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Toward noninvasive pressue estimation using ultrasound and phase-change contrast agents

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***Abstract*—It is well known now that 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). Since nanodroplet activation is highly dependent on the hydrostatic pressure exerted on it, any increases in this stabilizing force demands higher US energies to induce phase transition. Herein we explore this basic relationship as a potential direction for noninvasive pressure measurement and foundation of a new technology we are developing termed tumor interstitial pressure estimation using US (TIPE-US). TIPE-US was developed using a programmable US research scanner (Vantage 256, Verasonics Inc). A custom scan sequence was implemented and interleaved pulsed US transmissions for both PCCA activation and subsequent MB detection. An automated US pressure sweep is performed (peak negative pressures from 3 to 6 MPa, N = 200 discrete intervals), and US images are acquired at each increment. PCCAs were formulated in house using the popular condensation method. Pressurized samples were then studied using the TIPE-US system. The activation threshold required to convert PCCA from the liquid to gaseous state was recorded for various PCCA conditions. Overall, PCCA activation threshold was lowered with increasing sample temperature while PCCA concentration appeared to play no role.**

Keywords—microbubbles; nanodroplets; phase-change contrast agents, pressure estimation; ultrasound

I. Introduction

Tumor interstitial pressure (TIP) is often considerably elevated compared to normal tissues and can impede drug delivery and an effective treatment response. In order to accommodate the metabolic demands of a growing tumor, blood vessels from surrounding tissue grow into the tumor through a process known as angiogenesis. Unlike normal tissue, these newly formed blood vessels are disorganized, tortuous, and leaky. Owing to these abnormal blood vessels in addition to poor lymphatic drainage, high TIP levels can occur. Direct measurements from a large sample of human and animal subjects have demonstrated that interstitial pressure is 5 to 30 mmHg higher in cancerous tissue compared to normal tissue, although values exceeding 100 mmHg have been recorded [1]–[9]. Knowledge of TIP is clinically important and could be used to help justify administration of tumor pressure-lowering drugs prior to the primary treatment [10]. The ability to customize individual patient treatment based on TIP knowledge would allow one to then monitor treatment response throughout the chemotherapeutic cycles.

Interstitial pressures levels in tumor tissue can currently only be measured in the clinic with invasive wick-in-needle or micropuncture techniques [11]. While the wick-in-needle technique has shown promise in monitoring TIP changes in response to cancer treatment [12], this approach is vulnerable to clotting. The wick-in-needle technique also requires custom-made needles and a well-trained user to perform accurate and measurements. Conversely, micropuncture techniques only allow TIP measurements in superficial tissues. Further, glass micropipettes break easily and cause patient injury.

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 [13]–[19]. Noteworthy, 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 gaseous MBs [20], [21]. Herein we explore this basic relationship as a potential direction for noninvasive pressure measurement and the foundation of a new technology we are developing termed TIP estimation using US (TIPE-US). Our working hypothesis is that dysfunctional tumor microvascular networks result in elevated intratumoral pressure levels that will predictably impact PCCA activation and quantification using our TIPE-US method.

II. Materials and Methods

A. PCCA Preparation

A schematic illustrating our general procedure for preparing monodisperse PCCA is shown in Fig. 1. PCCAs were formulated in house using the popular condensation method [18]. Briefly, DSPC and DSPE-PEG-2000 were dissolved and mixed in chloroform 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 10% propylene glycol (v/v), and 10% (v/v) glycerol at 2 mg/mL. A 1.5 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) and then the vial was mechanically agitated (Vialmix shaker; Lantheus

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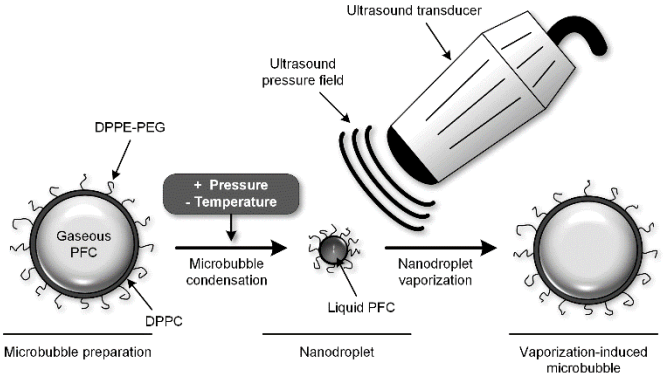

Fig. 1. Schematic for preparing a phase-change contrast agent (PCCA) by pressurization and slowly cooling to condense the gas inside the microbubbles (MBs). Once activated by ultrasound (US) energy, nanodroplets undergo a phase transition by vaporization back into highly echogenic MBs.

Medical Imaging, North Billerica, MA) to form MBs. Vial solutions were then pressurized using a 25 G needle and additional PFC gas. Once the onset of condensation was observed and nanodroplets formed, the pressure source was removed.

B. Hydrostatic Pressurization of PCCA

The impact of hydrostatic pressure (analogous to TIP) on the US activation threshold of PCCA was 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 18 G blunted needle. PCCA concentrations are referred to as low (0.5 μL/mL or 5×10^6 PCCA/mL), moderate (2.0 μL/mL or 20×10^6 PCCA/mL), and high (3.0 μL/mL or 30×10^6 PCCA/mL). Various levels of controlled hydrostatic pressure (10 to 100 mmHg in 10 mmHg increments, $N = 10$) were applied by injecting slightly more of the PCCA and saline solution into the Opticell plates. 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 or 37 °C.

C. TIPE-US Imaging System

The TIPE-US platform technology was developed using a programmable US research scanner (Vantage 256, Verasonics Inc, Kirkland, WA) equipped with an L11-4v array transducer. Standard grayscale US imaging was performed using a single focal zone placed at the level of the PCCA solution inside the Opticell plate. A custom scan sequence was used that interleaved pulsed US transmissions (5-cycle pulse with center frequency of 6.3) for both PCCA activation and detection. Since PCCA activation happens at a moderate peak negative pressure 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 short 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 to 100 mmHg) 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).

D. Image Processing

Custom MATLAB programs (Mathworks Inc, Natick, MA) 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 ± 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.

E. Stastical 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). 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 [22].

III. Results

Using our custom 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 mmHg. The series of contrast-enhanced US images clearly depict PCCA activation when exposed to a sufficiently high acoustic pressure. As 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 to 100 mmHg. Note once a sufficiently high US pressure is reached, MB destruction starts to offset the number of activated PCCA, and US image intensity decreases.

The new TIPE-US system was evaluated using a series of phantom studies. After TIPE-US image data was 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 μL/mL) and temperature (25 and 37 °C) on experimental findings. As summarized in Fig. 4A, changing PCCA concentration had no discernible impact on the

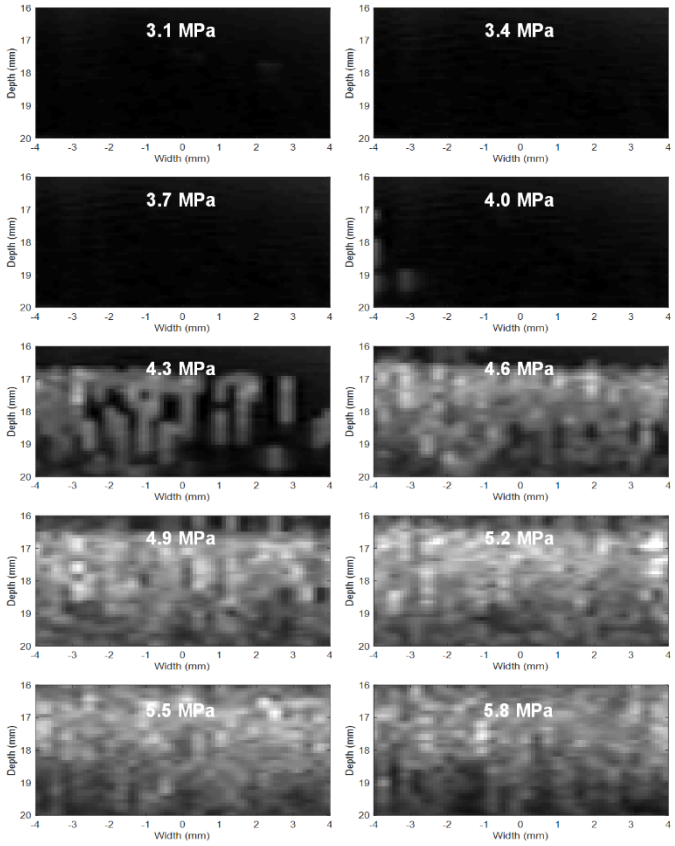

Fig 2. Experimental tumor interstitial pressure estimation using US (TIPE-US) results from PCCAs exposed to 50 mmHg of hydrostatic pressure. A series of contrast-enhanced US images are shown before and after activation when exposed to a sufficiently high negative US pressure (3.1 to 5.8 MPa) during the US imaging process.

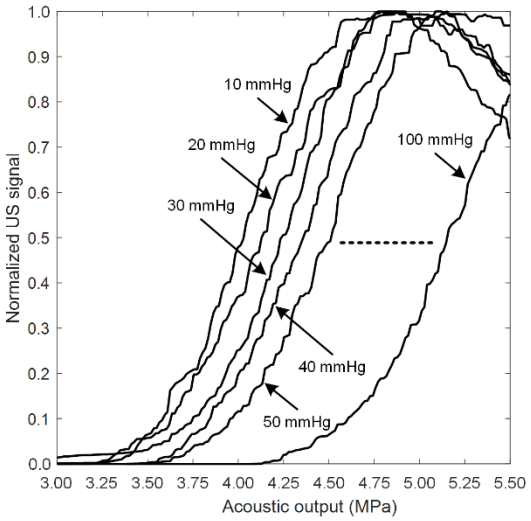

Fig. 3. Series of US image-derived PCCA activation curves plotted as a function of the hydrostatic pressure applied to the agents. Note the marked shift in the acoustic output required to induce PCCA activation as hydrostatic pressure is increased from 10 to 100 mmHg.

corresponding TIPE-US measurements ($p > 0.41$) and all three datasets exhibited a strong linear correlation with hydrostatic pressure ($R^2 > 0.81, p < 0.001$). Note that for a change in

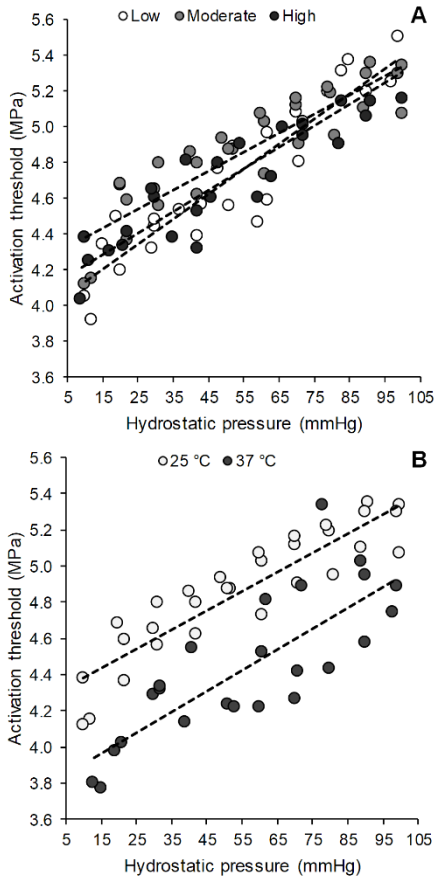

Fig 4. TIPE-US activation threshold data plotted as a function of hydrostatic pressure applied to the PCCA. Experimental data was collected after (A) variable PCCA concentration and temperature of 25 °C and (B) variable temperature for a moderate concentration of PCCA. Note the clear linear trends between hydrostatic pressure and PCCA activation that is temperature but not concentration dependent.

hydrostatic pressure from 10 to 100 mmHg, the corresponding PCCA activation threshold changed by approximately 1.5 MPa. As shown in Fig. 4B, after increasing the sample temperature from 25 to 37 °C, the PCCA activation threshold was considerably reduced ($p < 0.001$). US image data collected at both temperatures also had a positive correlation with hydrostatic pressure ($R^2 > 0.64, p < 0.001$). In practice, once the phase transition threshold is quantified, a TIP value can be derived via a lookup table that relates the observed PCCA activation pressure to the local hydrostatic pressure.

IV. Discussion

The impact of PCCA hydrostatic pressurization on the corresponding activation threshold was investigated. US image-derived activation curves were found to undergo distinct patterns of initial growth. Given the relationship between the hydrostatic pressure applied to the PCCA and US energy needed to induce phase transition from a liquid nanodroplet to an echogenic MB detectable during US imaging, this onset of activation may potentially be used as a gauge of hydrostatic pressure levels. While the PCCA activation threshold was

clearly reduced with increased sample temperature, it was not dependent on PCCA concentration.

Based on our *in vitro* measurements, 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. While we acknowledge that PCCA activation threshold detection was less precise at body temperature, this variance will be addressed in future work to improve accuracy during any *in vivo* study. Strategies may include PCCA use considerations so activation curves exhibit a sharper activation response [14] and a more sensitive contrast-enhanced US imaging mode [23]–[26]. An advanced TIPE-US system design to permit repeat measurements for statistical averaging could also help improve TIP estimation accuracy.

V. Conclusion

PCCA are a new 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 easily detected using US imaging. Data presented herein details 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 the temperature of the immersed PCCAs.

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