Real Time Ultrasound Molecular Imaging of Prostate Cancer with PSMA-targeted Nanobubbles – Supplementary Data

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Supplementary Material

Method

Preparation and characterization of contrast agents

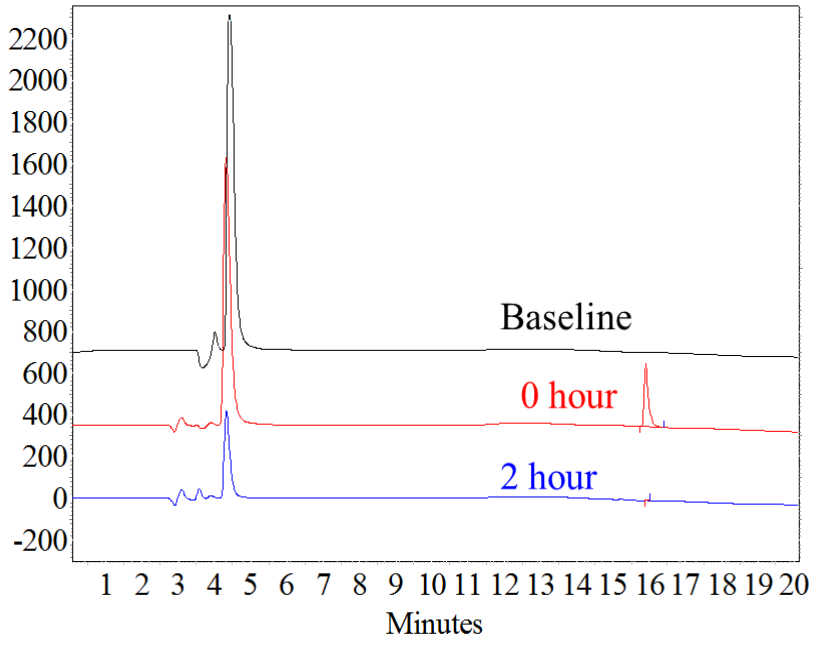
Lipid solution (10mg/mL) for nanobubbles was prepared by dissolving 1,2-dibehenoyl-sn-glycero-3-phosphocholine (DBPC, Avanti Polar Lipids Inc., Pelham, AL), 1,2-Dipalmitoyl-sn-glycero-3-Phosphate; DPPA, 1,2-dipalmitoyl-sn-glycero-3-phosphor ethanolamine; DPPE (Corden Pharma, Switzerland), and 1,2- distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-mPEG 2000, Laysan Lipids, Arab, AL) with 6:1:2:1 ratio in propylene glycol (PG, Sigma Aldrich, Milwaukee, WI) by heating and sonicating at 80°C. Mixture of glycerol (Gly, Acros Organics) and phosphate buffer solution (0.8 mL, Gibco, pH 7.4) preheated to 80°C was added and sonicated for 10 min at room temperature. The solution (1 mL) was transferred to a 3 mL headspace vial, capped with a rubber septum and aluminum seal. Air was replaced by octafluoropropane (C_3F_8, Electronic Fluorocarbons, LLC, PA) gas and activated by mechanical shaking with a VialMix shaker (Bristol-Myers Squibb Medical Imaging Inc., N. Billerica, MA) for 45s. Nanobubbles were isolated from the microbubbles by centrifugation at 50 rcf for 5 min with the headspace vial inverted, and the 100 μL NB solution withdrawn from a fixed distance of 5 mm from the bottom with a 21G needle[1-2].

PSMA-NB were prepared by adding DSPE-PEG-PSMA-1 (25μg/ml) to the initial lipid solution and followed the above protocol. To prepare DSPE-PEG-PSMA-1, PSMA-1 (from prof. James Basilion lab) was mixed with DSPE-PEG-MAL (1,2-distearoyl-sn-glycero-3-phospho ethanolamine-N-[methoxy (polyethylene glycol)-2000-Maleimide, Laysan Bio, Arab, AL) in 1:2

ratio at pH 8.0 in PBS. After combined, the mixture was vortexed thoroughly and was reacted for 4h on the vial rotator at 4°C. The product was lyophilized and the resultant powder was dissolved in PBS to obtain DSPE-PEG-PSMA-1 stock solution. Conjugation of DSPE-PEG-PSMA-1 was confirmed by High Performance Liquid Chromatography (HPLC) and MALDI TOF technique. HPLC was performed on a Shimadzu HPLC system equipped with a SPD-20A prominence UV/visible detector and monitored at a wavelength at 220 nm. Analytical HPLC was performed using an analytical Luna 5µ C18(2) 100A column (250mm × 4.6 mm × 5µm, Phenomenex) at a flow rate of 1.0 mL/min. Gradient used was 10% - 40% Acetonitirle against 0.1% TFA over 20 min.

The size distribution and concentration of NBs were characterized with resonant mass measurement (Archimedes®, Malvern Panalytical) as explained earlier[1-2]. Measurement was finalized after 1000 particles were measured. Data was exported from the Archimedes software (version 1.2) and analyzed for positive and negative counts[1]. Surface charge of the diluted NB solution (500X) was measure with an Anton Paar LitesizerTM 500.

A.



B.

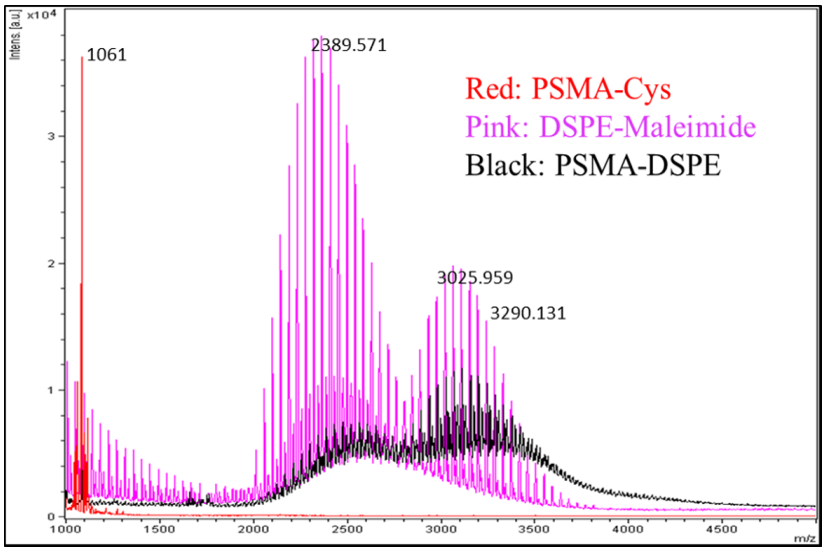


Figure S1. (A) MALDI-TOF MS analysis of DSPE-PSMA conjugation (B) HPLC analysis of PSMA-1 and DSPE-PSMA conjugation. Both methods confirm conjugation of PSMA to the DSPE lipid.

PSMA-Cys was conjugated to DSPE-PEG2000 lipids through reaction of –SH group in PSMA-Cys with DSPE-PEG2000-maleimide, and the molar ratio was 1:2. It has been reported that maleimide-thiol reaction happens quickly and easily. Two hours later, the peak of PSMA-Cys disappeared in HPLC indicating the completion of the reaction (**Figure S1A**). Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) results further confirmed the conjugation of PSMA-1 to the DSPE-PEG2000. DSPE-PEG2K (pink, **Figure S1B**) showed a cluster of peaks at around 2,200 due to polydispersity of DSPE-PEG2K; there was another cluster of peak at around 3,000, but with lower intensity, this might due to impurity of DSPE-PEG2K-maleimide. The molecular weight (MW) of PSMA-Cys is 1061 (red), the PSMA-1-DSPE conjugate should have MW at around 3,260. After reaction (black), MALDI-TOF-MS showed two clusters of peaks, one at ~2,200, and the other at ~3,200 and the intensity of the 3,200 peaks are higher than the 2,300 peaks. The 2,200 peaks were due to excess amount of DSPE-PEG2K-maleimide. The 3,200 peaks were partially overlapped with the 3,000 peaks of unreacted DSPE-PEG2K, however, the changes in intensity further confirmed successful conjugation of PSMA-Cys with DSPE-PEG2000-maleimde.

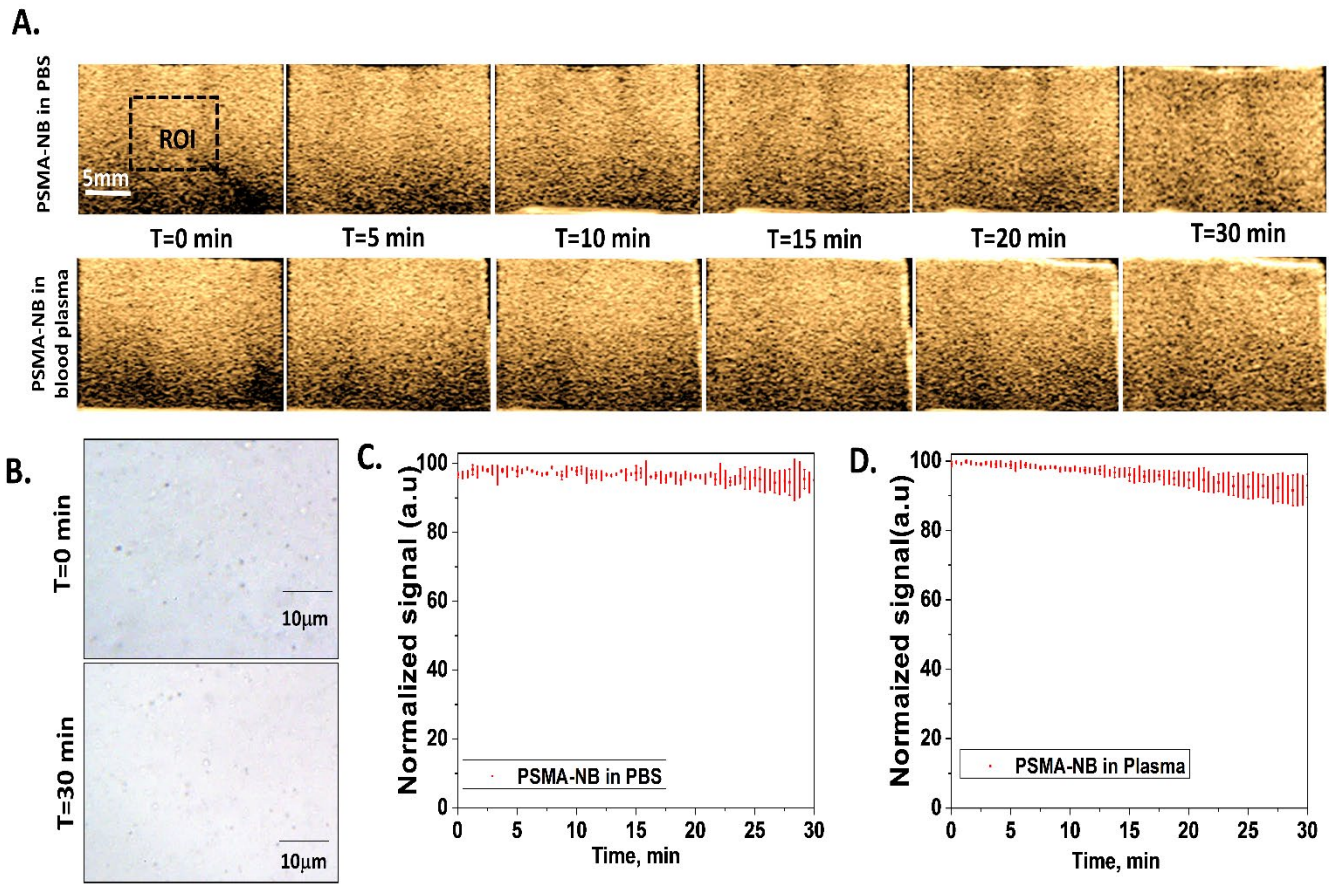


Figure S2. (A) Representative US images captured in contrast harmonic imaging (CHI) mode of PSMA-NB in PBS and blood plasma solution at MI= 0.1 and 12MHz. (b) Representative microscope images of PSMA-NB in blood plasma at t=o and t=30min (100X). (C) Stability curves o~~B~~f PSMA-NB in PBS at different time points. Mean ± s.d (n=2). (D) Stability curves of PSMA-NB in blood plasma at different time points. Mean ± s.d (n=3).

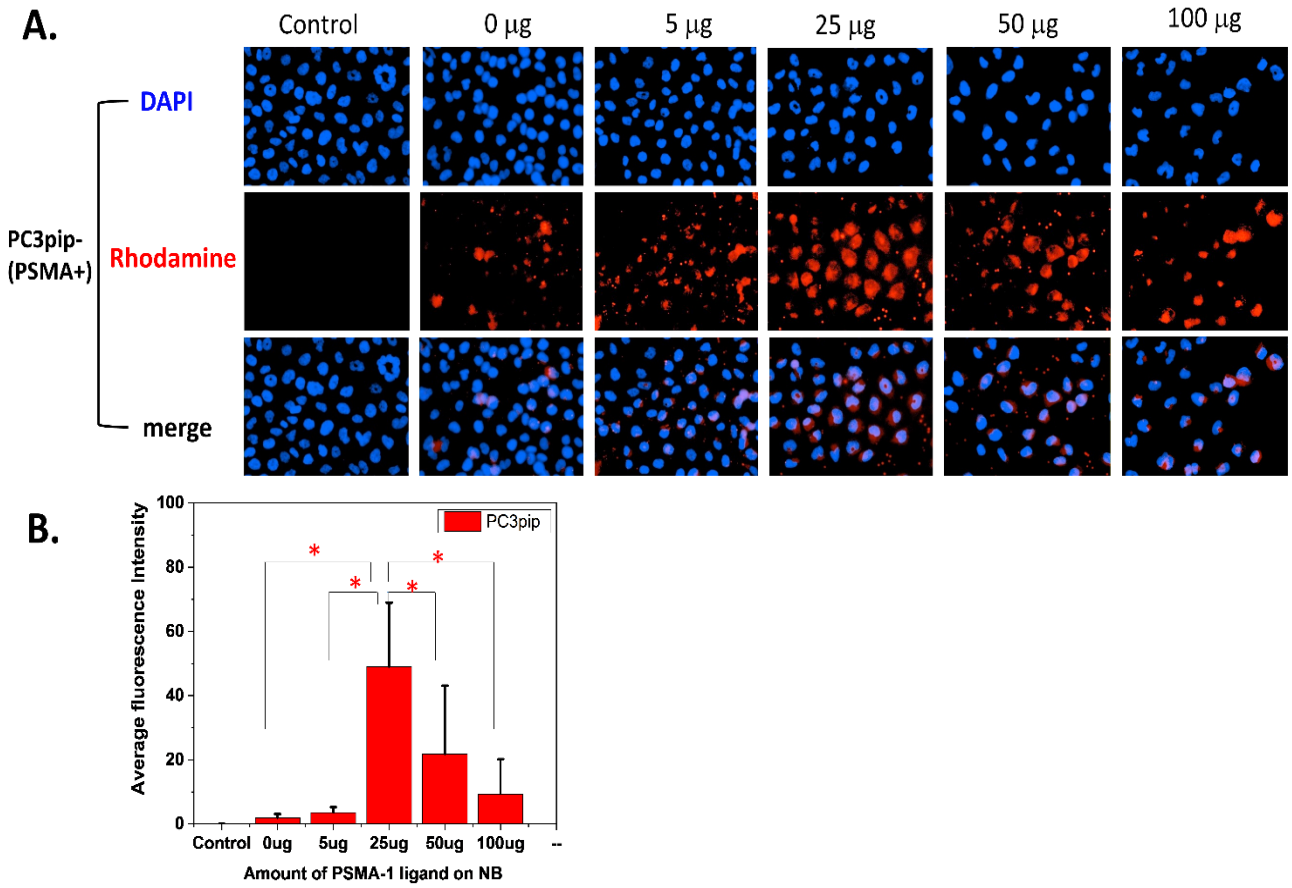


Figure S3. *In vitro* cellular uptake experiments reveal PSMA-NB selectively binds to the PSMA-positive PC3pip cells. (A) PSMA-positive PC3pip cells on coverslips were incubated with no NB (control), Rhodamine-NB or Rhodamine-PSMA-NB with different PSMA amounts for 1hr. Nuclei were stained using DAPI (blue) and uptake of Rhodamine tagged NB and PSMA-NB (red) was assessed by fluorescence microscopy. Images were taken at 40X. Representative images are shown from three independent experiments. (B) Optimization of PSMA amount in NB targeting. Use of 25 µg of PSMA-1 during conjugation shows significantly higher fluorescence signal than lower and higher concentrations. n=3, error bars represent mean ± s.d., * $P < 0.05$.

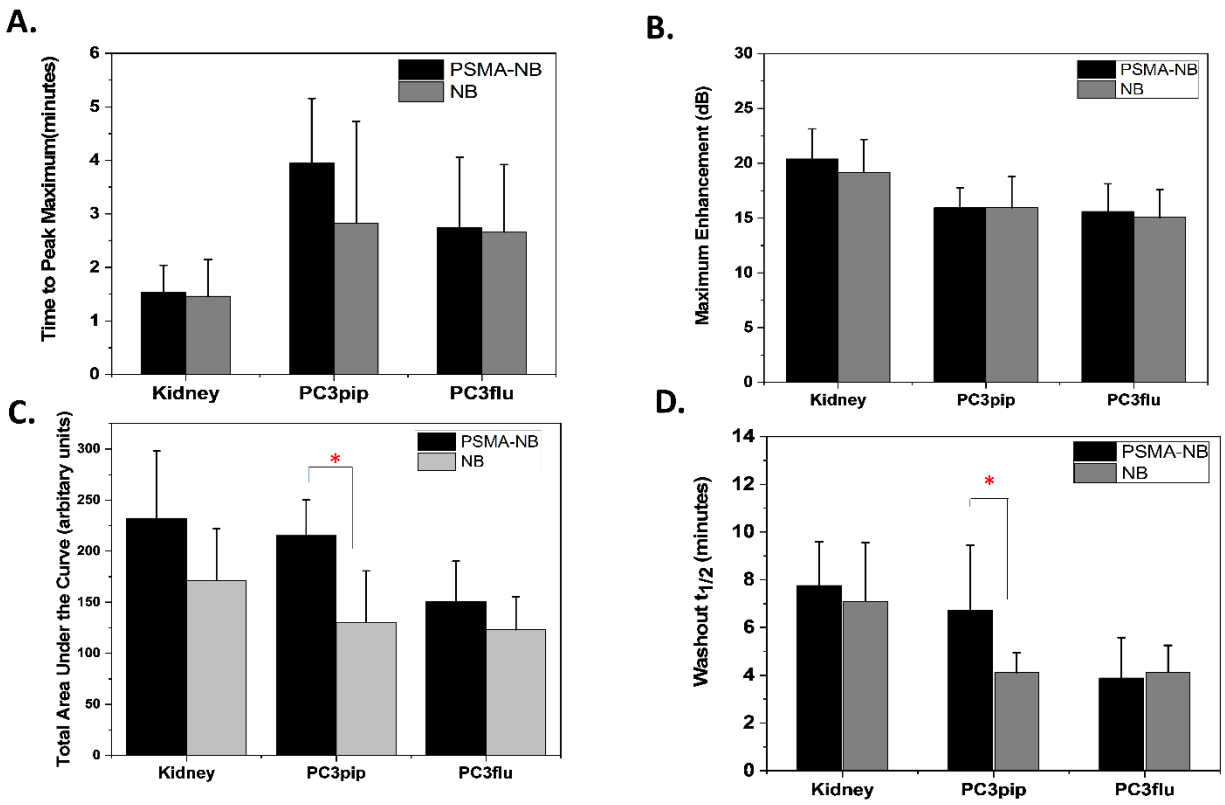


Figure S4. Quantitative *in vivo* parameters obtained from TIC analysis comparing kinetics of targeted (PSMA-NB) and untargeted (NB) bubbles. (A) Time to peak enhancement, (B) Maximum enhancement, (C) Total area under the curve (AUC), (d) Washout half-life. Significant differences ($p < 0.05$) were seen in the AUC and washout half-life between targeted and untargeted bubbles in the PSMA-expressing tumors (PC3pip).

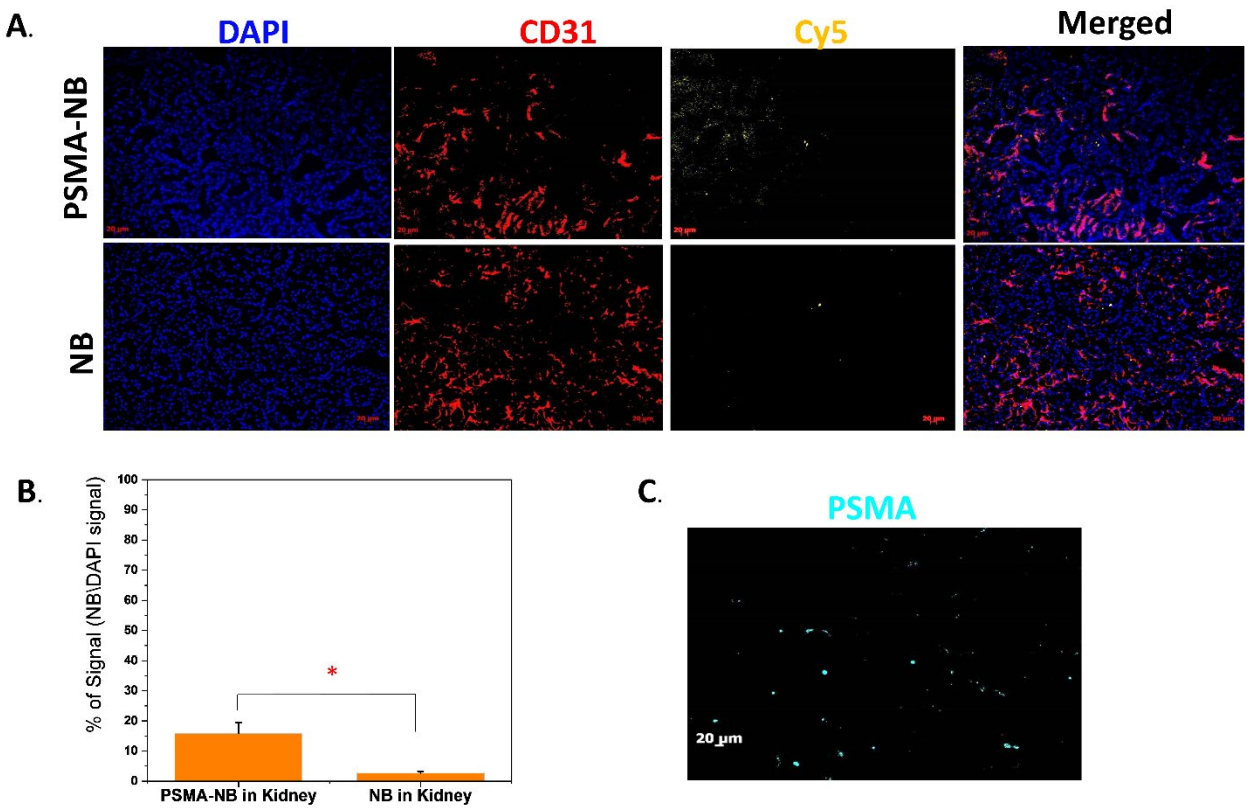


Figure S5. (A) Representative fluorescence images showing the bubble (yellow) and vasculature (red) distribution in Kidney. Residual Cy5.5 tagged PSMA-NB signal were seen in the kidney and its presence appears to be correlated to the location of vasculature (red). (B) The Cy5.5 signal intensity was quantified in these images and is shown here as a percentage of total cells in each kidney section field of view. PSMA-NB Cy5.5 signal was significantly different from the NB signal. (C) Representative fluorescence images showing the PSMA expression in the kidney (cyan).

References

1. Hernandez, C.; Abenojar, E. C.; Hadley, J.; de Leon, A. C.; Coyne, R.; Perera, R.; Gopalakrishnan, R.; Basilion, J. P.; Kolios, M. C.; Exner, A. A., Sink or float? Characterization of shell-stabilized bulk nanobubbles using a resonant mass measurement technique. *Nanoscale* **2019,** *11* (3), 851-855.
2. de Leon, A.; Perera, R.; Hernandez, C.; Cooley, M.; Jung, O.; Jeganathan, S.; Abenojar, E.; Fishbein, G.; Sojahrood, A. J.; Emerson, C. C.; Stewart, P. L.; Kolios, M. C.; Exner, A. A., Contrast enhanced ultrasound imaging by nature-inspired ultrastable echogenic nanobubbles. *Nanoscale* **2019,** *11* (33), 15647-15658.