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● *Original Contribution*

SINGLE-PARTICLE OPTICAL SIZING OF MICROBUBBLES

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Abstract—Single-particle optical sizing techniques are being used to determine the size distributions of microbubble ultrasound contrast agents and to study the dynamics of individual microbubbles during ultrasound stimulation. The goal of this study was to compare experimental light obscuration and scattering measurements of microbubble size distributions with predictions from generalized Lorenz-Mie scattering theory (GLMT). First, we illustrate that a mono-modal size distribution can be misrepresented by single-particle light obscuration measurements as multi-modal peaks because of non-linearities in the extinction cross section-versus-diameter curve. Next, polymer bead standards are measured to provide conversion factors between GLMT calculations and experimental flow cytometry scatter plots. GLMT calculations with these conversion factors accurately predict the characteristic Lissajous-like serpentine scattering plot measured by flow cytometry for microbubbles. We conclude that GLMT calculations can be combined with optical forward and side scatter measurements to accurately determine microbubble size. (E-mail: mark.borden@colorado.edu) © 2014 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Laser obscuration/extinction, Flow cytometry, Electro-impedance sensing, Generalized Lorenz-Mie theory, Lipid-coated microbubbles, Polystyrene beads.**

INTRODUCTION

Microbubbles are being used as intravascular contrast agents and molecular probes in ultrasound imaging (Inaba and Lindner 2012; Kiessling et al. 2012), drug delivery vehicles in ultrasound-assisted therapy (Sirsi and Borden 2012; Sutton et al. 2013), and as an oxygen gas carrier for ultrasound-targeted delivery (Kwan et al. 2012). The acoustic performance of a microbubble suspension depends strongly on its size distribution, and it is therefore important to identify proper methods of counting and sizing.

Sennoga et al. (2012) recently reported on a careful study to compare and contrast the precision of three microbubble sizing and counting methods: optical microscopy (OM), electro-impedance volumetric zone sensing (ES) and multiple-particle low-angle laser diffraction (LD). They tested these three methods for counting and sizing of the commercially available ultrasound contrast agent SonoVue (Bracco, Milan, Italy) and found that OM provided the best sizing reproducibility. ES provided the best counting repeatability, and LD gave the worst reproducibility in both sizing and counting. However, LD was more sensitive to sub-micron particles and reported substantially smaller mean diameters than the other methods. On the basis of these results, Sennoga et al. concluded that LD is not suitable for routine sizing and counting of microbubbles, and neither ES nor OM is superior for all microbubble sizing applications. Instead, they suggested that the choice of counting/sizing method should be based on the nature of the sample and desired physical characteristics.

Throughput is another important consideration for routine microbubble sizing. For example, OM is a relatively low-throughput method because it requires diluting and smearing the sample between glass slides, adjusting the microscope objective to find the correct focal plane, recording images and performing calibrated image analysis. Finding the correct focal plane with OM is particularly challenging, as pointed out by Sennoga et al. (2012), because movement of the focal plane through the sample volume disproportionately leads to inaccuracies in counting and sizing smaller microbubbles. In addition, Gelderblom (2012) recently described the difficulties in analyzing bright-field images owing to diffraction rings

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sometimes observed around a bubble, and concluded that fluorescence microscopy provides a more accurate and reproducible measurement of microbubble size. Owing to these constraints, there is significant demand for more convenient, high-throughput methods.

The dynamic range of the measurement technique is another important consideration. ES is higher throughput than OM, but it suffers from a narrower measurement range, typically 2% to 40% of the orifice diameter of the measurement tube (*e.g.,* 0.6–18 μm for a 30-μm-diameter orifice). Additionally, small orifices are susceptible to clogging by large particles in the sample.

Single-particle optical sizing (SPOS) methods can provide throughput similar to that of ES, but with a broader measurement range (*e.g.,* 0.5–100 μm) and less concern over clogging. For example, the AccuSizer (Particle Sizing Systems, Santa Barbara, CA, USA) is an SPOS system in which the particles are injected into a sample chamber, homogenized by stirring and pumped through an optical sensor. Particle sizing and counting are accomplished as an individual particle obstructs the optical path of the laser, causing a loss of light (obscuration) at the photodetector. Automated fluidics can be employed to dilute the sample and minimize coincident counts, and to purge and prime the system for the next sample measurement. It is possible that the flow systems in these devices can affect microbubble size distribution, particularly for microbubbles of low stability against dilution, although this has not been fully characterized. Regardless, several research laboratories employ single-particle light obscuration (LO) measurements to count and size microbubbles. Interestingly, LO measurements of microbubbles often give multi-modal size distributions (Feshitan et al. 2009). Microbubbles made by conventional emulsification techniques, such as sonication and mechanical agitation, are expected to exhibit a single-peak, log-normal size distribution. The mono-modal distribution often is observed for microbubbles measured by ES and OM (Feshitan et al. 2009; Sennoga et al. 2012), indicating that the multi-modal size distribution in LO is a measurement artifact.

Another variation of SPOS is flow cytometry (FC), which measures the optical forward and side scatter from a particle. In addition, FC provides the added functionality of detecting and quantifying the presence of fluorescence probes on the microbubble surface. A plot of FC forward scatter versus side scatter can be used to isolate measurements based on microbubble size (Chen and Borden 2010; Tu et al. 2011). The fluorescence intensity can then be used to measure microbubble surface modifications and bio-interactions (Chen and Borden 2011; Sirsi et al. 2012). FC measurements of microbubbles often yield a serpentine pattern on the scatter plot (Borden et al. 2007), and this pattern is highly reproducible between batches and instruments (Chen and Borden 2010). Previous FC measurements of polystyrene beads have exhibited similar patterns, or Lissajous-like curves, that were predicted by generalized Lorenz-Mie theory (Doornbos et al. 1994; Hoekstra et al. 1994). Previous work has also revealed intensity variations associated with bubbles in a fluid (Marston et al. 1988). The serpentine curve suggests that particles scatter light in certain directions non-uniquely, particularly for forward scatter. Microbubble scatter events are found to lie off of this serpentine pattern when they are non-spherical, for example, when they contain large surface folds (Chen and Borden 2010; Sirsi et al. 2013). It would be helpful to understand the physical origin of this curious serpentine pattern on the FC scatter plot for microbubbles and whether it is correlated with multi-modal size distributions obtained by light obscuration (*e.g.,* see Figs. 1 and 7).

Laser scattering and flow cytometry have also been used to investigate the radius-time dynamics of individual ultrasound-stimulated microbubbles. For example, Tu et al. (2009) used optical scattering measurements on SonoVue microbubbles to compare various shell models. Hsu et al. (2011) used laser scattering to investigate cycle-to-cycle dynamics of individual microbubbles. Tu et al. (2011) also developed an FC method for high-throughput characterization of microbubble shell properties and determination of size distributions. Such high-throughput optical scattering methods may provide useful data to supplement and examine single-bubble high-speed microscopy techniques over large populations (Chin et al. 2003; Dayton et al. 1999).

In this study, we employ generalized Lorenz-Mie scattering theory (GLMT) as a mathematical framework for the explanation of microbubble scattering phenomena measured by the two SPOS techniques: LO and FC. GLMT calculations allow prediction of the total light scattered and absorbed (light extinction) or the amount of light scattered within a solid angle window for a given particle size, assuming the microbubbles are spherical and non-absorbing. We illustrate that GLMT can be used to generate serpentine curves similar to those observed in FC measurements of microbubble populations. We also illustrate that GLMT can be used to explain the multi-modal peaks found in LO measurements.

THEORY

Several scattering models can be used to predict the light scatter from a particle, notably Rayleigh scattering, Mie theory and geometric optics. However, the validity of Rayleigh scattering and geometric optics depends on particle size compared with the wavelength of the