Quantification of Microspheres Appearance in Brain Vessels 
Implications for Residual Flow Velocity Measurements, Dose Calculations, and Potential Drug Delivery

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Background and Purpose—Characteristics of ultrasound-activated gaseous microspheres (µS) reflective of their size and quantities are needed for future dose-escalation and drug delivery trials.

Methods—A double-blind, interobserver-validated analysis of multi-gate power-motion Doppler µS traces included large (>8µ) µS from agitated saline injections in the right-to-left shunt (RLS) positive stroke patients and small (<5µ) µS from acute patients without shunts receiving thrombolysis and perflutren-lipid µS.

Results—In 101 µS traces from 50 RLS-positive and 10 thrombolysis + µS treated patients, a large µS passage had median maximum duration 30.8 ms (interquartile range [IQR] 22.0ms), multi-gate travel time (MGTT) 58.6±19.3 ms versus small µS: duration 8.3ms (IQR 4.3ms), MGTT 43.2±13.9ms, P<0.001. Small µS had higher embolus-to-blood ratio (EBR): 17.5 (IQR 9.3) versus 7.5 (IQR 4), P<0.001. Receiver-operating curve areas were: duration 0.998 (95% CI 0.968 to 1.000), MGTT 0.766 (0.672 to 0.859), and EBR (Embolois-to-Blood Ratio) 0.927 (0.871 to 0.982), P<0.001. A 15.1-ms duration discriminated size ranges with 98% to 99% accuracy. On average, 130 sequential large (range 51 to 260) and 500 (265–588) small µS can produce continuous flow enhancement for 4 seconds. Small µS velocities on m-mode in obstructed vessels (39.8±11.3 cm/s) were similar to large µS in patent vessels (40.8±11.5 cm/s; P=0.719) and higher than surrounding red blood cell velocities (28.8±13.8 cm/s, P<0.001).

Conclusions—With normal or reduced flow, activated µS passage duration through a small power motion Doppler gate can quantify the dose of delivered µS. Ultrasound can determine a minimum number of µS needed to achieve constant flow enhancement and targeted drug delivery. Propagation speed of µS smaller than red blood cells may reflect plasma flow velocities around acute occlusions. (Stroke. 2008;39:1476-1481.)

Key Words: microspheres ■ stroke ■ occlusion ■ transcranial Doppler
Subjects and Methods

At participating study centers, all patients presenting with acute ischemic stroke are screened for their eligibility for systemic thrombolysis. A fast-track diagnostic transcranial Doppler (TCD) ultrasound is performed in the emergency department to localize an arterial occlusion with no delay in the initiation of thrombolytic therapy. TCD detects residual blood flow signals around thrombus using angiographically validated criteria, and TCD monitoring of these signals is continued for ultrasound-assisted intravenous tissue plasminogen therapy (TPA) thrombolysis according to the CLOT-BUST trial protocol, combined with an IRB-approved experimental administration of perflutren-lipid transpulmonary-stable \( \mu S \). Details of TCD monitoring methods and results of this pilot safety study are published in a separate report. Institutional Review Board approvals were obtained at all participating centers, and all patients or proxies signed an informed consent.

Ultrasound can detect backscatter from a single \( \mu S \) and we recorded single \( \mu S \) traces within a few seconds from the beginning of \( \mu S \) infusion when dilution is maximum. Subsequent traces documented arrival of multiple clusters of \( \mu S \) and continuous “curtain”-like flow enhancement. Perflutren-lipid \( \mu S \) traces were termed “small” because their mean size is 1 to 2 \( \mu \) with 99\% of these \( \mu S \) being \(<7\mu\) and almost 100\% of \(<8\mu\) size given their stable passage through the lung circulation.

The comparison group consisted of air \( \mu S \) (Figure 1A) that lack engineered protective shells and are normally filtered out by lung circulation in patients without right-to-left shunts. Therefore, the size of most air \( \mu S \) is likely to be greater than 8 \( \mu \), making them larger.

Figure 1. A, PMD traces of a single “large \( \mu S \) (A) and “small” (B) are shown in the upper frame. Graphs in the lower frame show theoretical percent distribution of “small” and “large” \( \mu S \) relative to their size scale in microns, microphotographs of small and large \( \mu S \), erythrocyte, and a drawing of a lung capillary. B, PMD traces of microsphere intensity (dB, yellow circles) measurements in spectral (A) and M-mode (C) displays. PMD traces of intensity (dB) of blood flow in spectral (B) and M-mode (D) displays. C, Magnified view of a single large \( \mu S \) trace represents measurements of the time interval for \( \mu S \) display in M-mode (AB), distance of \( \mu S \) travel (BC), and maximum \( \mu S \) duration in a single small M-mode gate. Yellow horizontal line represents the depth at which the spectral Doppler fast Fourier transformation measurements were obtained (spectral display not shown). D, In vitro experiment in a closed-loop flow system. Saline (A) and bovine blood (B) flows through the loop monitored by a 2 MHz ultrasound transducer. Small (C) and large (D) \( \mu S \) traces from the in vitro experiment are shown with their respective duration on the Motion mode display.
than erythrocytes (6 to 8 μ) or the smallest lung capillaries (8 μ).16 Even if smaller air μS could exist, they are likely to be unstable and collapse early after injection. These characteristics make TCD testing with agitated saline at least equivalent to echocardiography for detection of right-to-left shunts such as patent foramen ovale.15–22 Experimental data suggest that perfusate-lipid μS larger than 5 μ are eliminated from the circulation by adhesion in capillaries,23 whereas air μS cannot be detected without a shunt (these are filtered out by lung capillaries of 8 to 12 μ diameter). Therefore, a theoretical overlap for the 5 to 8 μ range in both size groups becomes negligible (Figure 1A). Air μS were termed “large” and these traces were obtained in chronic stroke patients with patent middle cerebral arteries (MCA) who were diagnosed positive for right-to-left shunts (RLS) with routine diagnostic TCD testing. We used normal saline agitated with air according to standard diagnostic protocol (9 cc saline +1 cc air) as recommended by the International Consensus Group.17 We obtained single and multiple μS traces of both estimated sizes in the M1 segment of MCA on the multi-gate power-motion Doppler (PMD 100, Spencer Technologies). A single crystal pulsed wave 2 MHz TCD beam intercepted the M1 MCA at 40 to 60 mm depths (PMD 100, Spencer Technologies). A single crystal pulsed wave 2 MHz transcranial Doppler ultrasound (PMD 100, Spencer Technologies) transducer was aimed to intercept the tube at 5 cm distance from the transducer surface at a 30-degree angle of incidence. The same perfusate-lipid μS15,16 were used to obtain small μS traces. Because 1.0 mL of activated agent solution contains 1.2×1010 small μS, we performed serial dilutions with normal saline to achieve a total of less than 100 small μS per one milliliter of saline: (withdraw 0.1 mL of activated ultrasound contrast agent and dilute it with 10 mL normal saline) ×3. This solution was then slowly injected into the 65 mL circulating bovine blood in the closed loop to obtain single μS traces in vitro.

Large μS were produced by agitation of normal saline and air (9 cc +1 cc) through a 3-way stop-cock, identical to preparation for the right-to-left shunt testing in humans. This mixture was then injected slowly into the closed-loop flow system to obtain large μS traces. Image analyses were similar to those applied to μS traces from human subjects.

Statistical comparisons between ultrasound parameters for small and large μS were done with the unpaired t test or Mann-Whitney U test as indicated. We also used analysis of covariance (ANCOVA) to compare the maximum single-gate μS duration between groups after adjusting for distance of μS travel, time interval for μS display in Motion-mode, μS velocity, and peak blood flow velocity at μS appearance. Univariate and multivariate analyses with logistic regression were performed to identify predictors of large versus small microspheres. Significance was calculated by the likelihood ratio test. The odds ratio (OR) and 95% confidence intervals (CI) were computed with the small μS group as reference. Finally, the ability of Motion-mode and Doppler parameters to discriminate μS size ranges were examined by receiver-operating characteristic (ROC) curve analyses. The Statistical Package for Social Science (SPSS Inc, version 10.0 for Windows) was used for statistical analyses.

In Vitro Experiment
To validate our assumptions and findings from human subjects, we conducted an in vitro experiment in a closed-loop flow system mimicking the size of the arteries of the circle of Willis (Figure 1D). Plastic tubes resembling the MCA were immersed in a water tank at 37°C. Normal saline and bovine blood were perfused through the closed loop by a motorized pump that induced pulsatile and unidirectional flow. A 2-MHz transcranial Doppler ultrasound (PMD 100, Spencer Technologies) transducer was aimed to intercept the tube at 5 cm distance from the transducer surface at a 30-degree angle of incidence. We hypothesized that the maximum μS duration in a single small (3 mm) Motion-mode gate. This was termed maximum “μS duration” for brevity and displayed in milliseconds (ms); 2. Total duration of μS trajectory through multiple adjacent Motion-mode gates (multi-gate travel time-MGTT), in ms; 3. Total distance (mm) that μS traveled on the Motion-mode display in the M1 MCA (depth range 60 to 40 mm); and, 4. μS propagation velocity (distance divided by MGTT), in cm/s.

We measured (Figure 1C):

We hypothesized that the maximum μS duration in a single small (3 mm) Motion-mode gate is representative of μS size, and we tested the performance of other previously published ultrasound parameters listed above. We controlled for potential confounding factors such as time-corresponding blood flow velocity and intensity and μS propagation velocities. Still images of μS traces were analyzed using high-resolution color pixel software (Microsoft Digital Image Editor, 2006). This protocol was internally validated before the project for consistency between the raters blinded to μS size group and patient information. The intrarater and interrater reliability in the measurement of Motion-mode and Doppler parameters was assessed using intra-class correlation coefficients. All measurements performed by 2 blinded investigators showed an inter- and intraobserver agreement for maximum single-gate μS duration, μS velocity, Motion-mode, and Doppler EBR of 0.81 (0.87, 0.84)0.90, 0.80(0.85, and 0.86(0.89, respectively (n=30 μS samples).

Results
We analyzed a total of 101 single and multiple μS traces from 50 RLS-positive and 10 TPA+ μS treated patients. The median maximum μS duration in a single Motion-mode gate and multi-gate travel time (MGTT) of a single large μS (n=62) was 30.8 ms (interquartile range [IQR] 22.0 ms) and 58.6±19.3 ms compared to small μS (n=39): duration 8.3 ms (IQR 4.3 ms), MGTT 43.2±13.9 ms, P<0.001 (Table 1).

Embolois-to-Blood Ratio
Small μS had higher intensity ratios (EBR 17.5, IQR 9.3) as compared to large μS (7.5, IQR 4.0), P<0.001, because of reduced cerebral blood flow intensity with acute MCA occlusions (median 1.0 dB, interquartile range 0.2) compared to flow intensities with normal patency in the RLS-positive patients (median 4.0 dB, interquartile range 3.0, P<0.001).

Comparison of μS Duration and EBR
The receiver-operator curve (ROC) areas were: duration 0.989 (95% CI 0.968 to 1.000), MGTT 0.766 (0.672 to 0.859), and EBR 0.927 (0.871 to 0.982), all P<0.001, respectively (Table 2, Figure 2). After adjustment for all Motion-mode and Doppler parameters, including blood flow and μS velocities, a multivariable logistic regression model showed that maximum μS μS duration (OR per 1 ms
### Table 1. M-Mode and Doppler Parameters of Large and Small μS

| Variable                        | Large (n=62) | Small (n=39) | *P*
|---------------------------------|--------------|--------------|---
| M-mode                          |              |              |   |
| Distance of μS travel, mm       | 23.3±7.4     | 15.7±4.7     | <0.001|
| MGTT, msec                      | 58.6±19.3    | 43.2±13.9    | <0.001|
| μS velocity, cm/sec             | 40.8±11.5    | 39.8±11.3    | 0.719 |
| Maximum μS duration,* msec      | 30.8(22.0)   | 8.3(4.3)     | <0.001|
| Adjusted maximum μS duration**  | 27.3(24.8–29.9) | 15.5(12.1–18.9) | <0.001|
| μS intensity in M-Mode, dB      | 32.5±6.4     | 19.2±5.4     | <0.001|
| Blood intensity in M-Mode, dB   | 4.0(3.0)     | 1.0(0.2)     | <0.001|
| EBR (M-Mode)                    | 7.5(4.0)     | 17.5(9.3)    | <0.001|
| Doppler                         |              |              |   |
| Peak flow velocity at μS        | 45.7±17.0    | 28.8±13.8    | <0.001|
| μS intensity in Doppler         | 38.9±7.4     | 22.4±8.1     | <0.001|
| spectral display, dB            | 12.0(3.0)    | 6.0(2.0)     | <0.001|
| Blood intensity in Doppler      |              |              |   |
| spectral display, dB            |              |              |   |
| EBR (Doppler spectrum)          | 4(0.8)       | 4.5(1.1)     | 0.002 |

Variables with normal and skewed distribution are presented as mean±SD and median (interquartile range), respectively.

*Single gate. **Maximum μS duration was adjusted for the distance of μS travel, time interval for μS display in M-mode, μS velocity, and peak flow velocity at μS appearance by means of analysis of covariance (ANCOVA).

μS Numbers for Flow Enhancement

Because median maximum μS duration was 30.8 ms for large and 8.3 ms for small μS, the median μS number needed to arrive sequentially to produce continuous, or the curtain-like appearance of flow enhancement on a 4-second sweep was calculated as 130 (range 51 to 260) for large μS and 500 (range 265 to 588) for small μS.

### Table 2. ROC Curves of Doppler and M-Mode Parameters for Discrimination of μS Size Range

| Variable                        | ROC Area  | 95%CI       | *P*
|---------------------------------|-----------|-------------|---
| Maximum μS duration,* msec      | 0.989     | 0.968–1.000 | <0.001|
| μS trace length, mm             | 0.800     | 0.715–0.885 | <0.001|
| μS trace time on M-mode, msec   | 0.766     | 0.672–0.859 | <0.001|
| Peak flow velocity at μS        | 0.784     | 0.693–0.876 | <0.001|
| EBR (M-Mode)                    | 0.927     | 0.871–0.982 | <0.001|
| EBR (Doppler spectrum)          | 0.681     | 0.572–0.789 | 0.002 |

*Single gate.

μS Propagation and Vessel Patency

Controlling for the cerebral blood flow velocities and μS propagation speed showed that despite cerebral blood flow intensity and velocity reductions with acute MCA occlusions, small μS velocities in obstructed vessels (39.8±11.8 cm/s) were similar to large μS in patent vessels (40.8±11.5 cm/s, *P*=0.719). Furthermore, small μS were moving on an average 11 cm/s faster than the residual red blood cell flow at the time of μS appearance (28.8±13.8 cm/s, *P*<0.001), whereas large μS were moving at velocities similar to the surrounding cerebral blood flow (Table 1). Therefore, we further examined propagation of small μS in the unobstructed anterior cerebral arteries (ACA) in patients with persisting MCA occlusions. ACA flow signals were detected by motion-mode gates at 60 to 70 mm simultaneously with μS propagation in the obstructed MCAs. Small μS in the unobstructed vessels moved with a single-gate duration of 8.2 ms (n=10, IQR 1.5), which was similar to the duration in obstructed MCA vessels (8.3 ms, IQR 4.3, *P*>0.9). Additionally, small μS propagation velocities in patent ACAs were similar to small μS velocities in the obstructed MCAs and large μS velocities in patent vessels (small μS/patent ACA 38.9±13.7 versus small μS/unobstructed MCA 39.8±11.3 versus large μS/patent ACA 40.8±11.5 cm/s, *P*>0.9).

In Vitro Experiment

Single traces for both small and large μS in a controlled closed-loop flow system (Figure 1D) showed a median maximum duration for small μS (n=20) of 7.9 ms (IQR 1.6 ms) as compared to large μS traces (n=20): median 29.2 ms, IQR 19.9, *P*<0.001. Mean propagation velocities of small μS (45.5±12.4 cm/s) were similar to propagation velocities of large μS (37.6±18.1 cm/s, *P*=0.129). EBR could not be obtained because neither Motion-mode nor spectral display detected any background signals from circulating bovine blood. A longer than 15.1-ms single μS trace duration discriminated large from small μS with sensitivity 98.4%, specificity 100%, PPV 100%, NPV 97.5%, and 99% accuracy.

Discussion

Our study showed that in patients with normal or reduced flow conditions, the duration of activated μS passage through a small power motion Doppler gate correlates with μS size range and can provide preliminary estimates of μS numbers delivered to an intracranial vessel. While activating μS as potential therapeutic or drug delivery agents, real-time ultrasound monitoring can determine the minimum number of μS needed to achieve constant flow enhancement, a potentially useful finding for targeted drug delivery.

Our findings are in contrast to previous inconsistent results using EBR,9 intensity threshold measurements,8,10,25–26 and other analyses to characterize embolic signals.7,11,27–29 One potential reason is that we dichotomized μS size. Future applications of μS duration may show inconsistencies for calculating actual μS size particularly if μS propagate at different velocities. Furthermore, ultrasound traces may include clusters of μS. The ability of power Motion-mode to display intensity and duration of emboli tracks in multiple...
small contiguous gates\textsuperscript{30} may offer some technical solution to this problem, and it was encouraging that our in vitro experiment showed characteristics of $\mu$S similar to those observed in human studies.

One unexpected finding in our study is how small $\mu$S propagate in patent and obstructed vessels. Although small $\mu$S traveled in areas of slower blood flow velocities with acute arterial occlusions, their propagation speed was comparable to the speed of small and large $\mu$S that traveled through unobstructed vessels with higher blood flow velocities. In other words, $\mu$S propagation speed is comparable to the surrounding blood flow velocity in patent vessels, whereas small $\mu$S move faster than the residual blood flow around acute obstructions. This finding may indicate that perflutren-lipid $\mu$S smaller than erythrocytes may actually travel with either: (1) the speed that is dependent on the size of $\mu$S, the size of the residual lumen and pressure gradients across acute but incomplete obstructions; or (2) the speed of residual flow around thrombus that is so reduced that it could not be detected without $\mu$S presence. Of note, these velocity measurements were derived from $\mu$S traces on power motion-mode and not from spectral displays. The advantage of power motion-mode is its ability to track the front edge of the returned signal intensity over time. This makes $\mu$S velocity measurements independent of artifacts that occur with fast Fourier transformation on spectral analysis.

Because $\mu$S can change the signal-to-noise ratio and artificially increase velocities detected by spectral Doppler, an artifact such “blooming”\textsuperscript{31} could affect spectral measurements. However, $\mu$S traces were obtained from first-arriving microspheres at concentrations far less than those used for imaging (ie, 1.4 cc given as a bolus). Therefore, blooming at the very beginning of $\mu$S infusion should be minimal. If our findings are confirmed in subsequent clinical trials, $\mu$S may provide a tool for the residual flow assessment around and beyond acute arterial occlusions.

Our study had some additional limitations. Embolic material other than $\mu$S may be detected in cerebral arteries during thrombolysis for acute stroke. Acoustic properties and appearance of emboli on motion-mode and spectral TCD may resemble some $\mu$S features, thus possibly contaminating the sample. Arrival of multiple $\mu$S even at the beginning of infusion may not be completely excluded. Independent verification of our findings is needed as well as development of software for real-time quantification of $\mu$S during treatment. Finally, we compared the velocities of small and large $\mu$S in an setting mimicking the size of patent arteries of the circle of Willis and confirmed our in vivo findings in human subjects (small and large $\mu$S travel with similar velocities in patent vessels). However, our in vitro-experiment did not simulate occluded intracranial arteries, and therefore we were unable to compare velocities between small and large $\mu$S in obstructed vessel in an experimental setting.

In conclusion, ultrasound monitoring can be used for both activation of $\mu$S and estimation of the minimum dose of $\mu$S delivered to an intracranial vessel with flow enhancement. The ability of $\mu$S smaller than red blood cells to permeate around thrombus may represent plasma flow and provide measurement of its velocity. This information can be used to determine $\mu$S doses for potential targeted drug-delivery.

Table 3. Accuracy Parameters of $\mu$S Duration, M-Mode Intensity, and EBR for Discrimination of $\mu$S Size Range

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum $\mu$S duration &gt;15.1 ms</td>
<td>98.4%</td>
<td>100.0%</td>
<td>100%</td>
<td>97.5%</td>
<td>99.0%</td>
</tr>
<tr>
<td>M-Mode EBR&lt;13 dB</td>
<td>90.3%</td>
<td>84.6%</td>
<td>90.3%</td>
<td>84.6%</td>
<td>88.1%</td>
</tr>
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Figure 2. Boxplots of maximum $\mu$S duration and M-mode EBR. ROC curves demonstrate the ability of maximum $\mu$S duration (A) and M-mode EBR (B) to differentiate large from small microspheres.
Disclosures
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