Scientific Considerations for an In Vitro Bioequivalence Approach for Ultrasound Contrast Agent Products: How Size Distribution and Total Volume of Microbubbles Affect Its Echogenicity

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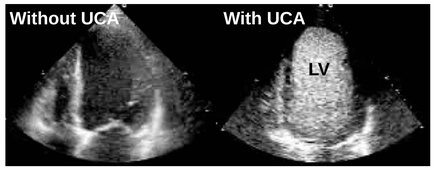
INTRODUCTION

Ultrasound contrast agents (UCAs), typically a suspension of gas microspheres (aka microbubbles), are injected intravenously to improve contrast in ultrasonography including suboptimal echocardiograms. There is a general correlation between acoustic signal efficiency, ultrasound frequency, as well as the size and concentration of gas microspheres. Because the performance of these products is not directly correlated with the concentration profile of the microsphere gas in the blood, developing and approving a generic UCA product based on traditional pharmacokinetic studies is challenging. To address this challenge, alternative methods are needed to assess the equivalence of generic UCA products and their reference listed drug product based on an understanding of the critical quality attributes with appropriate in vitro tests.

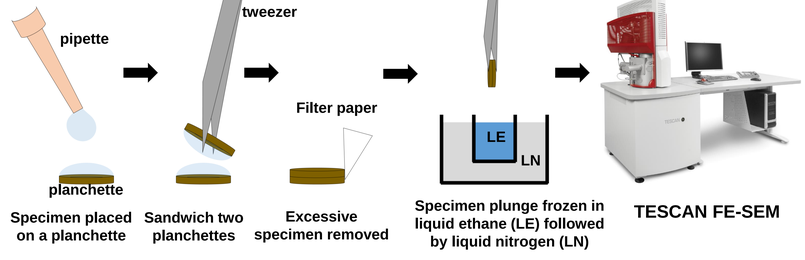


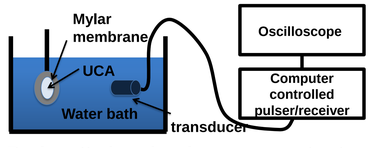
- Contrast Imaging (right) shows sharp delineation of the left ventricular endocardial borders compared to standard Imaging (left)

EXPERIMENTAL METHODS

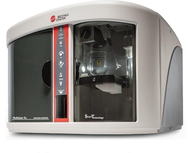
- Cryo scanning electron microscopy (cryo-SEM) was performed using a TESCAN FE-SEM equipped with a Leica EM GP grid plunge freezer, Leica EM VCT 500, Leica EM ACE 600, and Leica EM VCM.
- Size distribution and concentration was measured using a Multisizer 4 Coulter counter.
- The agitated suspension was fractionated by differential centrifugation.
- Three different shaking method were tested.
  - Vigorous hand shaking: ~ 4 shaking/sec for 20 sec.
  - Gentle hand shaking: ~ 1 shaking/sec for 20 sec.
  - Vortex shaking: ~ 3500rpm for 20 sec.
- Acoustic backscattered coefficient (BSC) measurement:
  - Two transducers (2.25 MHz & 5 MHz) were used to cover 1 – 7 MHz (clinically relevant frequency range).
  - The transducers transmitted a broadband pulse and received the gated RF signals backscattered from the UCA suspension.

Cryo-SEM sample preparation





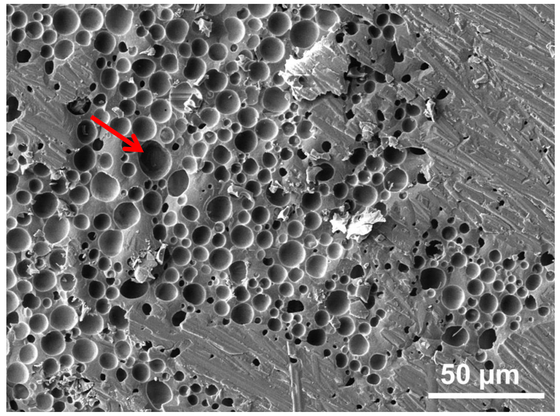
Backscattering signal measurement set-up



Multisizer 4

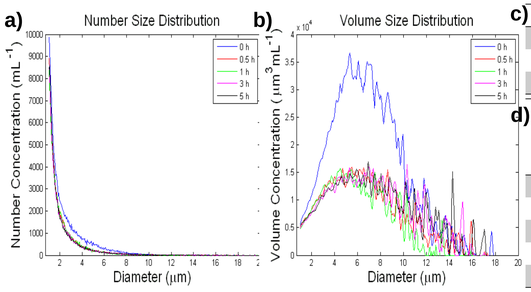
RESULTS

Cryo-SEM



- Representative cryo-SEM image revealed the hollow structure of a model UCA (approved sulfur hexafluoride lipid microspheres) that was originally filled with gas.
- Size distribution ranges from 1 – 10 µm, along with a rare presence of microbubbles > 10 µm (indicated with an arrow).

Particle Size Distribution of UCA Suspension



c)

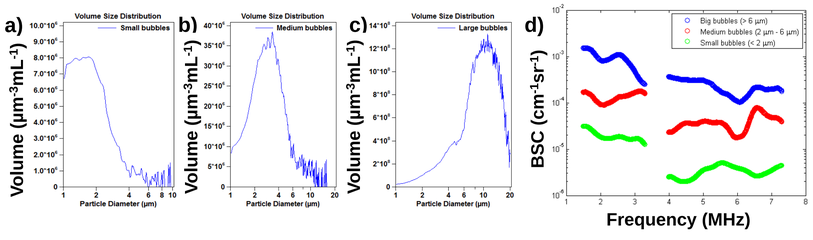
Diameter	Number (%)	Volume (%)
1 – 2 µm	64.2	4.7
2 – 6 µm	31.8	35.6
> 6 µm	3.9	59.7

d)

Time (h)	Number-weighted concentration (/mL)	Volume-weighted concentration (µm³/mL)
0	2.16×10^8	2.32×10^9
0.5	1.86×10^8	1.19×10^9
1	1.67×10^8	1.02×10^9
3	1.62×10^8	1.33×10^9
5	1.60×10^8	9.89×10^8

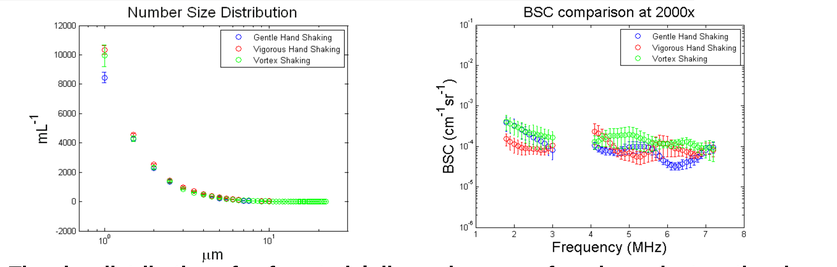
- Particle distribution showed that the majority (~64.2%) of the microspheres in the formulation were small (< 2 µm) but the majority (59.7%) of the particle volume fraction was from the large microspheres (> 6 µm). (c)
- Volume-weighted concentration was more sensitive to show the size distribution change over time. (a) – (b)
- Total microspheres concentration decreases in 30 min (14 % in number, 48.7 % in volume) and then remains relatively stable for 5 h. (d)
  - Unstable dispersions due to large size microspheres.

The Effect of Microspheres Size on BSC



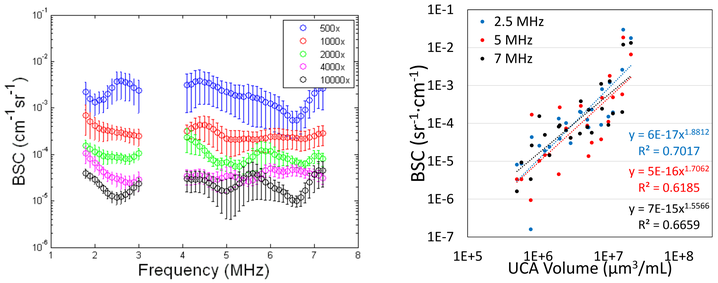
- Three microsphere populations of different size range (e.g. small, medium and large) were isolated from the same vial using differential centrifugation.
- Fractionated large microspheres (> 6 µm) had 2 orders of magnitude larger volume compared to small (< 2 µm) and medium microspheres (2 – 6 µm).
- <u>Microsphere size is an important factor in BSC of UCAs</u>.
  - With the same number-weighted concentration, large microspheres generated much higher backscattering signal compared to small microspheres.
  - Small microspheres contributed negligible acoustic effect in 1 – 7 MHz.

The Effect of Shaking Methods on Size Distribution and BSC



- The size distribution of soft material dispersions are often dependent on the shear force applied to make the dispersion. To test this, gas microspheres under increasing shear were prepared.
- Different shear (different shaking methods did not significantly affect the size distribution and BSC of UCA.

BSC of UCA suspension with series of dilution



- <u>Volume concentration is a key factor in acoustic properties.</u>
  - BSC decreased with decreased concentration (increased dilution factor).
- Power regression correlation between the volume concentration of microspheres and backscattering coefficient in the tested frequency range.
  - [BSC]=a·[microsphere volume]^b

CONCLUSIONS

- Cryo-SEM provided a indirect image of UCA with hollow microsphere structure in 1 – 10 µm size range.
- UCAs dispersions are unstable and quickly phase separate, therefore, the time analysis is performed after redispersion should be carefully considered during a quantitative characterization.
- Size and concentration (especially volume-weighted concentration) of UCA microspheres correlated with BSC, which directly relate to echogenicity.
- Microsphere preparation (shaking method) did not effect the size distribution or echogenicity of the dispersion formed.

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