ESTIMATION OF PROTEIN AGGREGATE DENSITY USING SEDIMENTATION COMBINED WITH MICRO-PARTICLE TRACKING

Richard Cavicchi, Dean Ripple, Jason King, Cayla Collett#

Biomolecular Measurement Division, NIST

#West Virginia Wesleyan College
Outline

1. Possible improvements to Flow Microscopy Analysis
   a. Shape effect
   b. New image processing algorithm
2. Aggregate Density measurements based on sedimentation
3. New Reference Materials
   a. Fluoropolymer aggregate simulants
   b. Other materials under development
Subvisible Particle Sizing Methods

Light obscuration (LO) \( \Delta I \sim \text{area} \)

Flow Imaging (FI) area

Electrical Sensing Zone (Coulter) (ESZ)
\( \Delta R \sim \text{volume} \)

Resonance Mass \( \Delta M \)

Particle tracking \( D \) (related to \( d_{\text{hydrodynamic}} \))
Measured diameter, concentration for polystyrene beads.

<table>
<thead>
<tr>
<th>Nominal dimension (µm)</th>
<th>ESZ (Multisizer)</th>
<th>FI Instrument A</th>
<th>FI Instrument B</th>
<th>LO</th>
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<tbody>
<tr>
<td></td>
<td>d (µm)</td>
<td>N (ml⁻¹)</td>
<td>d (µm)</td>
<td>N (ml⁻¹)</td>
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<tr>
<td>5</td>
<td>5.27</td>
<td>18672</td>
<td>5.20</td>
<td>20893</td>
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<td>10</td>
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<td>40</td>
<td>40.1</td>
<td>6288</td>
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</tr>
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</table>

Good agreement
For Protein aggregates, methods don’t agree...

Protein aggregates vary in size, shape, intensity...
Variable Threshold Method for improved Particle Boundary Detection

Can we get improved boundary detection without losing nearly transparent particles?

New algorithm, break analysis into two parts:

a) Find particles with a single low threshold
b) Evaluate particle boundaries using a threshold appropriate to the intensity of each individual particle
Variable Threshold Method for improved Particle Boundary Detection

Result:
Strategy for fragments

(a) Connect fragments
(b) Draw the convex hull
(c) and (d) Perform “AND” operation with original thresholded area
(e) Resulting boundary superimposed on image
Collage with overlays

Implemented in ImageJ/FIJI, open source software

Results table of analyzed particles

<table>
<thead>
<tr>
<th>Area</th>
<th>Mean</th>
<th>StdDev</th>
<th>Mode</th>
<th>Min</th>
<th>Max</th>
<th>X</th>
<th>Y</th>
<th>XM</th>
<th>YM</th>
<th>Perim.</th>
<th>BX</th>
<th>BY</th>
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<td>224.810</td>
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Results table of analyzed particles

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<tr>
<th>Area</th>
<th>%Area</th>
<th>RawIntDen</th>
<th>Slice</th>
<th>FeretX</th>
<th>FeretY</th>
<th>FeretAngle</th>
<th>MinFeret</th>
<th>AR</th>
<th>ARound</th>
<th>Solidity</th>
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<tr>
<td>2</td>
<td>1.752</td>
<td>100</td>
<td>4721</td>
<td>1</td>
<td>283</td>
<td>6</td>
<td>144.462</td>
<td>4.833</td>
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<td>0.428</td>
</tr>
</tbody>
</table>

Results table of analyzed particles
Collage strips of same beads analyzed with single threshold and Variable Threshold (red background) (Images obtained with Flowcam 10x)

Test of Variable Threshold Method
ECD 13.4 µm

Variable Threshold

10 µm

SEM image
Test of Variable Threshold Method protein aggregates
Possible improvements to Flow Microscopy Analysis

a. Shape effect, can use perimeter to improve equivalent circular diameter calculation for elongated particles.

b. Image processing algorithm yields tighter boundaries for intense particles, without losing counts on nearly transparent particles.
   i) better analysis for samples with varying particle properties
   ii) same instrument settings for calibration and operation
   iii) may be useful for improved classification of particles
What is the density of a protein aggregate and why is it important?

\[ \rho = \frac{M}{V} \]

Different methods characterize different aspects of size:

Flow imaging: spatial extent \((A\sim V)\)
Electrical Sensing Zone: liquid excluded volume \((V')\)
Resonance Mass: \(M\)
Particle Tracking: \(V_h\)

To compare particle counts above a certain size, or size distribution from these methods, a density value for protein aggregates is needed.
For a sphere \[ \Delta \rho = \frac{9 \mu v}{2gR^2} \]

\( \mu \) viscosity, \( v \) velocity, \( g = 9.8 \, \text{m/s}^2 \), \( R \) hydrodynamic radius of sphere

Measuring \( R \) from image, and measuring \( v \) by following images, can deduce \( \Delta \rho \)

To reduce convection, use Rectangular glass capillary 50 \( \mu \text{m} \) or 100 \( \mu \text{m} \) inside thickness \( \times 1 \, \text{mm} \), sealed at both ends
Aggregates from NIST MaB

100 μm
**Sedimentation: Stokes Law**

\[
\Delta \rho = \frac{9 \mu v}{2gR^2}
\]

Where \( \mu \) is viscosity, \( g \) is gravitation constant, \( v \) is the velocity (initial –final vertical position/time), and \( R \) is particle radius from image or Brownian trajectory analysis (below)

**Brownian Motion- Microparticle Tracking Analysis**

\[
<x^2> = 2Dt
\]

For a sphere

\[
D = \frac{k_BT}{6\pi \mu R}
\]

\( <x^2> \) is mean square displacement, calculated from particle trajectory (see D. Ernst & J. Kohler, Phys.Chem. Chem. Phys., 2013, 15, 845)
What is the density of a protein aggregate?

JAMES G. BARNARD, SATISH SINGH, THEODORE W. RANDOLPH, JOHN F. CARPENTER

1Department of Pharmaceutical Sciences, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado 80045
2Biotherapeutics Pharmaceutical Sciences, Pfizer Inc., 700 Chesterfield Parkway West, Chesterfield, Missouri 63017
3Department of Chemical and Biological Engineering, University of Colorado, Boulder, Colorado 80309

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0.03 g/mL has been reported. Because protein aggregates will contain both protein and water, their density is expected to be bounded by the density of water (1.0) and the density of the monomeric protein. Thus, we arbitrarily assumed that each particle contained (by volume) 75% of protein and 25% of water. To arrive at a mass value, the volume of a sphere for each size bin was multiplied by the protein volume fraction estimate (0.75) and the protein density value (1.43 g/mL). This mass value was multiplied by the number of particles per milliliter detected in a given size bin to give the mass per milliliter per bin values (Eq. 1). These mass estimates were then integrated over the entire particle size range to provide the integral and total mass of the sample.

Estimated protein mass per size bin

\[ = (0.75) \times \text{(Volume)} \times (1.43 \text{ g/mL}) \times \text{(Number of particles)} \]  

(2) \[ \text{from MFI can be further used to develop mathematical models to improve calculated estimates of particle volume, surface area, and mass.} \]

In the Barnard et al. method,\textsuperscript{17} it was assumed that protein particles were spherical in nature and that particle density was composed of 75% protein and 25% water. A protein density of 1.43 g/mL (determined by Quillin and Matthews)\textsuperscript{37} was also used in their calculations. For our method, we not only used MFI data to account for the nonspherical nature of the particles, we also implemented some updated assumptions about the composition of protein particles. First, we used a mAb density value of 1.41 g/mL. This value was obtained using an equation developed by Fischer et al. that accounts for the empirical dependency of protein density on its molecular weight.\textsuperscript{40} Second, we estimated the volume fraction of protein in a protein particle to be 0.2 based on several considerations: (1) protein crystals have large interstitial volumes that are around 50% of the total volume\textsuperscript{42}; (2) using an RI of 1.41\textsuperscript{28} and \( dn/dc \) of 0.19 mL/g\textsuperscript{43} at 589 nm for protein particles, we can estimate the concentration of protein in a particle suspended in water to be \( \sim 0.4 \) g/mL (\( \sim 28\% \) protein volume fraction) because the relationship has been shown to be linear for other proteins at much greater concentrations\textsuperscript{44,48}; and (3) the density of IgG layers adsorbed to different surfaces was measured to be 1.05–1.10 g/mL,\textsuperscript{46} which corresponds to volume fractions of \( \sim 10\%–24\% \) in pure water. By assuming 0.2 to be the volume fraction of protein in a particle, the protein mass values calculated using the Barnard et al. method\textsuperscript{17} are reduced by \( \sim 3.75\)-fold. It would be beneficial in the future to develop an analytical method to determine the average volume fraction of protein contained within particles generated from different stresses to further improve the accuracy of these particle mass calculation methods.

The particle mass calculation used in this work is referred to as the E-V method. On the basis of observation that subviscible particles tend to range from fiber like to roughly spherical,
3 mm Polystyrene beads in water
3 mm Polystyrene beads in water, background subtracted
3 mm Polystyrene beads in water, with tracks

Particles analyzed by custom ImageJ plugin
Tracks determined using Trackmate
Bead trajectories - Microparticle tracking analysis

Polystyrene beads in water
Results 1 µm bead

[Graphs showing data distribution for median tracking diameter, sedimentation velocity, and image diameter against tracking length.]
Results 2 μm bead
Results 5 μm bead
Results 10 µm bead
<table>
<thead>
<tr>
<th>Beads: Image Diameter vs Tracking Diameter</th>
</tr>
</thead>
</table>

```
<table>
<thead>
<tr>
<th>Beads: Density by MSD &amp; Image (after diam correction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSD</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>10</td>
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```
Beads: Image Diameter vs Tracking Diameter

Beads: Density by MSD & Image

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<th>MSD</th>
<th>Image</th>
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<td>51.9681</td>
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<td>43.1026</td>
<td>41.0793</td>
</tr>
<tr>
<td>10</td>
<td>53.313</td>
<td>43.664</td>
</tr>
</tbody>
</table>
Aggregates from NIST MaB with tracks

Particles analyzed by custom ImageJ plugin
Tracks determined using Trackmate
Average density \(\sim 1040 \text{ kg/m}^3\)

Aggregate density increases with decreasing size
Conclusions

- Improvements in analysis can provide better accuracy in particle sizing for Flow imaging: variable threshold plugin for ImageJ/FIJI
- Particle density critical for relating counts from different instruments
- Microparticle tracking gives useful dimensions up to ~5 μm
- Results based on sedimentation of 1μm-7 μm particles indicates a density of 1040 kg/m³, with density increasing with decreasing size. This density is lower than generally assumed in the literature.
Test of Variable Threshold Method

Silica beads $n=1.33$ fluid

Silica beads $n=1.43$ fluid

Variable Threshold
Protein aggregate at different focal distances