Impact of hydrostatic pressure on phase-change contrast agent activation by pulsed ultrasound

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(Received 20 November 2018; revised 10 May 2019; accepted 23 May 2019; published online 14 June 2019)

A phase-change contrast agent (PCCA) can be activated from a liquid (nanodroplet) state using pulsed ultrasound (US) energy to form a larger highly echogenic microbubble (MB). PCCA activation is dependent on the ambient pressure of the surrounding media, so any increase in hydrostatic pressure demands higher US energies to phase transition. In this paper, the authors explore this basic relationship as a potential direction for noninvasive pressure measurement and foundation of a unique technology the authors are developing termed tumor interstitial pressure estimation using ultrasound (TIPE-US). TIPE-US was developed using a programmable US research scanner. A custom scan sequence interleaved pulsed US transmissions for both PCCA activation and detection. An automated US pressure sweep was applied, and US images were acquired at each increment. Various hydrostatic pressures were applied to PCCA samples. Pressurized samples were imaged using the TIPE-US system. The activation threshold required to convert PCCA from the liquid to gaseous state was recorded for various US and PCCA conditions. Given the relationship between the hydrostatic pressure applied to the PCCA and US energy needed for activation, phase transition can be used as a surrogate of hydrostatic pressure. Consistent with theoretical predictions, the PCCA activation threshold was lowered with increasing sample temperature and by decreasing the frequency of US exposure, but it was not impacted by PCCA concentration.

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Pages: 3457–3466

I. INTRODUCTION

Tumor interstitial pressure (TIP) is often remarkably high compared to normal tissue and can impede the systemic delivery of anticancer drug treatment. Normal interstitial pressure is less than 0 mm Hg, which allows fluid and molecular transport from circulation to the tissue spaces. Tumors, like healthy tissues, need oxygen and nutrients to survive. In order to accommodate the demands of a growing tumor, blood vessels from surrounding tissue begin to grow into the tumor, a well-studied process known as angiogenesis (Folkman, 1995). Yet, unlike normal tissue, these newly formed blood vessels are disorganized, tortuous, and leaky (Jain, 2013). In addition to poor lymphatic drainage, high TIP is also a result of these abnormal blood vessels, which leak fluid and proteins into the area between tumor cells (Stohrer *et al.*, 2000). Direct measurements from a large sample of human (Chaudary *et al.*, 2012; Milosevic *et al.*, 2001; Nathanson and Nelson, 1994; Taghian *et al.*, 2005; Willett *et al.*, 2004) and animal (Chaudary *et al.*, 2012; Ferretti *et al.*, 2009; Geretti *et al.*, 2015; Halldorsdottir *et al.*, 2017; Heine *et al.*, 2012; Kłosowska-Wardega *et al.*, 2009; Lunt *et al.*, 2008; Murakami *et al.*, 2013; Pietras *et al.*, 2002; Reigstad *et al.*, 2016) subjects have demonstrated that interstitial pressure was found to be 5–30 mm Hg higher in cancerous tissue compared to normal tissue, although values exceeding 100 mm Hg have been recorded. Noteworthy, elevated TIP levels reduce blood flow and compromise

anticancer drug delivery (Jain, 1994). Knowledge of TIP is clinically important and could be used to help justify administration of tumor pressure-lowering drugs prior to the primary treatment (Goel *et al.*, 2011). The ability to customize individual patient treatment based on TIP knowledge would allow one to then monitor treatment response throughout the chemotherapeutic cycles.

Currently, TIP can only be measured in the clinic with invasive wick-in-needle or micropuncture techniques (Heldin *et al.*, 2004). TIP studies using the wick-in-needle technique have been conducted in cancers of the breast, cervix, head, neck, lymph nodes, and more (Curti *et al.*, 1993; Gutmann *et al.*, 1992; Jain *et al.*, 2007; Less *et al.*, 1992; Milosevic *et al.*, 2014). While the wick-in-needle technique has shown promise in monitoring TIP changes in response to cancer treatment (Taghian *et al.*, 2005), this approach is vulnerable to clotting if there is extravasated blood within the tissue. The wick-in-needle technique also requires custom-made needles and a well-trained user to perform accurate and consistent measurements. Conversely, micropuncture techniques do not allow for measurements in deep tissues; usually recordings can only be made down to a few millimeters from the surface. Furthermore, the glass micropipette breaks very easily during tissue penetrations and from the slightest patient motion.

A phase-change contrast agent (PCCA) that can be selectively activated from a liquid (nanodroplet) state using pulsed ultrasound (US) energy to form a larger highly echogenic microbubble (MB) has been described by several different groups (Choudhury *et al.*, 2017; de Gracia Lux *et al.*, 2017; Li *et al.*, 2015; Mountford *et al.*, 2014; Rapoport *et al.*, 2011;

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Sheeran *et al.*, 2012; Williams *et al.*, 2013). This perfluorocarbon (PFC)-based contrast agent permits dynamic contrast-enhanced US (DCE-US) imaging of both the intravascular and extravascular tumor spaces (Puett *et al.*, 2014; Williams *et al.*, 2013). It is important to note that PCCA activation is dependent on the ambient pressure of the surrounding media, so any increase in media stiffness or hydrostatic pressure demands higher US energies to phase transition these liquid nanodroplets into a gaseous MB (Hoyt *et al.*, 2016). In this paper, we explore this basic relationship as a potential direction for noninvasive pressure measurement and the scientific foundation of a new technology we are developing termed TIPE estimation using US (TIPE-US). Our basic assumption is that dysfunctional tumor microvascular networks result in an increase in both intravascular and extravascular pressure levels, and these elevated pressures will impact PCCA activation in perfused tumor regions. After introducing the mathematical theory behind TIPE-US, we introduce preliminary results from simulation and experimentation that explore the influence of nanodroplet concentration, temperature, and select US system settings on PCCA activation.

II. THEORY

Here we briefly detail the mathematical framework behind TIPE-US in relation to the chemical and physical properties of the PCCAs used to generate the reporter signal. Central to our TIPE-US system is the nucleation of the PCCA and phase transition from a liquid nanodroplet into a gaseous MB that is detectable using US. In the presence of US wave energy, this nanodroplet vaporization process occurs beyond a critical peak negative pressure, which is called the vaporization or activation threshold. Importantly, the activation threshold depends on the properties of the nanodroplet PFC liquid core and phospholipid coating, ambient conditions, and US excitation parameters (Aliabouzar *et al.*, 2018; Hoyt *et al.*, 2016; Kripfgans *et al.*, 2004; Martz *et al.*, 2011; Sheeran *et al.*, 2012; Shpak *et al.*, 2014).

Basic nucleation theory can be used to model the transition of a liquid nanodroplet into a gaseous phase (Blander and Katz, 1975). This theory also helps predict the activation threshold and dependence on temperature, material properties, and US parameters. In blood circulation, when the nanodroplet core is exposed to a negative pressure, the liquid faces tensile stresses that induce metastable equilibrium. Upon achieving a sufficient degree of metastability, nanodroplet nucleation is initiated and bulk phase transition produces a gaseous MB (accompanied by a 4- to 5-fold change in agent diameter d). In basic nucleation theory, the number of nucleation events in a liquid is proportional to the probability of formation of a critical cluster containing x gas molecules. Assuming an energy barrier W that must be overcome to initiate phase transition, the following is an equation governing PCCA nucleation (Blander and Katz, 1975; Shpak *et al.*, 2013):

$$J = J_0 \exp\left(\frac{-W}{kT}\right), \quad (1)$$

where J is the nucleation rate per unit volume per unit time and k is the Boltzmann constant. Also, in Eq. (1),

$$J_0 = n\sqrt{2\sigma/\pi m}, \quad (2)$$

where n is the number density of liquid molecules, m is the molecular mass, and σ the surface tension at the liquid-gas interface. Equation (2) assumes that the energies of the liquid PFC molecules obey a Maxwell-Boltzmann distribution. Given a pressure-temperature coexistence between the gas and liquid PFC phases, the vapor pressure p_v inside the nanodroplet as a function of temperature T is

$$p_v(T) = p_0 \exp\left[\frac{Lm}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right], \quad (3)$$

where T_0 is the boiling point at atmospheric pressure p_0 , L the heat of vaporization, and R is the ideal gas constant. From Eqs. (1) and (3), the liquid pressure p_l as a function of J is (Miles *et al.*, 2016)

$$p_l(J) = p_0 \exp\left[\frac{Lm}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] - \sqrt{\frac{16\pi\sigma^3}{3kT\ln(J_0/J)}}. \quad (4)$$

To appreciate Eq. (4), we first consider an appropriate expression for the nucleation rate that describes the probability of a nucleation site occurring inside the nanodroplet during exposure to negative US pressure. Given a weak dependency of the local nucleation pressure on nucleation rate (Miles *et al.*, 2016), we assume a quasi-static approximation for J and define the following rate criteria for nucleation to occur:

$$J > J_T \cong \frac{1}{V\tau}, \quad (5)$$

where the spherical nanodroplet volume V is

$$V = \pi d^3/6 \quad (6)$$

and τ the time that the nanodroplets are exposed to negative pressure responsible for phase transition. For an US pulse of frequency f and N cycles, $\tau \cong N/4f$. At lower US frequencies, nanodroplets are exposed to a longer duration of negative pressure resulting in earlier activation. Defining the local nucleation (activation) pressure threshold to be $p_T = p_l(J_T)$, we can assume that nucleation and nanodroplet activation occurs when and where $p_T > p_l$. As the hydrostatic (intratumoral) pressure exerted on a nanodroplet increases, Eq. (4) indicates that this equates to an increase in the liquid pressure and thus, an increase in the magnitude of the pressure activation threshold. Equation (4) also predicts that increases in PCCA temperature or agent size and decreases in frequency of US pulsed exposure will further decrease the nanodroplet activation threshold.

III. METHODOLOGY

A. Simulations

To evaluate PCCA activation along theoretical lines, a simulation program was developed using MATLAB (Mathworks Inc, Natick, MA). Using the mathematical framework detailed

in the Theory section, this simulation predicts the PCCA activation threshold via the nanodroplet nucleation rate and dependence on the material properties of the PCCAs, hydrostatic pressure, temperature, and pulsed US parameters. All default parameters used in simulation are listed in Table I unless otherwise stated.

B. PCCA preparation

PCCA agents were formulated in house using the popular condensation method (Sheeran *et al.*, 2012). Briefly, DSPC and DSPE-PEG-2000 (NOF Corp., Shibuya-ku, Tokyo, Japan) were dissolved and mixed in chloroform (Fisher Scientific, Waltham, WA) at appropriate molar ratios (90% DSPC, 10% DSPE-PEG2000) and dried by nitrogen evaporation. Lipid films were then hydrated in phosphate-buffered saline (PBS) with 20% propylene glycol (v/v), and 20% (v/v) glycerol (Fisher Scientific) at 2 mg/mL. A 2 mL sample of the resulting solution was added to 3 mL vials and the headspace of the vial was gas-exchanged with decafluorobutane (DFB; boiling point, -2.0°C to -1.1°C ; Fluoromed, Round Rock, TX) and then the vial was mechanically agitated (Vialmix shaker; Lantheus Medical Imaging, North Billerica, MA) to form MBs. Sealed vials were cooled to -16°C using a dry ice cooled 2-methylbutane bath. After sample cooling, a 16G needle was used to pierce the septum and expose the cooled MBs to atmospheric pressure, causing the agents to condense into nanodroplets. PCCA size and concentration was measured using small samples from vial solutions. Before activation, PCCA sizing was performed using dynamic light scattering (DLS) (Malvern Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) and after activation using optical microscopy measurements of physical diameter.

C. Hydrostatic pressurization of PCCA

The effects of hydrostatic pressure (analogous to TIP) on the US activation threshold of PCCA were investigated. A fixed concentration of PCCA in PBS was loaded into a 50-mL syringe and then injected into acoustically transparent and sealed liquid sample holders (Opticell, Thermo Fisher Scientific, Waltham, MA) via an 18G blunted needle. PCCA

concentrations are referred to as low ($0.5\text{ }\mu\text{L/mL}$ or 5×10^6 PCCA/mL), moderate ($2.0\text{ }\mu\text{L/mL}$ or 20×10^6 PCCA/mL), and high ($3.0\text{ }\mu\text{L/mL}$ or 30×10^6 PCCA/mL). Various levels of controlled hydrostatic pressure ($10\text{--}100\text{ mm Hg}$ in 10 mm Hg increments, $N=10$) were applied by injecting slightly more of the PCCA and saline solution into the Opticell plates. These Opticell plates are composed of two $75\text{ }\mu\text{m}$ thick membranes, 2 mm apart, with increased spacing following pressurization. Hydrostatic pressures were confirmed using a calibrated needle-based pressure monitoring device (Stryker, Kalamazoo, MI). Pressurized plates were then submerged into a heated isothermal water bath at 25°C or 37°C .

D. TIPE-US platform technology

The TIPE-US platform technology was developed using a programmable US research scanner (Vantage 256, Verasonics Inc., Kirkland, WA) equipped with an L11-4v or CL15-7 linear array transducer for the lower and higher US frequency studies, respectively. Standard grayscale US imaging was performed using a single focal zone placed at the level of the PCCA solution inside the Opticell plate (fixed depth of 1.7 cm , $f\text{-number}=1.0$). A custom scan sequence was used that interleaved pulsed US transmissions (5-cycle pulse with center frequency of 6.3 or 8.9 MHz) for both PCCA activation and detection. Since PCCA activation happens at a moderate peak negative pressure (p_T) on the order of 5 MPa , technology design included an automated US pressure sweep that encompassed this variable activation threshold. The entire scan sequence starts by transmitting a low-pressure US pulse and after a 10 ms delay, a sequence of 50 US image frames are acquired at a frame rate of 30 Hz . The PCCA activation pulse amplitude is then incremented by nominal value 15 kPa and another 50 frames of US data are recorded. This acquisition sequence is rapidly repeated for a user-defined range of acoustic outputs (e.g., peak negative pressures from 3 to 6 MPa , $N=200$ discrete intervals) and completes in entirety within minutes of initiation. Given the relationship between the hydrostatic pressure applied to the PCCA (i.e., $10\text{--}100\text{ mm Hg}$) and US energy needed to induce phase transition from a liquid nanodroplet to a highly echogenic MB, this onset of activation can be used as an indicator of hydrostatic pressure variation. Acoustic output measurements from the TIPE-US system were performed using a calibrated hydrophone setup (AIMS III, Onda Corp, Sunnyvale, CA).

Custom MATLAB programs were developed to process the US image stacks offline. Following user selection of a region-of-interest (ROI), US image sequences are summarized for each discrete US output and recorded as mean $\pm$ SD. US image-derived PCCA activation curves are then generated, normalized by the first measurement, and median filtered using a 1D kernel of size 15 samples. This data filtering was performed to smooth activation curves. Given an 8-bit US image dynamic range, the activation threshold was taken as the first US pressure observed that produced a corresponding average ROI amplitude of 30 . Note that this threshold was subjectively selected and deemed sufficient for *in vitro* US images from both low and high PCCA concentration conditions.

TABLE I. List of assumed values used in simulation for parameters related to phase-change contrast agent (PCCA) formulation using lipid-shelled decafluorobutane and ultrasound (US) exposure.

Description	Parameter	Value	Units
Ambient temperature	T	25 or 37	$^{\circ}\text{C}$
Atmospheric pressure	p_0	101×10^3	Pa
Boiling temperature	T_0	271.15	K
Boltzmann constant	k	1.38×10^{-23}	$\text{m}^2/\text{kg} \cdot \text{s}^2 \cdot \text{K}$
Heat of vaporization	L	9.7×10^4	J/kg
Ideal gas constant	R	8.314	J/mol $\cdot$ K
Molar weight	m	0.238	kg/mol
Nanodroplet diameter	d	220×10^9	m
Number density	n	5.0×10^9	
Surface tension	σ	5×10^{-3}	N/m
US pulse frequency	f	6.3	MHz
US pulse length	N	5	cycles

E. Statistical analysis

All experiments were performed in triplicate. A linear regression analysis was used to assess any trends between the hydrostatic pressure applied to the PCCA in solution and the US energy needed to induce MB formation (i.e., activation threshold). The residual standard error (RSE) of this statistical test was used as an indicator of measurement accuracy. A Wilcoxon signed rank test was used to compare data sets collected under varying conditions. A p -value less than 0.05 was considered statistically significant. All analyses were performed using R software (R Core Team, 2016).

IV. RESULTS

Conditions that impact PCCA activation were analyzed in simulation. The relationship between the hydrostatic pressure (for a fixed boiling temperature) applied to a PFC-based PCCA and the corresponding US energy required for activation from a liquid nanodroplet to gaseous MB state is plotted in Fig. 1(A). In practice, data such as this might be used as a lookup table to derive *in vivo* TIP-US measures. Given a fixed US pulse frequency of 6.3 MHz, the PCCA activation threshold is inversely related to agent size, Fig. 1(B). This implies that larger PCCAs will activate much sooner than smaller nanodroplets. For a PCCA that is 220 nm in size, theoretical results also reveal that as the center frequency of the US pulses increase from 1 to 20 MHz, there is an increase in the nucleation pressure required for PCCA activation, Fig. 1(C). This trend appears to asymptotically approach a plateau value at higher US pulse frequencies. Also highlighted by the simulated data presented in Fig. 1, when PCCA temperature was increased from room (25 °C) to body level (37 °C), there was a discernible decrease in the nucleation pressure necessary to activate the PCCAs.

Our TIPE-US strategy requires a PCCA that is stable at body temperature but can be activated by US energy exposure to undergo a phase transition from a liquid nanodroplet to a highly echogenic MB contrast agent also detectable using US. These lipid-shelled and PFC-based agents can be formulated using established laboratory methods. After PCCA preparation and sizing, these nanodroplets were found to be about 220 nm in diameter before activation and MBs of size 0.9 μm after activation.

Using our TIPE-US technology, an automated scan sequence rapidly interleaved pulsed US transmission for both PCCA exposure to increasing acoustic pressures and detection of any activated agents. Presented in Fig. 2 are US images of PCCA that have been hydrostatically pressurized to 50 mm Hg. The series of contrast-enhanced US images clearly depict PCCA activation (nucleation) when exposed to a sufficiently high acoustic pressure. As further detailed in Fig. 3, US image-derived PCCA activation curves reveal a progressive shift in output required to induce PCCA activation as hydrostatic pressure is increased from 10 (1.33 kPa) to 100 mm Hg (13.3 kPa), which is a physiologically relevant range commonly found in tumor tissue. Note that once a sufficiently high US pressure is reached, MB destruction more than offsets the number of activated PCCA and the US image intensity starts to decrease.

FIG. 1. Simulation results illustrating the temperature-dependent relationship between the PCCA activation threshold (nucleation pressure) and (A) hydrostatic pressure applied to the nanodroplets for a fixed PCCA size of 220 nm and ultrasound (US) frequency of 6.3 MHz, (B) PCCA size for a fixed US frequency of 6.3 MHz and hydrostatic pressure of 0 mm Hg, and (C) US frequency for a fixed PCCA size of 220 nm and hydrostatic pressure of 0 mm Hg.

The new TIPE-US system was evaluated using a series of *in vitro* phantom studies. These experiments involved diluting PCCA in saline and then injecting the solution into acoustically transparent and sealed plates. Hydrostatic

FIG. 2. Experimental TIPE-US results from PCCAs exposed to 50 mm Hg of hydrostatic pressure. A series of contrast-enhanced US images are shown before and after activation when exposed to a sufficiently high negative US pressure (3.1 to 5.8 MPa as noted in the subimage header) during the US imaging process.

pressurization was controlled by volume transfer of this stock solution. After TIPE-US image data were collected, analysis of the PCCA activation was performed. During these initial *in vitro* studies, we evaluated the effects of PCCA concentration (0.5, 2.0, and 3.0 $\mu\text{L/mL}$), temperature (25°C and 37°C), and US frequency during exposure (6.3 and 8.9 MHz). Note that all phantom studies were conducted at default temperature of 25°C using an US frequency of 6.3 MHz and moderate concentration of PCCA unless otherwise stated. As summarized in Fig. 4(A), changing PCCA concentration had no discernible impact on the corresponding TIPE-US measurements ($p > 0.41$) and all three datasets exhibited a strong linear correlation with hydrostatic pressure ($r > 0.90$, $p < 0.001$). Note that for a change in hydrostatic (ambient) pressure from 10 to 100 mm Hg, the corresponding

PCCA activation threshold changed by approximately 1.5 MPa. As highlighted in Fig. 4(B), after increasing the sample temperature from 25°C to 37°C, the PCCA activation threshold was considerably reduced ($p < 0.001$) as predicted by Eq. (4). US image data collected at both temperatures also had a positive correlation with hydrostatic pressure ($r > 0.80$, $p < 0.001$). Measures at body temperature were noticeably less accurate. Last, when the US frequency used for PCCA activation was increased from 6.3 to 8.9 MHz, the activation threshold was reduced significantly ($p < 0.001$) as detailed in Fig. 4(C) and predicted by Eq. (4). This observation is attributed in part to the increased time that the nano-droplets were exposed to negative US pressure. When reviewing the slopes of the linear regression lines, $\Delta y/\Delta x = 0.011 \pm 0.001$ and 0.023 ± 0.003 for the 6.3 and 8.9 MHz

FIG. 3. Series of US image-derived PCCA activation curves plotted as a function of the hydrostatic pressure applied to the agents. Note the marked shift in the acoustic output required to induce PCCA activation as hydrostatic pressure is increased from 10 to 100 mm Hg.

US data, respectively. Compared to data collected using higher frequency US pulsing and PCCA exposure, the activation threshold measurements from the lower US frequency data had a lower variance (RSE = 0.45 and 0.14, respectively, 68.9% change). Consistent with other measurements, data collected at both US frequencies had a positive correlation with the hydrostatic pressure ($r > 0.85$, $p < 0.001$). In practice, once the activation threshold is quantified, a TIP value can be derived via a lookup table that linearly relates the observed PCCA nucleation pressure to the local hydrostatic pressure experienced by the circulating agents.

V. DISCUSSION

The influence of PCCA hydrostatic pressurization on the nanodroplet activation threshold has been investigated. US image-derived activation curves were found to undergo distinct patterns of initial growth and then saturation. Given the relationship between the hydrostatic pressure applied to the PCCA (i.e., 10 to 100 mm Hg) and US energy needed to induce phase transition from a liquid nanodroplet to a highly echogenic MB, this onset of activation may potentially be used as a gauge of hydrostatic pressure levels. While the PCCA activation threshold was shown to be reduced when increasing sample temperature or decreasing the frequency of US exposure, it was not dependent on PCCA concentration. A comparison of simulated and experimental findings reveals that the impact of US pulse frequency on PCCA activation are directly related and more pronounced in the *in vitro* data. While the exact mechanism for this disparity needs to be explored in future work, we hypothesize that the deviation from theory at higher acoustic frequencies may be due in part to increased attenuation as US propagates through the PFC liquid nanodroplet suspension proximal to the focal zone.

FIG. 4. *In vitro* TIPE-US activation threshold data plotted as a function of hydrostatic pressure applied to the PCCA. Experimental data were collected after (A) variable PCCA concentration for a fixed US frequency of 6.3 MHz and temperature of 25 °C, (B) variable temperature for a moderate concentration of PCCA and US frequency of 6.3 MHz, and (C) US frequency for a moderate concentration of PCCA and temperature of 25 °C. Note the clear linear trends between hydrostatic pressure and PCCA activation that is both temperature and frequency dependent.

The PCCA activation threshold measurements acquired using US imaging at 6.3 and 8.9 MHz exhibited slopes of $\Delta y/\Delta x = 0.011 \pm 0.001$ and 0.023 ± 0.003 , respectively. An increased slope would suggest improved sensitivity for TIPE-US measurements and a lower measurement variance would improve accuracy. Given knowledge of linear regression equations and coefficient standard errors, hydrostatic pressure can be estimated from PCCA activation thresholds. For example, using an US frequency of 6.3 MHz to obtain a threshold measurement of 4.4 MPa, hydrostatic pressure can be estimated to be 20.0 ± 7.1 mm Hg. Likewise, a threshold value of 6.1 MPa obtained using an US frequency of 8.9 MHz yields a hydrostatic pressure estimate of 20.0 ± 7.5 mm Hg. As these examples suggest, improvements in TIPE-US measurement accuracy will produce a hydrostatic pressure estimate with a more precise confidence interval.

The relationship between US pulse frequency and the PCCA activation threshold has been an ongoing discussion in the literature. Using larger micrometer-sized PFC droplets, an early report showed that the activation threshold decreased when the agents were exposed to US pulses of increased frequency (Kripfgans *et al.*, 2004). A similar trend in PCCA activation thresholds was found when using smaller PFC-based nanodroplets (Aliabouzar *et al.*, 2018; Sheeran *et al.*, 2013; Sheeran *et al.*, 2016), which agreed with our findings and what nucleation theory predicts. Several studies have also demonstrated that PCCA activation thresholds are considerably reduced when using larger PCCAs (Kripfgans *et al.*, 2004; Miles *et al.*, 2016; Schad and Hynynen, 2010; Shpak *et al.*, 2014), going from room to body temperatures (Schad and Hynynen, 2010; Williams *et al.*, 2013), or by incorporating PFC compounds with lower boiling points during PCCA formulation (de Gracia Lux *et al.*, 2017; Matsunaga *et al.*, 2012; Sheeran *et al.*, 2011). These tradeoffs are important considerations when contemplating PCCA for *in vivo* studies and use within the output capabilities of common clinical US systems.

For the experimental data presented, PCCA sizing was performed using a DLS system for standardization of the PCCA fabrication process to reliably predict final PCCA size after condensation to initial MB counts. While we know the PCCA samples had larger micrometer-sized nanodroplets (determined by optical diffraction patterns during microscopy imaging), an additional characterization of these larger agents was not performed. These larger PCCA are known to exhibit a lower activation threshold if not removed (Sheeran *et al.*, 2016) and could help nucleate smaller nanodroplets. The effect of any premature activation should be investigated in future studies.

PCCAs were previously embedded in phantom materials of different stiffness (Williams *et al.*, 2013), which is analogous to hydrostatic pressurization, and motivated our early work on TIPE-US (Hoyt *et al.*, 2016). In short, using PCCA that were 221 ± 12 nm in diameter before activation, this study demonstrated that increasing the stiffness of the surrounding material linearly increased the PCCA activation threshold. They further observed that the MBs produced in stiffer phantom materials were smaller in size compared to

those formed in softer phantoms. Using a comparable experiment setup to ours, the effect of hydrostatic pressure on PCCA activation was also investigated (Rojas *et al.*, 2019). Although the PCCAs were fabricated using a condensation approach as also detailed herein, activation thresholds were noticeably lower, which may be attributed to the use of US pulses at a lower *f*-number and increased focusing effect on the PCCAs (Shpak *et al.*, 2014). In contrast to our findings, this group found no distinguishing trend between the hydrostatic pressures applied to the PCCA samples and activation thresholds. One possible explanation may reside in how the PCCA activation threshold was measured. Rojas *et al.* (2019) calculated this threshold value by finding the first US pressure whereby image intensity became higher than 10% of its maximum value. At higher hydrostatic pressures, the MBs formed after PCCA evaporation can be considerably smaller in size (Williams *et al.*, 2013). This reduced scatterer cross-sectional diameter will decrease the backscattered US signal and could complicate PCCA activation threshold quantification if the maximum image intensities are not probably normalized. In contrast, we calculated PCCA activation thresholds as the first US pressure observed that produced a corresponding mean image intensity of 30, which was independent of the maximum image intensity. One additional possible explanation for the disparity in experimental findings may be due to the US pressure resolution used, which was 250 kPa versus our 15 kPa. Our experience has revealed that a fine US pressure sweep is critical for accurately detecting measurable differences in PCCA activation thresholds.

Based on the *in vitro* measurements presented herein, we propose the novel TIPE-US technique, which has the potential to be a noninvasive approach to measure absolute values and changes in intratumoral pressure. It is important to note that PCCA activation threshold detection was less precise at body temperature and this variance will need to be reduced in future work to improve *in vivo* accuracy. Strategies may include improved PCCA design, so activation curves exhibit a much sharper activation response (de Gracia Lux *et al.*, 2017) (cf. Fig. 3) and TIPE-US system modification to permit repeated measurements for statistical averaging and variance reduction (Hoyt *et al.*, 2016). In practice, this US imaging technology could estimate intratumoral pressure by first, detection of the PCCA activation threshold, and then use of a lookup table to convert this threshold to a known hydrostatic pressure value.

Currently, TIP can only be measured in the clinic with invasive wick-in-needle or micropuncture techniques (Heldin *et al.*, 2004). These approaches pose the risk of leaving a foreign body in human tissues and are therefore subject to limitations for measuring TIP safely in deep tissues. Consequently, an US-based technology, termed subharmonic aided pressure estimation (SHAPE), has evolved for performing remote TIP measurements. In short, changes in hydrostatic pressure affects the nonlinear response of flowing intravascular MB contrast agents leading to a correlation between TIP levels and subharmonic signals acquired using a dedicated US system (Forsberg *et al.*, 2005). Initial *in vivo* studies in five swine with naturally occurring melanomas showed that SHAPE and

reference standard TIP measurements were in good agreement ($r = 0.79$, $p < 0.05$) (Halldorsdottir *et al.*, 2014). A more recent experiment in rats with breast cancer xenografts also demonstrated a linear relationship between TIP and SHAPE measurements ($r = 0.69$, $p < 0.01$) (Halldorsdottir *et al.*, 2017). Albeit based on a small sample size, it has been concluded that SHAPE has potential to help predict response to neoadjuvant chemotherapy for breast cancer as early as completion of one cycle of therapy (Nam *et al.*, 2017). Despite these encouraging results, the average error associated with these *in vivo* US-based SHAPE measurements has been reported to exceed 6 mm Hg (Halldorsdottir *et al.*, 2017) so improvements in pressure estimation technology and accuracy may be needed if this treatment indicator is to be adopted in clinical practice. Further, one needs to carefully optimize the US parameters (e.g., excitation frequency and amplitude) for optimum SHAPE performance (Katiyar *et al.*, 2011). These limitations aside, SHAPE highlights the potential of medical imaging-based solutions to help address the challenge of performing noninvasive TIP measurements for clinical applications.

Cancer growth can result in abnormal blood flow and increased intratumoral fluid pressure. The abnormal blood flow not only impedes drug delivery, but the resulting hypoxia also aids tumor invasion, metastasis, inflammation, fibrosis, and treatment resistance (Stylianopoulos *et al.*, 2018). While tumor microcirculation and TIP may lead to a heterogeneous distribution of PCCAs in the tumor space, we hypothesize that enough agents would be present to allow use of TIPE-US. While TIP is spatially uniform throughout the tumor center, it does tend to progressively drop near its periphery (Boucher *et al.*, 1990; DiResta *et al.*, 1993), which strengthens the argument of having sufficient PCCA penetration for TIPE-US measurements if only in the tumor periphery.

Herein we demonstrated that PCCA can be activated from a liquid nanodroplet to a gaseous MB state using pulse US. Previous studies have demonstrated that the final MB size is dependent on the gas concentration in the surrounding fluid (Radhakrishnan *et al.*, 2016). It is reasonable to assume that PCCA activation in hypoxic tumors (having depressed oxygen tension) may result in MBs that are smaller in size than those formed under normal blood oxygen levels. While this situation may impact the overall amplitude of the US image-derived PCCA activation curves, TIPE-US measurements should be minimally impacted. In our detailed approach, as the PCCA are exposed to repeated US pulses of a progressively increasing peak negative pressure, the activation threshold is taken as the first US pressure observed that produces a corresponding average ROI amplitude of a certain value and is independent of the maximum achieved signal.

A new laboratory method for the formation of monodisperse PCCAs was recently detailed (de Gracia Lux *et al.*, 2017). This production technique utilized small volume microfluidization to yield reliable, narrowly dispersed PFC liquid-filled phospholipid-coated nanodroplets that are stable at body temperature for a period of days. These monodisperse PCCAs exhibited a much more defined activation threshold so future work might investigate integration with our TIPE-US technology. Further, improvements in intratumoral

pressure estimation sensitivity and accuracy using TIPE-US may also be realized by integration of MB-sensitive imaging functionality (Ghosh *et al.*, 2017; Ghosh *et al.*, 2019; Hoyt *et al.*, 2015; Saini and Hoyt, 2014) and volumetric US imaging to incorporate a larger sample from 3D space (Hoyt *et al.*, 2012a,b; Mahoney *et al.*, 2014). While different formulation strategies exist, a recent scientific publication detailed how PCCAs can be generated directly from commercially available MB products (Sheeran *et al.*, 2017), which could help accelerate clinical translation of any TIPE-US system.

VI. CONCLUSION

PCCA are a class of US contrast media that can be selectively triggered to undergo a phase transition from a liquid nanodroplet state to a highly echogenic MB that can be detected using US imaging methods. Data presented herein detail how the US negative pressure required to induce PCCA activation is related to the hydrostatic pressure exerted on the agents. As demonstrated, this activation is further impacted by US system level parameters (e.g., frequency) and PCCA conditions (e.g., temperature). Overall, these relationships are exploited by our new TIPE-US technology, which may prove useful for measuring intratumoral pressure levels impacting anticancer drug delivery and effective treatment.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health (NIH) Grant Nos. K25EB017222, R21CA212851, R01CA235756, R01EB025841, and Cancer Prevention Research Institute of Texas (CPRIT) Grant No. RP180670.

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