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Ultrasound-stimulated phase-change contrast agents for transepithelial delivery of macromolecules, towards gastrointestinal drug delivery

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21 **ABSTRACT**

22 The gastrointestinal (GI) tract presents a notoriously difficult barrier for macromolecular drug delivery,
23 especially for biologics. Herein, we demonstrate that ultrasound-stimulated phase change contrast agents
24 (PCCAs) can transiently disrupt Caco-2 monolayers and improve the transepithelial transport of a
25 macromolecular model drug. With ultrasound treatment in the presence of PCCAs, we achieved a
26 maximum of 44±15% transepithelial delivery of 70 kDa FITC-dextran, compared to negligible delivery
27 through sham control monolayers. Among all tested rarefactional pressures (300-600 kPa), dextran
28 delivery efficiency was consistently greatest at 300 kPa. To explore this unexpected finding, we quantified
29 stable and inertial cavitation energy generated by various ultrasound exposure conditions. In general,
30 lower pressures resulted in more persistent cavitation activity over 30 second exposure times, which may
31 explain the enhanced dextran delivery efficiency. Thus, a unique advantage of using low boiling point
32 PCCAs for this application is that the same low-pressure pulses can be used to induce vaporization and
33 provide maximal delivery.

34

35 **KEYWORDS**

36 Ultrasound; Transepithelial Delivery; Acoustic Droplet Vaporization; Phase-change contrast agents;
37 Nanodroplet

38 INTRODUCTION

39 The gastrointestinal (GI) tract has evolved to efficiently breakdown food products, allow nutrient
40 uptake, and simultaneously provide a robust defense against ingested toxins and pathogens (Barrett 2012,
41 Schoellhammer, et al. 2016). This presents a complex barrier for both local and systemic drug delivery
42 through the GI tract, particularly for biologics (e.g., therapeutic peptides, proteins, and nucleic acids)
43 (Goldberg and Gomez-Orellana 2003). The first hurdle is maintaining drug stability in the degradative
44 environment. This often requires specialized formulations to protect the active pharmaceutical ingredient
45 from (1) the harsh and fluctuating acidic pH and (2) an array of degradative species and enzymes (Moss,
46 et al. 2017). If drug integrity is maintained, the second hurdle is permeability. The GI tract is blanketed
47 with mucus that traps and protects the underlying epithelium from foreign particles (Cone 2009).
48 Furthermore, GI epithelial cells are linked by tight junctions that restrict paracellular transport of large
49 molecules (> 200 Da), and the hydrophilic nature of most biologics limits passive transcellular diffusion
50 (Goldberg and Gomez-Orellana 2003). Due to these challenges, generally only small molecule drugs can
51 be delivered through the GI tract.

52 Therapeutic ultrasound has recently been proposed as a universal method to physically enhance
53 GI permeability to macromolecular drugs (Schoellhammer, et al. 2017, Schoellhammer, et al. 2016,
54 Schoellhammer, et al. 2017, Schoellhammer, et al. 2015, Schoellhammer and Traverso 2016). This
55 technique involves colonic insertion of an ultrasound probe with co-administration of a medicated enema
56 (Schoellhammer and Traverso 2016). Low-frequency (≤ 100 kHz) ultrasound exposure for one minute or
57 less has been shown to significantly enhance macromolecule delivery into surrounding GI tissue
58 (Schoellhammer, et al. 2017, Schoellhammer, et al. 2017). This technology has the potential to transform
59 the treatment landscape of several GI diseases including ulcerative colitis, cancers of the GI tract,
60 infection, and Crohn's disease, and offers the following unique benefits (Schoellhammer, et al. 2017,

61 Schoellhammer, et al. 2016, Schoellhammer, et al. 2015). First, rapid delivery limits concerns of drug
62 degradation by intestinal enzymes, eliminating the need for complex protective formulations. Second, this
63 technique may allow for local GI delivery of biologic drugs previously restricted to intravenous
64 administration (including peptides, proteins, monoclonal antibodies, **nucleic acids**), which would expand
65 therapeutic options for GI diseases. **Finally**, concentrated local delivery has the potential to enhance drug
66 efficacy and reduce systemic toxicities.

67 The mechanism of ultrasound-mediated drug delivery involves ***in situ* generation and acoustic**
68 **excitation of vapor cavities** near a biological barrier **through the process of cavitation** (Lentacker, et al.
69 2014, Miller, et al. 2002, Schoellhammer and Traverso 2016). When **a fluid is** subjected to ultrasound of
70 sufficient energy, vapor cavities are created (**cavitation**). These bubbles oscillate with the passing acoustic
71 wave (stable cavitation) and cause flow disturbances in the surrounding fluid, **and this ‘microstreaming’**
72 **exerts shear stress on nearby structures** (Kooiman, et al. 2014, Pereno, et al. 2018). With sufficient acoustic
73 amplitude, the bubble becomes unstable, violently collapses (inertial cavitation), and creates a fluid
74 microjet towards nearby boundaries. Stresses exerted by these physical phenomena transiently enhance
75 the permeability of biological barriers (Coussios, et al. 2016, Escoffre and Bouakaz 2018, Kooiman, et al.
76 2014, Miller, et al. 2002, Postema, et al. 2005). In addition to enhanced permeability, acoustic cavitation
77 and radiation force enhance the transport of molecules through newly permeabilized membranes
78 (Bhatnagar, et al. 2014).

79 The likelihood of ultrasound to induce cavitation is related to the wave’s mechanical index (MI)
80 which is defined as the peak rarefactional pressure (in MPa) divided by the square root of the center
81 frequency (in MHz) (Apfel and Holland 1991, Bhatnagar, et al. 2014). To achieve ultrasound-mediated
82 drug delivery, low frequencies (≤ 100 kHz) and consequently high MIs (>2) are often needed
83 (Schoellhammer, et al. 2017, Schoellhammer, et al. 2017, Schoellhammer, et al. 2015). However,

84 introducing exogenous cavitation nuclei into the acoustic field substantially lowers the energy required to
85 induce cavitation-mediated bioeffects as initial bubble formation is no longer required (Bhatnagar, et al.
86 2014). This can be achieved through the use of microbubble-based ultrasound contrast agents.
87 Microbubbles are 1-10 μm gas-filled particles stabilized by a lipid, protein or polymer shell (Sirsi and
88 Borden 2009). When subjected to ultrasound, they oscillate and/or collapse, providing biological
89 permeabilization similar to that described above, but with acoustic parameters in line with what is
90 employed for clinical diagnostic imaging (i.e. with MIs less than the U.S. Food and Drug Administration's
91 limit of 1.9) (Delalande, et al. 2011, Lentacker, et al. 2014). Indeed, drug delivery by this technique can
92 be achieved with diagnostic ultrasound imaging **equipment**, which provides the added benefit of
93 simultaneous ultrasound image guidance (Kotopoulos, et al. 2013).

94 The use of ultrasound-stimulated microbubbles for drug delivery has been studied extensively for
95 the disruption of vascular barriers (Martin and Dayton 2013, Sennoga, et al. 2017). For applications such
96 as opening the blood brain barrier (Burgess and Hynynen 2014, Wu, et al. 2017, Zhao, et al. 2013) or
97 enhancing vascular permeability within solid tumors (Mullick Chowdhury, et al. 2017, Qin, et al. 2016),
98 microbubbles are administered intravenously and are stimulated with an external ultrasound source. We
99 envision an adaptation of this technology for GI drug delivery, which would involve co-administration of
100 contrast agents with a medicated enema in the colon followed by ultrasound stimulation using an
101 endoscopic probe. With the use of contrast agents, we believe that higher frequencies and lower pressures
102 may be used compared to the previously described, contrast-free methods of ultrasound-mediated GI drug
103 delivery. This approach may theoretically localize bioeffects more precisely, as permeabilization would
104 only occur where contrast agents are present (e.g., at the GI wall). This would reduce concerns of
105 unsuppressed cavitation activity outside the desired treatment area (e.g., surrounding tissues).

106 As a first step towards this goal, we studied the dynamics of epithelial monolayer disruption and
107 recovery *in vitro* using phase-change ultrasound contrast agents (PCCAs) stimulated with 1.0 MHz
108 ultrasound pulses. PCCAs are liquid perfluorocarbon-filled particles that can be vaporized into
109 acoustically active microbubbles with the application of an ultrasound pulse of sufficient amplitude. In
110 the liquid state, PCCAs are characterized by nanometer-range size distributions (100-300 nm) that may
111 allow more thorough permeation through the GI mucus mesh compared to microbubbles (0.5-10 μm (Sirsi
112 and Borden 2009)). Once vaporized, PCCAs form microbubbles several microns in diameter that can be
113 utilized to enhance drug delivery.

114 Typically, PCCAs are created using perfluorocarbons with bulk boiling points near body
115 temperature (e.g. dodecafluoropentane, b.p. = 29 °C). These agents require acoustic pressures in the range
116 of 3-6 MPa to initiate vaporization (Burgess and Porter 2015, Williams, et al. 2013), which may induce
117 undesired tissue bioeffects (e.g., cell lysis or tissue heating) for applications where transient drug delivery
118 is desired. We have developed a low boiling point formulation of PCCAs filled with octofluoropropane
119 (OFP), which has a bulk boiling point of -36.7°C. These agents can be vaporized with peak negative
120 pressures as low as 300 kPa (at 1.0 MHz) (Fix, et al. 2017) and with clinically available hardware. We
121 believe that this low boiling point PCCA formulation is ideal for GI drug delivery applications where
122 transient permeabilization is desired without permanent tissue damage.

123 The primary objective of this study is to demonstrate the potential of using ultrasound-stimulated
124 PCCAs to cause transient disruption of confluent colorectal adenocarcinoma (Caco-2) epithelial
125 monolayers and enhance the permeation of a model macromolecular drug. This represents a first step
126 towards the goal of *in vivo* GI drug delivery applications.

127

128 MATERIALS AND METHODS

Phase-change ultrasound contrast agent (PCCA) fabrication and characterization

PCCAs were generated as previously described (Sheeran, et al. 2011, Sheeran, et al. 2012). Briefly, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene-glycol)-2000 (DSPE-PEG2000) (both lipids >99% purity, Avanti Polar Lipids, Alabaster, AL, USA) were combined in a 9:1 molar ratio and solubilized in a phosphate-buffered saline (PBS)-based solution (1× PBS: 11.9 mM phosphates, 137 mM sodium chloride, 2.7 mM potassium chloride) containing 15% propylene glycol (v/v) (≥99.5% purity, Fisher Chemical, Waltham, MA, USA) and 5% glycerol (v/v) (≥99.5% purity, Fisher Chemical, Waltham, MA, USA) for a final combined concentration of 1.0 mg/mL. Aliquots (1.5 mL) of this solution were dispensed into 3.0 mL glass vials, the headspace air was replaced with octofluoropropane (OFP) gas (99.8% purity, FluoroMed, Round Rock, TX, USA), and microbubbles were generated via shaking for 45 seconds in a VialMix device, which operates at a frequency of 4530 ± 100 oscillations per minute (Bristol-Myers-Squibb, New York, NY, USA). Finally, PCCAs were formed via microbubble condensation through simultaneous cooling and pressurization of the microbubble suspension (Sheeran, et al. 2012). The size distribution of resultant PCCAs was characterized using a NanoSight NS500 (Malvern Instruments, Westborough, MA, USA) (detection capability 50-2000 nm) (n=3 representative vials).

Do note that during *in vitro* experiments performed at 37°C, a portion of the PCCA sample is lost to spontaneous vaporization due to the very low boiling point of OFP. However, we previously showed that stimulating cells with ultrasound and OFP-filled PCCAs below the vaporization threshold of the PCCAs does not cause barrier permeabilization and does not result in substantial cavitation activity (Fix, et al. 2017). This indicates that spontaneous vaporization alone likely does not contribute a substantial enough bubble population to influence drug delivery outcome. Furthermore, *in vivo* imaging studies from our group have shown that while some spontaneous vaporization occurs after intravenous injection of OFP

PCCAs, substantial vaporization can be achieved in a controllable manner via ultrasound-induced vaporization (Sheeran, et al. 2015).

PCCA vaporization captured via high-speed optical microscopy

PCCA vaporization events were captured at a range of acoustic settings using high-speed optical microscopy, using a previously described experimental set-up (Sheeran, et al. 2013). Briefly, a temperature-controlled water bath (37°C) was mounted on an inverted microscope with a 100× water immersion objective (Olympus IX71, Center Valley, PA, USA), and the microscope was interfaced with a high-speed camera to capture vaporization events (FastCam SA1.1, Photron USA, Inc., San Diego CA, USA).

First, a calibrated needle hydrophone (HNA-0400, Onda Corp., Sunnyvale, CA, USA) was aligned to the optical focus of the microscope and subsequently used to characterize the peak negative pressure (PNP) of a 1.0 MHz unfocused piston transducer (IP0102HP, Valpey Fisher Corp., Hopkinton, MA, USA). The transducer was excited with sinusoidal pulses generated by an arbitrary function generator (AFG3021, Tektronix, Inc., Beaverton, OR, USA) and amplified approximately 55 dB with a power amplifier (A500, ENI, Rochester, NY, USA). After the transducer was calibrated, the hydrophone was replaced with a 200-μm inner diameter microcellulose tube (Spectrum Labs, Inc., Rancho Dominguez, CA, USA), which was also aligned to the optical focus of the microscope. This process ensured that contents of the microcellulose tube that were visible in the microscope focus would experience the acoustic pressure calibrated at that location. The microscope field of view was 130 μm × 130 μm.

PCCAs were diluted 1:4 in PBS and perfused through the microcellulose tube. Injection of the PCCAs was followed by a brief waiting period to allow particles in the field of view to become nearly stationary, which minimized blurring in the captured images. Previous work has shown that PCCA

175 vaporization is a pressure-threshold dependent phenomenon, independent of pulse length when sub-
176 millisecond pulses are used (Fabiilli, et al. 2009, Lo, et al. 2007). Therefore, to determine an approximate
177 pressure threshold for vaporization, PCCAs were exposed to single 20-cycle acoustic pulses with PNPs
178 of 100, 200, 300, 400, 500, or 600 kPa.

179 A synchronization pulse from the function generator was used to trigger the high-speed camera
180 and initiate video recording at 500 frames per second. Videos were set to begin recording just before the
181 triggered ultrasound pulse, which allowed complete PCCA vaporization events within the optical field of
182 view to be captured. Videos were reviewed offline using ImageJ to determine whether or not vaporization
183 was achieved at the various acoustic excitation parameters (National Institutes of Health, Bethesda, MD,
184 USA).

185 Cell culture

186 Human colorectal adenocarcinoma (Caco-2) cells were chosen as an *in vitro* model of intestinal
187 absorption. When Caco-2 monolayers are grown to confluence, they differentiate, developing polarity and
188 tight junctions between cells. This accurately mimics the human intestinal epithelial barrier, making Caco-
189 2 permeability testing a reliable tool for predicting *in vivo* intestinal absorption of various compounds (van
190 Breemen and Li 2005).

191 Mycoplasma-free caco-2 cells were purchased from the American Type Culture Collection
192 (ATCC) and cultured using minimum essential medium without L-glutamine (Corning Inc., Corning, NY,
193 USA) supplemented with 100 U/mL penicillin/streptomycin, 1× non-essential amino acid supplement
194 (NEAA), 1× sodium pyruvate, 1× L-glutamate, and 20% heat-inactivated fetal bovine serum (FBS)
195 (VWR, Radnor, PA, USA) in a humidified, 37°C incubator with 5% CO₂ atmosphere. Low passage
196 number (passage 15) Caco-2 cells were seeded at 150,000 cells per well onto Transwell supports with 0.4
197 µm pores (12-well plate, 12 mm membrane diameter, 1.12 cm² cell growth area, Corning Inc., Corning,

198 NY, USA) and allowed to grow for approximately 10 days to form confluent monolayers. Monolayer
199 integrity was regularly monitored via the measurement of transepithelial electrical resistance (TEER)
200 (World Precision Instruments, Sarasota, FL, USA). Monolayers were used for permeability experiments
201 after TEER values had reached at least 500 Ohm (560 Ohm·cm²), which is similar to TEER values
202 reported in the literature for this model (Kudsiova and Lawrence 2008, Srinivasan, et al. 2015).
203 Furthermore, above this threshold, macromolecular tracers (e.g., the 70 kDa dextran used here) are unable
204 to permeate through the Caco-2 monolayers.

205 **Ultrasound alignment strategy**

206 Caco-2 monolayers were sonicated from the apical side using a 1.0 MHz unfocused piston
207 transducer (diameter = 8 mm, -6 dB ‘focal spot’ = ~6 mm diameter, IP0102HP, Valpey Fisher Corp.,
208 Hopkinton, MA, USA). A custom water bath was designed to (1) facilitate consistent alignment of the
209 transducer above the center of each well and (2) reduce acoustic reflections from the base of the well plate
210 (Figure 1). The 14 cm×10 cm×8.5 cm (L×W×H) water bath was created using 3.2 mm acrylic sheets. For
211 each experiment, a 12-well culture plate was placed on an internal ledge within the bath, providing
212 coupling between the bottom of the well plate and the pre-heated 37°C water below. **Water was maintained**
213 **at 37°C for the duration of the experiment by placing the apparatus on a heated plate. Coupling between**
214 **the well plate and water** was designed to minimize acoustic reflections that would have otherwise occurred
215 at the air-plastic interface, **although we do note that it is unlikely that this strategy entirely eliminated**
216 **reflections and standing waves.** A lid for the water bath was created with 1.0 cm-diameter circular holes
217 centered above each well. This served as a method to hold the ultrasound transducer centered within each
218 well at a consistent height above the cells (6 mm). The full design of this water bath can be found in the
219 supplemental materials, which includes complete instructions for fabrication (available in Mendeley Data
220 repository online).

Ultrasound-mediated FITC-dextran delivery through epithelial monolayers

Twelve-well plates containing confluent Caco-2 monolayers on Transwell® supports were positioned in the custom water bath as described above. Complete cell media (400 µl) containing PCCAs (0.5 µl; 1.75×10^9 particles/mL; roughly matching the circulating concentration of OFP-PCCAs used as vascular ultrasound contrast agents (Sheeran, et al. 2015)) and 70 kDa FITC-dextran (50 µg total; 0.125 mg/mL; similar in concentration to prior reports of colonic delivery of fluorescent dextran in mice (Schoellhammer, et al. 2017)) was added to the donor chamber (apical side) of a well to simulate absorption from the intestinal lumen. The 70 kDa dextran was chosen to model macromolecular drugs typically unable to pass the GI epithelial barrier. Subsequently, the 1.0 MHz transducer was placed in center of the well and excited with amplified sinusoidal signals from an arbitrary function generator (AFG3021C, Tektronics, Inc., Beaverton, OR, USA and 3100LA Power Amplifier, ENI, Rochester, NY, USA) (Figure 1). A pulse repetition frequency of 5000 Hz and exposure time of 30 seconds was used for all conditions. Pulse lengths of 20 cycles (10% duty cycle) or 40 cycles (20% duty cycle) were employed, and peak negative pressures were varied from 300 to 600 kPa. Control samples were exposed to (1) sham treatment (without PCCAs or ultrasound), (2) PCCAs only, and (3) ultrasound only (using the highest energy condition of 600 kPa and 40 cycle pulse length). A summary of the conditions tested can be found in Table 1, including experimental replicates for each group.

Samples (100 µl) were collected from the receiving chamber (basolateral side) at the following time points after treatment and replaced with cell media: ~5 min, 3 hr, 8 hr, 24 hr, 48 hr, and 72 hr. Dextran concentration was determined via fluorescence intensity (excitation: 485 nm, emission: 528 nm) using a Cytation 5 Plate Reader (BioTek, Winooski, VT, USA). Percent dextran delivery was calculated based on the mass initially added to the donor compartment, correcting for the mass removed through sampling at

each time point and considering 100% to be the receiving well concentration if equilibrium had been established between both compartments.

TEER values were recorded at the same time points and used to monitor monolayer disruption and recovery dynamics. For all experiments, monolayers with a TEER value of at least 560 Ohms·cm² were considered intact, and monolayers below this threshold were considered disrupted. “Time to monolayer recovery” was calculated as a summary metric to quantify the degree of monolayer disruption caused by sonication. For monolayers that recovered by the last measurement time point (72 hours), time to recovery was calculated as the time that TEER values remained below 560 Ohms·cm², using a linear interpolation between measured time points. Monolayers that never dropped below 560 Ohms·cm² therefore had a time to monolayer recovery of 0 hours.

Detection of cavitation signals and their persistence during 30 second ultrasound exposures

Cavitation is thought to be the main mechanical driver of biological barrier permeabilization with ultrasound. As such, we were interested in characterizing the generation and persistence of stable and inertial cavitation energy generated by ultrasound-stimulated PCCAs during 30 second exposures.

We detected cavitation signals using a method adapted from our previous work (Fix, et al. 2017). PCCAs (1.75×10⁹ particles/mL) were suspended in 800 µl of 37°C PBS in a disposable polystyrene cuvette (One-way transmission factor at 1.0 MHz = 93±2% (n=10); thus, attenuation ≅ 7%) (FisherBrand, Thermo Fisher Scientific, Waltham, MA, USA). The unfocused 1.0 MHz transducer was shallowly submerged in the PCCA suspension and set to transmit the same ultrasound pulses that were used for transepithelial delivery (Table 1) with the only difference being that the pulse repetition frequency was reduced to 2000 Hz. Unfortunately, hardware limitations prevented us from saving data with a pulse repetition frequency of 5000 Hz to match the cell experiments. We do not believe that this discrepancy significantly alters the interpretation of our results, as discussed in subsequent sections.

266 A separate, single element, spherically focused transducer (7.5 MHz nominal frequency, measured
267 center frequency = 8.740 MHz, diameter = 19 mm, focal distance = 50 mm, -6 dB bandwidth = 1.270
268 MHz) (IL0706HP, Valpey Fisher Corp., Hopkinton, MA, USA) was arranged perpendicular to the
269 transmit transducer with its focus positioned within the PCCA solution. Acoustic signals received by this
270 transducer were passed through a receive amplifier (BR-640A, RITEC, Inc., Warwick, RI, USA) with 27
271 dB gain and a 500 kHz high pass filter. Signals were subsequently digitized using a 12-bit analog-to-
272 digital conversion card with a sampling rate of 200 MHz (GaGe model #CSE1222, DynamicSignals LLC,
273 Lockport, IL, USA) installed in a computer (Dell, Round Rock, TX, USA) running a custom acquisition
274 program (LabVIEW, National Instruments Corp., Austin, TX, USA).

275 We saved signals over a total of 30 seconds to monitor the dynamics of cavitation energy generated
276 from the acoustically-stimulated PCCAs. Ultrasound was transmitted continuously throughout the 30-
277 second period; however, signals were saved intermittently to reduce data to a manageable size. Each
278 second, 20 individual signals (i.e., 10 ms of data) were saved followed by a 990 ms saving delay. This
279 was repeated every second for a total of 30 seconds.

280 All saved data was post-processed using an adaptation of our previously described post-processing
281 analysis script in MATLAB (MathWorks Inc., Natick, MA, USA) to quantify stable and inertial cavitation
282 doses (Fix, et al. 2017). Briefly, for each individual radiofrequency line, the PCCA signal was selected by
283 applying a 30 μ s window from the point corresponding to the beginning of the acoustic pulse. Fast Fourier
284 transform was used to convert individual time-domain signals to the frequency domain in order to estimate
285 the level of stable and inertial cavitation. For stable cavitation, the area under curve (AUC) of the second
286 harmonic component was calculated considering a spectral window from 1.9 to 2.1 MHz. For inertial
287 cavitation, the broadband emission was quantified by calculating the AUC of frequency content ranging
288 from 5.25 to 7.75 MHz (while excluding the 6 and 7 MHz harmonic components). Stable and inertial

cavitation doses were then calculated by normalizing AUC values obtained for PCCAs with those calculated for a PBS-filled cuvette (without PCCAs) exposed to the same acoustic parameters. This was repeated for three independent vials of PCCAs; average cavitation doses (relative AUC) with inter-vial standard deviation are reported. Finally, average of relative AUC for stable and inertial cavitation are plotted over the 30 second period, and total cavitation dose for each was calculated by taking the area under these curves.

Statistical analyses

All statistical analyses were performed in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). P-values of <0.05 were considered statistically significant, and data are presented as average $\pm$ standard deviation throughout this work.

To evaluate which ultrasound parameters resulted in statistically significant dextran delivery, we performed a two-way repeated-measures ANOVA with respect to time and acoustic treatment. At each timepoint, the mean of each treatment group was compared to the mean of the sham control at the same time point via Dunnett's multiple comparisons testing. The same statistical procedure was applied to analyze changes in TEER values over time in various ultrasound treatment groups vs. time point-respective sham controls.

Cumulative dextran delivery achieved at 72 hours was analyzed via an additional two-way ANOVA with respect to acoustic pressure and pulse length. The purpose of this test was to evaluate the extent to which dextran delivery is affected by these factors (i.e. the percent of variability in dextran delivery that can be attributed to pressure vs. number of cycles). Following this two-way ANOVA, two multiple comparisons tests were performed. First, a Sidak multiple comparison test was used to compare mean delivery efficiency between 20 and 40 cycle cases at each pressure. Second, a Tukey multiple

comparison test was used to compare mean delivery efficiency between all rarefactional pressures separately for the 20 and 40 cycle cases.

The relationship between monolayer disruption and percent dextran delivery was assessed by calculating the correlation between time to monolayer recovery and cumulative percent dextran delivery at the 72-hour time point. Spearman correlation coefficients (r) and p -values are reported.

Linear regressions were performed to assess the relationships between pressure and (1) total stable cavitation dose, (2) total inertial cavitation dose, and (3) the sum of total stable and inertial cavitation. This was done separately for 20-cycle and 40-cycle conditions. Again, goodness-of-fit (r^2) and p -values are reported.

RESULTS AND DISCUSSION

PCCA characterization

The PCCA formulation was characterized by a polydisperse size distribution with an average concentration of $(1.4 \pm 0.2) \times 10^{12}$ particles/mL and mean particle size of 170 ± 20 nm ($n=3$ independent vials) (Figure 2A). Figure 2B shows representative optical microscopy images captured before ($t = -2$ ms) and after ($t = 8$ ms) a single 20-cycle ultrasound pulse at $t = 0$ ms. We found that PCCAs consistently vaporized into microbubbles at and above a peak negative pressure of 300 kPa, but showed little to no activation at lower pressures (at 1.0 MHz), which is consistent with our previous findings (Fix, et al. 2017). Qualitatively, we observed an increase in the number of bubbles generated with a single ultrasound pulse as pressure was increased from 300 kPa to 600 kPa (Figure 2A). From these experiments, we concluded that the pressure threshold for PCCA vaporization is between 100 – 300 kPa at 1.0 MHz, and therefore pressures at or above 300 kPa were used for all subsequent experiments.

Ultrasound-mediated FITC-dextran delivery through epithelial monolayers

334 As expected, 70 kDa dextran was not able to penetrate sham-treated Caco-2 monolayers over the
335 72 hr incubation period, confirming that effective barrier function had been established in our model
336 (Figure 3A). Similarly, we did not observe appreciable delivery through control monolayers treated with
337 (1) PCCAs only (no ultrasound) or (2) ultrasound only (no PCCAs, ultrasound stimulation at the highest
338 setting of 600 kPa and 40 cycles) (Figure 3A). This indicated that neither our agents nor ultrasound alone
339 altered monolayer integrity with the parameters used in our study. Correspondingly, TEER values for all
340 control groups remained well above the previously chosen threshold for an intact monolayer (560
341 $\text{Ohm}\cdot\text{cm}^2$) throughout the 72-hour observation period, further demonstrating that control treatments did
342 not significantly alter membrane integrity (Figure 4A). Neither PCCAs alone nor ultrasound alone resulted
343 in statistically significant changes in TEER values at any time point compared to the time-point respective
344 sham controls (Table 2).

345 When ultrasound was combined with PCCAs we were able to achieve significant delivery (vs.
346 time-point respective sham controls) of both dextran sizes with select parameter combinations (Figure 3B
347 and 3C). When substantial delivery was achieved, it most often reached statistically significant levels
348 between 8-24 hours post-treatment with cumulative percent delivery increasing slightly thereafter. We
349 note that we do not see appreciable dextran accumulation before the 8 hr time point in any case, and we
350 speculate that the lag time may be due to the diffusional characteristics of the dextran molecules. Future
351 experiments will look into the origin of this lag time with greater detail. In general, the dextran delivery
352 results correspond with TEER values indicating significant monolayer disruption (Figure 4 and Table 2).
353 For all ultrasound-stimulated PCCA treatments, we observed an immediate and statistically significant
354 reduction in TEER, with all groups characterized by average TEER values in the range of 200-280
355 $\text{Ohm}\cdot\text{cm}^2$ approximately 5 minutes post-treatment (Figure 4 and Table 2). TEER values tended to recover
356 within 24-48 hours post-treatment, regaining values $\geq 560 \text{ Ohm}\cdot\text{cm}^2$. The 300-kPa treatment groups were

the only conditions that did not show 100% recovery of all monolayers by the 72-hour time point. For the 300 kPa treatment groups, we achieved 33% and 50% recovery for the 20 and 40 cycle cases, respectively.

Treatment groups diverged with respect to monolayer recovery time. To investigate this further, we interpolated “time to monolayer recovery” for each treatment (i.e., time for a monolayer to regain a TEER value $\geq 560 \text{ Ohm}\cdot\text{cm}^2$). This summary value, which captures the monolayer recovery dynamics, correlated well with the cumulative percent dextran delivered ($r = 0.8475$, $p < 0.0001$) (Figure 5). Biologically, the main factor dictating dextran delivery efficiency appears to be how long the monolayers remain leaky.

In terms of acoustic parameters, rarefactional pressure has a greater influence on dextran delivery outcome than pulse length. To test this observation statistically, we performed a two-way ANOVA to quantify the relative contributions of acoustic pressure and pulse length to overall variation in cumulative dextran delivery (72 hr time point). We found that pressure ($p = 0.0002$) but *not* pulse length ($p = 0.1476$) was a significant source of variability in percent dextran delivery, with pressure accounting for 53.5% of the total variation compared to 3.5% attributed to pulse length. This can be assessed visually in Figure 6, where the cumulative percent delivery varies substantially with respect to pressure but remains fairly consistent between pulse lengths. Indeed, multiple comparison testing showed that differences in cumulative dextran delivery between 20 and 40 cycle conditions were statistically insignificant at each rarefactional pressure.

The most interesting and unexpected finding was that the 300 kPa exposure conditions consistently provided significantly greater dextran delivery compared to higher-pressure groups, while the higher-pressure conditions (400, 500, and 600 kPa) provided similar results (statistically insignificantly different) (Figure 6). These findings are counter to our initial hypothesis that as acoustic energy was increased, a greater proportion of PCCAs would vaporize into microbubbles and cavitation dose would be more

pronounced, together leading to more dramatic biological effects and ultimately greater dextran delivery efficiency. We hypothesize that these unexpected findings may be explained by differences in cavitation *persistence* between ultrasound exposure conditions, as is explored in the subsequent section.

Persistence of cavitation over 30 second exposures

In our experiments, as would be the case for GI drug delivery applications, the same sample of PCCAs were stimulated for the duration of the ultrasound exposure time (30 seconds in this study). Therefore, at high pressure, PCCAs may be rapidly converted to microbubbles but also subsequently destroyed via inertial cavitation. This would limit the effective cavitation duration to a fraction of the ultrasound on time (30 s). Conversely, if PCCAs were being vaporized more slowly and were able to persist for longer at lower pressures, the effects of microbubble cavitation would have been felt by the cells for a longer period of time, enhancing the biological effect.

To test this hypothesis, we quantified stable and inertial cavitation dose generated from acoustically-stimulated PCCAs *in vitro* over the course of 30 seconds. All parameters matched those of our dextran delivery experiments except for pulse repetition frequency, which was reduced to 2000 Hz.

For all acoustic exposure conditions (300-600 kPa with 20 and 40 cycle pulse lengths), we observed substantial stable and inertial cavitation immediately after the ultrasound transmission was initiated (Figure 7A-D). For 20 cycle pulse lengths, there is a clear separation in cavitation dynamics with respect to rarefactional pressures, with lower pressures resulting in more sustained stable and inertial cavitation compared to the higher pressures. The lower pressures (especially 300 kPa) provide sustained stable and inertial cavitation activity over the entire 30 second exposure, while cavitation dose decreases dramatically within the first 15 s for the higher pressures (especially 600 kPa) (Figure 7A & 7C). This trend is also observed for stable cavitation dose with respect to pressure at 40 cycles (Figure 7B). This is less pronounced for inertial cavitation dose at 40 cycles, as cavitation dose decreases more rapidly for all

403 pressures (Figure 7D). In general, these data demonstrate that both peak rarefactional pressure and pulse
404 length influence cavitation persistence for PCCAs, as has previously been reported for microbubbles
405 (Pouliopoulos, et al. 2016). The lower pulse repetition frequency used for these experiments compared to
406 the dextran delivery experiments may have biased results slightly. At a higher pulse repetition frequency,
407 cavitation dose would be expected to decrease more rapidly for all conditions, thus this effect is likely
408 more pronounced in our cell experiments at higher pulse repetition frequency.

409 In general, we hypothesize that two main factors contribute to the inverse trend between total
410 cavitation dose and pressure. First, we qualitatively observed that PCCA vaporization efficiency increases
411 with increasing pressure. This can be observed in the representative images shown in Figure 2, where
412 more bubbles are generated after a single ultrasound pulse at 600 kPa vs. 300 kPa. This qualitative finding
413 is supported by previous work showing that relative PCCA vaporization efficiency increases with
414 increasing pressure (Reznik, et al. 2013, Wu, et al. 2018). As a result, it will take longer to vaporize all
415 PCCAs in the sample with lower pressures, contributing to sustained cavitation activity over the 30-second
416 exposure time. Secondly, as pressure increases, we believe that generated microbubbles will be destroyed
417 more rapidly, ultimately limiting microbubble lifetime and cavitation persistence. A similar inverse trend
418 between generated microbubble stability/survival and pressure has previously been described by Reznik
419 et al. (Reznik, et al. 2013).

420 To quantitatively analyze the trends in acoustic cavitation with respect to pressure, we first
421 calculated the cumulative stable cavitation dose (SCD) and cumulative inertial cavitation dose (ICD) by
422 calculating the area under each of the cavitation curves over the 30 s ultrasound exposure. This provided
423 single values summarizing the total SCD and ICD generated by each condition. We also summed these
424 cumulative SCD and ICD values to provide an estimate of the overall cavitation dose generated per
425 condition (SCD+ICD). Linear regressions were performed between each of these summary values and

426 acoustic pressure (Figure 7E-F). For the 20 cycle cases, we find statistically significant inverse trends
427 between pressure and total SCD+ICD and total SCD ($p = 0.0007 - 0.0100$). For the 40 cycle cases, all
428 three summary cavitation metrics were found to significantly and inversely trend with pressure
429 ($p = 0.0049 - 0.0100$). For both pulse lengths, 300 kPa qualitatively provided the greatest cumulative
430 SCD, ICD and SCD+ICD compared to all higher-pressure groups, which corresponds with our finding
431 that 300 kPa results in the greatest dextran delivery efficiency.

432 **Limitations**

433 Total cavitation dose alone cannot predict dextran delivery efficiency as evidenced by the
434 discrepancy we find between 20 and 40 cycle conditions: dextran delivery outcomes are comparable across
435 pulse lengths, while total cavitation doses were in general lower for 40 cycle conditions compared to
436 respective 20 cycle conditions. For the 20 cycle conditions, we find a significant correlation between
437 cumulative dextran delivery and (1) total SCD (Pearson $r = 0.9624$, $p = 0.0376$) and (2) total SCD+ICD
438 (Pearson $r = 0.9638$, $p = 0.0362$). However, for the 40 cycle cases we do not find significant correlations
439 between dextran delivery and any of the total cavitation metrics. This discrepancy may be due to various
440 confounding variables that were not captured by the total cavitation dose metric. For instance, we
441 hypothesize that peak cavitation dose and the balance between stable and inertial cavitation may be
442 important variables contributing to biological outcome.

443 Furthermore, acoustic pressure and pulse length are known to influence the stability and size
444 distribution of microbubbles generated by PCCA vaporization (Reznik, et al. 2013, Sheeran, et al. 2013),
445 and differences in microbubble populations may have influenced dextran delivery efficiency. The
446 proportion of small microbubbles generated by PCCA vaporization has previously been show to increase
447 with increasing acoustic pressure (Fix, et al. 2017, Sheeran, et al. 2013), which is attributed to the inverse
448 relationship between vaporization pressure threshold and PCCA size (Sheeran and Dayton 2012, Sheeran,

et al. 2013). Additionally, the likelihood of microbubble fusion (i.e., generation of larger microbubbles) increases with increasing pulse length (Sheeran, et al. 2013). Furthermore, differences in bubble aggregation and coalescence due to secondary Bjerknes forces may alter the number and size of bubbles between treatment groups. These expected differences in microbubble size distribution as a function of acoustic excitation parameters may influence monolayer disruption efficiency.

After ultrasound-mediated monolayer disruption, TEER value analysis indicated that it typically took 24-48 hours for the epithelial cells to regain their integrity. While recovery mechanism was not explicitly studied in this work, this rather long recovery time may indicate that recovery is due to cell repopulation and growth rather than resealing of transiently opened tight junctions between cells. A limitation of this study is that we did not quantify cell death after treatment. Furthermore, not all monolayers treated with the 300 kPa conditions recovered within the 72-hour observation window. We do not claim that we have found optimal acoustic parameters for transepithelial delivery *in vivo*. Rather, this study provides an encouraging proof of principle demonstration that transepithelial delivery is feasible with ultrasound stimulated PCCAs and supports further optimization of acoustic parameters to maximize transient disruption while minimizing recovery time and cell death.

It is important to note that cell-based assays of intestinal permeabilization are known to overestimate damage compared to the what would be observed in viable intestinal tissue (in the context of chemically induced permeability enhancement) (McCartney, et al. 2016, Petersen, et al. 2013). This is attributed to the lack of complete intestinal repair mechanisms and protective mucus in simple cell culture assays. As a result, any future optimization of acoustic parameters should be carefully validated (e.g., through the *in situ* intestinal perfusion (Escribano, et al. 2012)) to ensure that the chosen parameters are effective and safe when working with viable intestinal tissue. One of the greatest safety concern for intestinal barrier disruption *in vivo* is the risk of facilitating absorption of harmful bystanders such as

bacteria, viruses, and toxins (McCartney, et al. 2016). This risk should be carefully evaluated and minimized upon *in vivo* translation.

Future directions – potential for ultrasound-mediated GI drug delivery with PCCAs

The data presented herein represent a first step towards the ultimate goal of ultrasound-mediated GI drug delivery with PCCAs, and the finding that lower pressure results in greater delivery efficiency is important for successful *in vivo* translation. In the *in vivo* setting, we envision co-administering a medicated enema with a solution of PCCAs. Subsequently, an endoscopic ultrasound probe would be inserted and used to stimulate PCCAs for GI permeabilization. Without natural replenishment of the PCCAs (e.g., through blood flow), achieving persistent cavitation by limiting the destruction of generated microbubbles will be of utmost importance.

This is in contrast to an analogous application where ultrasound-stimulated contrast agents are used to enhance drug delivery through vascular barriers. For vascular disruption, microbubbles are administered intravenously, and blood flow provides continuous replenishment of intact microbubbles. Indeed, delivery efficiency has been shown to increase with increasing pressure for this application (Chen and Konofagou 2014, Liu, et al. 2012, Wang, et al. 2015), and microbubble reperfusion into the focal zone can be achieved by alternating sonication with rest time (Mullick Chowdhury, et al. 2016, Wang, et al. 2015) or theoretically by using short pulses and long pulse repetition periods (Shamout, et al. 2015). However, using high pressures for vascular permeabilization is associated with an increased risk of undesirable bioeffects (Baseri, et al. 2010), pointing to another motivation for using reduced pressures for any drug delivery application. To this end, Pouliopoulos and colleagues have demonstrated the value of redefining the acoustic pulses typically used for vascular permeabilization to provide sustained, controllable, and safe cavitation activity with low pressures and short pulse lengths (Pouliopoulos, et al. 2014, Pouliopoulos, et al. 2016).

495 The likelihood that ultrasound will induce cavitation is related to the mechanical index (MI = peak
496 rarefactional pressure divided by center frequency) of the transmitted pulse. The United States Food and
497 Drug Administration stipulates that MI cannot exceed 1.9 for clinical diagnostic ultrasound, which is
498 intended to prevent cavitation-mediated biological effects in the absence of exogenous contrast agents.
499 Therefore, when ultrasound-mediated GI permeabilization is performed in the absence of contrast agents,
500 MIs above this threshold (MI~2.2 – 3.3) are often employed to generate the cavitation activity necessary
501 for permeabilization (Schoellhammer, et al. 2017, Schoellhammer, et al. 2017, Schoellhammer, et al.
502 2015). (Note: these MIs were estimated based on the reported acoustic intensities for *ex vivo* experiments,
503 assuming plane wave transmission, an acoustic impedance of water of $1.48 \times 10^6 \text{ kg} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, negligible
504 tissue attenuation, and that the reported intensity represented instantaneous acoustic intensity.) While
505 these protocols have generally been described as safe in preclinical studies (Schoellhammer, et al. 2017,
506 Schoellhammer, et al. 2015), cavitation-mediated biological effects could be induced in tissues beyond
507 the GI epithelium. Conversely, we were able to achieve efficient drug delivery at a mechanical index of
508 0.3 (300 kPa at 1.0 MHz), which is well below the FDA limit of MI = 1.9, indicating that biological effects
509 will likely only be induced in areas with direct contact to the contrast agents. This claim is supported by
510 the absence of monolayer disruption (as evidenced by stably high TEER values and impermeability to
511 dextran) when cells were stimulated with ultrasound alone in our experiments. This offers an opportunity
512 for improved treatment localization and the potential for enhanced safety compared to ultrasound-
513 mediated GI drug delivery in the absence of contrast agents.

514 In the GI space, we believe low boiling point PCCAs will provide greater success than standard
515 microbubbles. Unlike microbubble contrast agents, the small size of PCCAs may allow permeation
516 through the GI mucus mesh and permeabilization of the underlying GI epithelium. In future studies, we

will test the penetration of PCCAs through mucus and optimize acoustic parameters for GI drug delivery in a model that more accurately captures the *in vivo* setting (e.g., *in situ* intestinal perfusion).

The use of ultrasound for drug delivery offers an opportunity for simultaneous image guidance. This will be particularly important for GI drug delivery applications where site-specific (e.g., immunotherapy delivery to a colon tumor) rather than global GI drug delivery is desired. Ultrasound image guidance could be achieved in one of two ways. First, it may be possible to achieve ultrasound imaging and subsequent drug delivery with clinically available endoscopic ultrasound probes (e.g. with 5 MHz center frequency). A similar approach has been published for externally applied ultrasound for drug delivery to pancreatic tumors with a clinically available machine (Dimcevski, et al. 2016, Kotopoulis, et al. 2013). Perhaps more exciting is the prospect of developing dual frequency endoscopic ultrasound probes that incorporate aligned low frequency elements for optimal drug delivery and high frequency elements for high-resolution ultrasound imaging. The prospect of high-frequency and therefore high-resolution image guidance may be particularly advantageous for small animal studies where anatomical features are difficult to resolve with relatively low-frequency imaging. Dual frequency transducers are currently under development for contrast-enhanced intravascular and intracavity imaging (Kim, et al. 2015, Lindsey, et al. 2017, Wang, et al. 2017), which could be modified for image-guided therapy purposes. A dual frequency approach can also be employed to initiate PCCA phase change at high frequency and detect the unique acoustic signature of PCCA vaporization at low frequency (Arena, et al. 2015). This would offer a method to image the efficiency and duration of PCCA vaporization events during the treatment.

CONCLUSIONS

We have demonstrated efficient delivery of a macromolecular drug mimic (70 kDa FITC-dextran) through otherwise impermeable Caco-2 epithelial monolayers using ultrasound-stimulated PCCAs. We

found that the lowest pressure conditions (300 kPa) consistently provided the greatest dextran delivery efficiency (vs. 400-600 kPa), which is explained in part by the observation that cavitation is more persistent during ultrasound exposure at lower pressures. While we believe that persistent cavitation activity is important for achieving efficient epithelial disruption, we have not fully explored other acoustic metrics that may also play a role, such as acoustic radiation force, peak cavitation dose, and variable size distributions of acoustically generated microbubbles. Further experimentation should be conducted to evaluate the relative importance of these variables and their contribution to dextran delivery outcome. Insight gleaned from these experiments will allow for rational and thorough optimization of acoustic parameters for *in vivo* drug delivery through the GI epithelial barrier.

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P.A.D declares that he is a co-inventor on a patent describing the formulation of low-boiling point perfluorocarbon agents and a co-founder of Triangle Biotechnology, a company that has licensed this patent. All the other authors declare that they have no competing interests.

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723

724 **Figure 1: (Color online) Sonication strategy.** Colorectal adenocarcinoma (Caco-2) cells were cultured
725 on permeable Transwell supports. Phase-change contrast agents (PCCAs) and dextran were added to the
726 donor chamber before sonication and samples were collected from the receiving compartment over the
727 following 72 hours post-ultrasound treatment. The twelve-well culture plates were positioned in a custom
728 water bath and coupled to the 37°C water below before treatment. A lid with circular holes was used to
729 align the ultrasound transducer in the center of each well at a consistent height above the cells.

730

731 **Figure 2: (A) PCCA characterization.** The size distribution and concentration of PCCAs were
732 characterized using a Nanosight. The PCCA formulation was characterized by a polydisperse size
733 distribution with an average concentration of $(1.4 \pm 0.2) \times 10^{12}$ particles/mL and mean particle size of
734 170 ± 20 nm (n=3 independent vials). **(B) Acoustic PCCA vaporization.** Representative high speed optical
735 microscopy images showing PCCA vaporization as a function of rarefactional pressure. Considerable
736 PCCA vaporization is observed at and above 300 kPa, with the number of generated microbubbles
737 increasing with increasing pressure. **Scale bar = 10 μ m**

738 **Abbreviations:** PCCA – phase change contrast agent; kPa – kilopascal

739

740 **Figure 3: Dextran delivery through Caco-2 monolayers.** Dextran delivery is presented as a percentage
741 of the maximum dextran mass that would have been found in the receiving compartment if equilibrium
742 had been achieved between the donor and receiver Transwell compartments (i.e. if there was no barrier
743 between chambers). (A) Negligible amounts of the 70 kDa dextran permeated through control treated
744 monolayers over the 72-hour incubation period. When monolayers were sonicated in the presence of
745 PCCAs, significant delivery was achieved with select acoustic parameters (B and C). Stars indicate the
746 results of Dunnett's multiple comparison testing at each timepoint comparing mean percent dextran

delivery of each group to that of the sham control for that timepoint after two-way ANOVA. Data is presented as mean $\pm$ SD. A total of n=3 replicates were performed for all conditions except the 300 kPa-40 cycle condition, which was repeated n=6 times. The three experimental replicates were performed on independent 12-well Transwell plates.

Key: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

Figure 4: (Color online) Transepithelial resistance (TEER) values before and after monolayer sonication. Control treated cells did not show any significant change in TEER values after manipulation, and TEER values remained above 560 Ohm $\cdot$ cm² (green dashed line) throughout the 72-hour observation period, indicating maintained monolayer integrity (A). For all monolayers treated with ultrasound and PCCAs, a significant decrease in TEER values was recorded immediately (~5 min) after treatment (B and C). Most of these monolayers recovered (regained TEER values $\geq$ 560 Ohm $\cdot$ cm² within 24-48 hours, with the exception of some monolayers treated with 300 kPa and either 20 or 40 cycle pulse lengths. Data is presented as mean $\pm$ SD. A total of n=3 replicates were performed for all conditions except the 300 kPa-40 cycle condition, which was repeated n=6 times. The three experimental replicates were performed on independent 12-well Transwell plates.

Figure 5: Correlation between cumulative dextran delivery and time to monolayer recovery. A significant correlation was found between percent dextran delivery at 72 hours and interpolated time to monolayer recovery.

Figure 6: Influence of pulse length and rarefactional pressure on overall dextran delivery outcome.

Dextran delivery efficiency was comparable for 20 cycle and 40 cycle groups at each rarefactional pressure (statistically insignificantly different). The 300 kPa conditions consistently provided the greatest dextran delivery outcomes compared to all higher-pressure conditions. Higher pressure conditions (400 – 600 kPa) provided similar delivery outcomes to each other. Statistical comparisons between pressures are shown with red dashed lines and # symbols for the 20-cycle case and with blue dotted lines and * symbols for the 40-cycle case.

Key: * and # $p \leq 0.05$; *** $p \leq 0.001$

Figure 7: (Color online) Generation of stable and inertial cavitation and trends with rarefactional

pressure. (A-D) Acoustic stimulation of PCCAs resulted in the generation of substantial stable cavitation (SCD) and inertial cavitation (ICD) for all acoustic conditions tested. The persistence of this cavitation over 30 seconds varied between conditions. In general, SCD and ICD was most persistent at the low-pressure conditions (300 or 400 kPa) with short pulse length (20 cycles). Total SCD and ICD dose was calculated as the area under the cavitation curves over 30 seconds. (E) For the 20 cycle conditions, we found significant inverse trend between rarefactional pressure and total SCD+ICD dose and SCD dose. (F) This trend held true for SCD+ICD, SCD and ICD doses for the 40 cycle conditions. All data presented as mean $\pm$ SD with n=3 replicates.

789 **Table 1: Summary of conditions tested.** Summary of the acoustic conditions tested for ultrasound-
790 mediated Caco-2 permeabilization with PCCAs, including the number of experimental replicates
791 performed.

792 **Abbreviations:** PCCA – phase change contrast agent; kPa – kilopascal; kDa – kilodalton; US –
793 ultrasound; Y – yes; N – no; NA – not applicable

794

Condition	PCCA (Y/N)	Pressure (kPa)	Cycles (#)	Dextran size (kDa)	Experimental replicates
1	Y	300	20	70	3
2		400			3
3		500			3
4		600			3
5	Y	300	40	70	6
6		400			3
7		500			3
8		600			3
9 - sham	N	NA	NA	70	3
10 - US only	N	600	40	70	3
11 - PCCA only	Y	NA	NA	70	3

811 **Table 2: Statistics describing transepithelial resistance (TEER) values before and after monolayer**
812 **sonication.** Stars indicate the results of Dunnett's multiple comparison testing at each timepoint
813 comparing mean TEER value of each group to that of the sham control for that timepoint after two-way
814 ANOVA.

815 **Key:** * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

816 **Abbreviations:** PCCA – phase change contrast agent; kPa – kilopascal; US – ultrasound; cyc – cycles.

Significantly different than respective sham control?							
Time point (hours)							
Group	pre	0	3	8	24	48	72
US only	ns	ns	ns	ns	ns	ns	ns

PCCA only	ns	ns	ns	ns	ns	ns	ns
600 kPa - 20 cyc	ns	****	****	****	***	ns	ns
500 kPa - 20 cyc	ns	****	****	****	****	**	ns
400 kPa - 20 cyc	ns	****	****	****	****	****	ns
300 kPa - 20 cyc	ns	****	****	****	****	****	****
600 kPa - 40 cyc	ns	****	****	****	****	*	ns
500 kPa - 40 cyc	ns	****	****	****	****	***	ns
400 kPa - 40 cyc	ns	****	****	****	**	ns	ns
300 kPa - 40 cyc	ns	****	****	****	****	****	****

A. Size distribution

B. PCCA vaporization

A. Controls

B. 20 cycles

C. 40 cycles

Time (hours)

A. Controls

B. 20 cycles

C. 40 cycles

Time (hours)

Cumulative % delivery
of dextran at 72 hrs

20 Cycles

40 Cycles