Characterization of Nanobubble Ultrasound Contrast Agents in Human Whole Blood

Michaela B. Cooley[1], Eric C. Abenojar[1], Anirban Sen Gupta[1], Michael C. Kolios[2], Agata A. Exner[1]
[1]Case Western Reserve University, [2]Ryerson University

Statement of Purpose: Ultrasound contrast agents (UCAs) are being explored in preclinical and clinical applications ranging from targeted theranostic imaging of tumors to early diagnosis of diabetes.[1,2] UCAs can be either in the nanoscale (nanobubbles, NBs ~100–500 nm diameter) or microscale (microbubbles, MBs ~1–10µm diameter) and consist of a gas core with an ultra-flexible, compressible shell made of lipids, polymers, or proteins. Due to their sub-micron size, NBs have been shown to extravasate through hyperpermeable vasculature.[1] Some formulations of NBs have also shown significantly longer circulation time *in vivo* compared to MBs.[3] The reasons for the longer circulation time are not fully elucidated. One hypothesis is that these processes are aided by nanoparticle adsorption onto red blood cells (RBCs), delaying nanoparticle clearance.[4,5] Here, we examine the potential role of NB adsorption onto RBCs and how this process affects NB ultrasound imaging *in vitro*.

Methods: Lipid shell stabilized C_3F_8 NBs (~$4.07x10^{11}$ NBs/mL, 274±8 nm diameter) were formulated via mechanical amalgamation and isolation based on differential centrifugation as previously described.[6] MBs (~$1.18x10^6$ MBs/mL, 1.0±0.02 µm diameter) with the same lipid shell as NBs were formulated as previously described.[7] UCAs were mixed with solutions of a) fresh human whole blood (IRB Number: 09-90-195), b) RBCs in phosphate buffered saline (PBS) (45:55%), c) plasma in PBS (55:45%), d) recombined RBCs and plasma (45:55%), or e) PBS. Imaging with a clinical ultrasound system in contrast harmonic mode (12 MHz transducer, MI: 0.1, 1 fps) was performed in agarose, tissue-mimicking phantoms with an inlet for the solution of UCAs (**Figure 1A**). Ultrasound signal enhancement over time was analyzed. Autocorrelation analysis and decorrelation time were used to measure the random Brownian-like motion of NBs; decorrelation time was defined as the time shift required to reach a correlation coefficient of 0.5. NB solutions with whole blood were imaged using light microscopy, fluorescent microscopy (Cy5.5-labeled), and scanning electron microscopy (SEM, ThermoFisher Apreo 2S).

Results: **Figure 1A** shows the experimental setup and representative SEM and fluorescent microscopy images of NBs in whole blood. **Figure 1B** shows the percent change in ultrasound signal intensity over 500s when NBs are in the presence of PBS, whole blood, isolated RBCs, isolated plasma, or recombined plasma + RBCs. **Figure 1C** and **1D** shows decorrelation time, representing how random NB or MB motion is. A longer decorrelation time indicates less random motion. Our results clearly demonstrate that: (i) NBs localize to the RBC surface in whole blood; (ii) there is a distinct signal increase when NBs are in the presence of whole blood (22.8±13.1%); (iii) NBs in separated blood components and PBS do not show an increase in signal enhancement (RBCs: 1.9±12.2%, Plasma: -6.1±6.4%, PBS:-1.7±3.2%); (iv) NBs, but not MBs, show less random movement in the presence of whole blood and this does not occur with PBS, isolated RBCs, or isolated plasma.

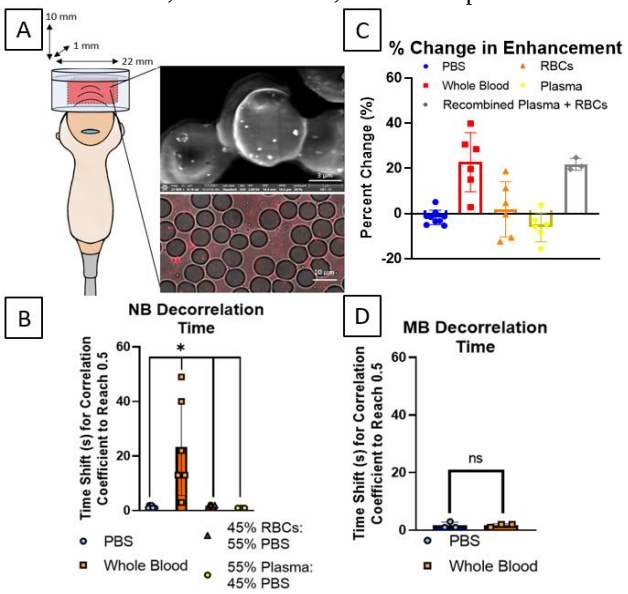

Figure 1. [A] Experimental setup with representative SEM and fluorescent microscopy images; **[B]** Percent change in signal intensity over 500s of imaging; **[C]** NB decorrelation time; **[D]** MB decorrelation time

Conclusions: The results demonstrate, directly through microscopy, that NBs localize to the RBC surface. This interaction may cause the maximum ultrasound signal enhancement generated by NBs to increase and stabilize over time. Adsorption likely does not occur unless both RBCs and plasma are present in the surrounding medium, which is why there is no increase in signal enhancement or decorrelation time with separated blood components. There is a clear distinction between MB and NB decorrelation time, indicating that any interaction between NBs and RBCs is likely size dependent. These experimental findings provide valuable information regarding the behavior of ultra-flexible and compressible lipid nanoparticles in human whole blood and how interactions with RBCs, in the presence of plasma, may affect NB *in vivo* behavior. This work could help explain the extended circulation time of NBs *in vivo* that could be exploited for numerous avenues of research (e.g. improving targeted theranostic imaging of tumors and early diabetes diagnosis).

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