum Half-Life and Preserves the Receptor-Binding Ability of mmTRAIL with Minimal Stimulation of PEG-Specific Antibodies. *Molecular pharmaceutics* 2017.

- OPTISON™. [package insert]. GE Healthcare Inc., Princeton, NJ 5 2012.
- Paefgen V, Doleschel D, Kiessling F. Evolution of contrast agents for ultrasound imaging and ultrasound-mediated drug delivery. *Frontiers in Pharmacology* 2015; 6.
- Palmowski M, Huppert J, Ladewig G, Hauff P, Reinhardt M, Mueller MM, Woenne EC, Jenne JW, Maurer M, Kauffmann GW, Semmler W, Kiessling F. Molecular profiling of angiogenesis with targeted ultrasound imaging: early assessment of antiangiogenic therapy effects. *Molecular cancer therapeutics* 2008; 7:101–9. [PubMed: 18202013]
- Pascual M, Schifferli JA. Another function of erythrocytes: transport of circulating immune complexes. *Infusionsther Transfusionsmed* 1995; 22:310–5. [PubMed: 8924746]
- Salmaso S, Caliceti P. Stealth properties to improve therapeutic efficacy of drug nanocarriers. *Journal of drug delivery* 2013; 2013:374252. [PubMed: 23533769]
- Schirin-Sokhan R, Winograd R, Roderburg C, Bubenzer J, do ON, Guggenberger D, Hecker H, Trautwein C, Tischendorf JJ. Response evaluation of chemotherapy in metastatic colorectal cancer by contrast enhanced ultrasound. *World journal of gastroenterology* 2012; 18:541–5. [PubMed: 22363120]
- Shek PN, Heath TD. Immune response mediated by liposome-associated protein antigens. III. Immunogenicity of bovine serum albumin covalently coupled to vesicle surface. *Immunology* 1983; 50:101–6. [PubMed: 6193054]
- Shek PN, Lopez NG, Heath TD. Immune response mediated by liposome-associated protein antigens. IV. Modulation of antibody formation by vesicle-encapsulated methotrexate. *Immunology* 1986; 57:153–7. [PubMed: 3510967]
- Shelton SE, Lindsey BD, Tsuruta JK, Foster FS, Dayton PA. Molecular Acoustic Angiography: A New Technique for High-resolution Superharmonic Ultrasound Molecular Imaging. *Ultrasound in medicine & biology* 2016; 42:769–81. [PubMed: 26678155]
- Spivak I, Rix A, Schmitz G, Fokong S, Iranzo O, Lederle W, Kiessling F. Low-Dose Molecular Ultrasound Imaging with E-Selectin-Targeted PBCA Microbubbles. *Molecular imaging and biology : the official publication of the Academy of Molecular Imaging* 2016; 18:180–90. [PubMed: 26391990]
- Sroda K, Rydlewski J, Langner M, Kozubek A, Grzybek M, Sikorski AF. Repeated injections of PEG-PE liposomes generate anti-PEG antibodies. *Cellular & molecular biology letters* 2005; 10:37–47. [PubMed: 15809678]
- Steffan AM, Gendrault JL, McCuskey RS, McCuskey PA, Kirn A. Phagocytosis, an unrecognized property of murine endothelial liver cells. *Hepatology* 1986; 6:830–6. [PubMed: 3758936]
- Streeter JE, Herrera-Loeza SG, Neel NF, Yeh JJ, Dayton PA. A comparative evaluation of ultrasound molecular imaging, perfusion imaging, and volume measurements in evaluating response to therapy in patient-derived xenografts. *Technology in cancer research & treatment* 2013; 12:311–21. [PubMed: 23369156]
- Suzuki T, Ichihara M, Hyodo K, Yamamoto E, Ishida T, Kiwada H, Ishihara H, Kikuchi H. Accelerated blood clearance of PEGylated liposomes containing doxorubicin upon repeated administration to dogs. *International journal of pharmaceutics* 2012; 436:636–43. [PubMed: 22850293]
- Tardi PG, Swartz EN, Harasym TO, Cullis PR, Bally MB. An immune response to ovalbumin covalently coupled to liposomes is prevented when the liposomes used contain doxorubicin. *J Immunol Methods* 1997; 210:137–48. [PubMed: 9520297]
- Tartis MS, Kruse DE, Zheng H, Zhang H, Kheirloomoom A, Marik J, Ferrara KW. Dynamic microPET imaging of ultrasound contrast agents and lipid delivery. *Journal of controlled release : official journal of the Controlled Release Society* 2008; 131:160–66. [PubMed: 18718854]
- Ueda N, Nagira H, Sannomiya N, Ikunishi S, Hattori Y, Kamida A, Koyanagi Y, Shimabayashi K, Sato K, Saito H, Hirooka Y. Contrast-Enhanced Ultrasonography in Evaluation of the Therapeutic Effect of Chemotherapy for Patients with Liver Metastases. *Yonago acta medica* 2016; 59:255–61. [PubMed: 28070162]
- Walday P, Tolleshaug H, Gjøen T, Kindberg GM, Berg T, Skotland T, Holtz E. Biodistributions of air-filled albumin microspheres in rats and pigs. *Biochemical Journal* 1994; 299:437–43. [PubMed: 8172604]

- Xu H, Ye F, Hu M, Yin P, Zhang W, Li Y, Yu X, Deng Y. Influence of phospholipid types and animal models on the accelerated blood clearance phenomenon of PEGylated liposomes upon repeated injection. *Drug delivery* 2015; 22:598–607. [PubMed: 24524364]
- Yanagisawa K, Moriyasu F, Miyahara T, Yuki M, Iijima H. Phagocytosis of ultrasound contrast agent microbubbles by Kupffer cells. *Ultrasound in medicine & biology* 2007; 33:318–25. [PubMed: 17207907]
- Yang Q, Jacobs TM, McCallen JD, Moore DT, Huckaby JT, Edelstein J, Lai SK. Analysis of pre-existing IgG and IgM antibodies against polyethylene glycol (PEG) in the general population. *Anal Chem* 2016.
- Yang Q, Lai SK. Anti-PEG immunity: emergence, characteristics, and unaddressed questions. *Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology* 2015; 7:655–77. [PubMed: 25707913]
- Zhang C, Fan K, Ma X, Wei D. Impact of large aggregated uricases and PEG diol on accelerated blood clearance of PEGylated canine uricase. *PloS one* 2012; 7:e39659. [PubMed: 22745806]
- Zhang H, Tam S, Ingham ES, Mahakian LM, Lai C-Y, Tumbale SK, Teesalu T, Hubbard NE, Borowsky AD, Ferrara KW. Ultrasound molecular imaging of tumor angiogenesis with a neuropilin-1-targeted microbubble. *Biomaterials* 2015; 56:104–13. [PubMed: 25934284]
- Zhang H, Tam S, Ingham ES, Mahakian LM, Lai CY, Tumbale SK, Teesalu T, Hubbard NE, Borowsky AD, Ferrara KW. Ultrasound molecular imaging of tumor angiogenesis with a neuropilin-1-targeted microbubble. *Biomaterials* 2015; 56:104–13. [PubMed: 25934284]
- Zhou B, Xiong Z, Zhu J, Shen M, Tang G, Peng C, Shi X. PEGylated polyethylenimine-entrapped gold nanoparticles loaded with gadolinium for dual-mode CT/MR imaging applications. *Nanomedicine (London, England)* 2016; 11:1639–52.

Figure 1:

Timeline detailing experiment 2. Free PEG competition to recover circulatory persistence of house-MBs. On days 0, 7, 22, and 24, animals underwent contrast ultrasound imaging to quantify microbubble circulatory persistence. On days 1, 2, and 3, animals received a dose of house-MBs without imaging to induce the ABC effect. On days 7 and 24, free PEG (or saline) was administered prior to the house-MB dose and contrast imaging.

Figure 2:

Representative B-mode and contrast images of rat kidneys from experiment 1. Kidneys are outlined in white. As shown in the day 0 panel, contrast washes out uniformly from all visible tissues in naïve rats, and some signal from the house-MBs is still observable at the 10-min time point. The ABC effect becomes apparent on day 3 and persists throughout the study. At these later time points we see rapid washout of contrast from the kidney, and redistribution into the liver (white arrows). Shadowing was observed post-contrast injection and is noted with white arrowheads. Bar = 5 mm.

Figure 3:

Average time intensity curves from experiment 1. (A) Time intensity curves obtained from animals receiving repeat doses of polyethylene glycol (PEG)-containing house-MBs over 28 d. Curve labels (D0-D28) indicate time in days since the initial dose. (B) Comparison of the initial contrast time intensity curve to those obtained 28 days later in animals that received multiple doses of contrast (purple) and those that received a single prior dose (blue).

Figure 4:

Changes in house-MB pharmacokinetic parameters over 30 d of repeat dosing. A)

Elimination rate (k_e); B) Half-life ($t_{1/2}$); C) Area under the curve (AUC). Symbol key: ** p

0.01; *** p 0.001; **** p 0.0001

Figure 5:

Quantification of serum anti-PEG antibody levels and results of a competitive ELISA using free mPEG-DSPE (experiment 1). (A) Serum anti-PEG IgG is shown to peak 7–14 d after the initial dose of house-MBs and decrease by the 28-d time point. (B) Serum anti-PEG IgM is shown to peak on day 7 for multiple and single dose groups. Anti-PEG IgM was found to decrease at subsequent time points. C and D) For those samples showing high anti-PEG IgM or anti-PEG IgG antibody expression, a competitive ELISA assay was performed to confirm PEG specificity. IgM and IgG binding to the PEG-coated ELISA plate was shown to decrease substantially when serum was incubated in the presence of free mPEG-DSPE (0.9–0.99 mg/ml). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. ELISA = enzyme-linked immunosorbent assay, Ig = immunoglobulin, DSPE-mPEG2000 = 1,2,-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene-glycol)-2000.

Figure 6.

Competition with 2,200 mpk free PEG20 kDa prolongs house-MB circulation in animals exhibiting the ABC effect. (A) Initial time intensity curve of house-MBs (red) shows slow clearance compared to that of control animals on day 24 (grey). When animals were pre-dosed with 2,200 mpk free PEG, the microbubble time intensity curve (black) showed a long circulation profile similar to that seen on day 0, indicating successful competition of binding between anti-PEG antibodies and microbubbles. (B-D) After competition on day 24, k_e and $t_{1/2}$ values were not statistically different from their respective initial values. After competition on day 24, AUC was still significantly lower than the initial value, however, competition did significantly increase AUC compared to control animals that received saline injections on day 24. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure 7.

(A) Average time intensity curves obtained from animals receiving multiple injections of Definity over 30 d. Curve labels (D0-D30) indicate time in days since the initial dose. (B) Average time intensity curves from animals receiving multiple injections of Optison over 30 d.

Figure 8.

Change in areas under the curves (A), half-lives (B), elimination rates (C) through time for Definity when administered repeatedly over a 30-d period. Change in areas under the curves (D), half-lives (E), elimination rates (F) through time for Optison when administered repeatedly over a 30-d period. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure 9.

Enzyme-linked immunosorbent assay detection of serum anti-PEG IgM (A) and anti-PEG IgG (B) antibodies for animals repeatedly dosed with Definity or Optison over 30 d. PEG = polyethylene glycol; Ig = immunoglobulin

Table 1:

Summary of key parameters describing the persistence of house-MBs in circulation from experiment 1.

	k_e (min ⁻¹)		$t_{1/2}$ (min)		AUC (I × min)	
Treatment day	Avg. ± SD	Different from initial?	Avg. ± SD	Different from initial?	Avg. ± SD	Different from initial?
Day 0	0.18 ± 0.02	--	3.8 ± 0.3	--	320 ± 30	--
Day 1	0.17 ± 0.03	No - ns	4.2 ± 0.8	No - ns	370 ± 60	No - ns
Day 2	0.24 ± 0.02	No - ns	3.0 ± 0.3	Yes **	230 ± 30	Yes ***
Day 3	0.32 ± 0.06	No - ns	2.2 ± 0.4	Yes ****	160 ± 40	Yes ****
Day 7	0.4 ± 0.1	Yes **	1.8 ± 0.5	Yes ****	90 ± 30	Yes ****
Day 14	0.8 ± 0.3	Yes ****	0.9 ± 0.2	Yes ****	50 ± 20	Yes ****
Day 28 - Multiple	0.7 ± 0.2	Yes ****	1.0 ± 0.3	Yes ****	50 ± 20	Yes ****
Day 28 - Single	0.42 ± 0.04	Yes *	1.7 ± 0.1	Yes ****	150 ± 20	Yes ****

Multiple = animals that received a house-MB dose at each time point, Single = animals that received house-MB dosing only on days 0 and 28 of the study, k_e = elimination rate, $t_{1/2}$ = half-life, AUC = area under the curve, House-MB = homemade polyethylene glycol-containing microbubbles, SD = standard deviation. Not significant (ns) $p > 0.05$;

*
p 0.05;

**
p 0.01;

p 0.001;

p 0.0001