Beyond Aggregates: Light Scattering Tools for Biophysical Characterization and Quantitation

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About Wyatt Technology Corporation

- Founded in 1982 by Dr. Philip J. Wyatt to commercialize multi-angle light scattering (MALS)
- Award-winning, robust, low maintenance, easy to use instruments that have been validated by thousands of peer-reviewed publications
- Leading provider of light scattering instruments for solution-based characterization of macromolecules and nanoparticles: *molar mass, size, charge, & interactions*
- Pioneer of SEC-MALS and FFF-MALS, now standard analytical tools in protein, biopharma, biopolymer, synthetic polymer labs and more
- Pioneer of plate-based dynamic light scattering (DLS), an essential technology for high-throughput protein and nanoparticle formulation
Light scattering provides critical attributes

<table>
<thead>
<tr>
<th>Static light scattering</th>
<th>Dynamic light scattering</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Identity</strong></td>
<td></td>
</tr>
<tr>
<td>✔ Molar mass</td>
<td>✔ Hydrodynamic size</td>
</tr>
<tr>
<td>✔ Size (RMS radius)</td>
<td>✔ Conformation</td>
</tr>
<tr>
<td>✔ Conjugation/loading</td>
<td></td>
</tr>
<tr>
<td>✔ Extinction coefficient</td>
<td></td>
</tr>
<tr>
<td>✔ Concentration</td>
<td></td>
</tr>
<tr>
<td><strong>Quality</strong></td>
<td></td>
</tr>
<tr>
<td>✔ Aggregate amount and size</td>
<td>✔ Aggregate size</td>
</tr>
<tr>
<td>✔ Fragment amount and size</td>
<td>✔ Viscosity</td>
</tr>
<tr>
<td>✔ Heterogeneity</td>
<td></td>
</tr>
<tr>
<td><strong>Stability</strong></td>
<td></td>
</tr>
<tr>
<td>✔ Reversible associations</td>
<td>✔ Diffusion interaction parameter</td>
</tr>
<tr>
<td>✔ Second virial coefficient</td>
<td>✔ Transition temperatures</td>
</tr>
</tbody>
</table>
Multi-Angle Light Scattering

The variation of scattered light with scattering angle is proportional to the average size of the scattering molecules.

The amount of light scattered at 0° is directly proportional to the molar mass and mass concentration:

\[ I_{\text{scattered}} \propto M \cdot c \cdot \left(\frac{dn}{dc}\right)^2 \]

Isotropic scattering

Small particles
(R < 10 nm)

Anisotropic scattering

large particles
(R > 10 nm)
Dynamic light scattering (DLS)

Brownian motion of particles in solution

Decay rate ∝ Diffusion coefficient

Avalanche Photodiode

Autocorrelation Analysis

Stokes-Einstein Relationship

Light Intensity Fluctuations

Hydrodynamic Radius

6.5 nm (IgG)
Typical MALS hardware setup and applications

SEC-MALS of biomolecules
- Proteins, polysaccharides, nucleic acids, conjugates
- Measure monomer and aggregate molar mass, molar mass distribution and polydispersity ($M_w/M_n$)
- Characterize branching and degree of conjugation

Eclipse AF4-MALS of BioNPs
- Viral vectors, EVs/exosomes, lipid or other NPs
- Isolate, identify, and quantify nanoparticle size, molar mass, and concentration
- Characterize shape/structure and payload/cargo content
Example:
Online Molar Mass and RMS Radius

Light scattering and refractive index data are measured for each eluting slice to yield *absolute* molecular weight, $R_g$, and (with DLS) $R_h$.

$I \propto M_w c$

$M_w = 122 \text{ kDa}$

$R_{\text{rms}} \propto \text{slope}$

$R_g = 11.5 \text{ nm}$
Example:
Online Molar Mass and RMS Radius

Light scattering and refractive index data are measured for each eluting slice to yield *absolute* molecular weight, $R_g$, and (with DLS) $R_h$. 
Case Study 1: Adenovirus

Measure size of monomer and aggregates
Quantify number of aggregates
Relate aggregation to stress conditions
Adenovirus

- Small amount of aggregates will not be detected by batch DLS.
- $R_g$ and $R_h$ can be measured by MALS and online DLS, respectively.
## Quantitation: size, particle counts, aggregate%

<table>
<thead>
<tr>
<th>Monomer</th>
<th>Aggregate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radius (nm)</td>
</tr>
<tr>
<td>Run 1</td>
<td>43.7</td>
</tr>
<tr>
<td>Run 2</td>
<td>44</td>
</tr>
<tr>
<td>Average</td>
<td>43.9</td>
</tr>
</tbody>
</table>

Good reproducibility was obtained for both sizing and particle counting, despite very low amount of aggregate.

MALSS provides more accurate quantitation of aggregate than traditional UV method.

- UV peak area may overestimate the percentage of large aggregates
- Scattering contribution in UV data is significant for particle radius >50 nm
How do stressors change the vector?

Freeze-thaw
- Isolate and quantify virus size with Eclipse AF4-MALS
- Sensitive and robust aggregation assessment

Fresh vs. aged
- Elution time and peak shape are not representative of size distribution
- Eclipse fractionation with DAWN MALS detector quantifies absolute size

Buffer effects
- Measure differences in size distribution between buffers
- Quantify number of particles: $1.7 \times 10^{10}$ for each case
Case Study 2: Adeno-associated virus (AAV)

Comparison of separation techniques
Quantify genetic payload
Determine structure
AAV by SEC-MALS and Eclipse AF4-MALS

SEC-MALS

- SEC may be able to resolve monomer and oligomer
- SEC is not the right tool to quantify large aggregates

Eclipse AF4-MALS

- AF4 provides better separation of confirmation of aggregate %
- HMW aggregates visible by AF4-MALS may be removed by SEC column
Quantify genetic payload

SEC/FFF cannot resolve empty and filled AAVs, but the apparent MW data from MALS and dRI may correlate to the percentage of full AAV
AAV structure by SEC with multi-detection

Confirm payload and measure shape

$R_g$ from MALS

$R_h$ from DLS

$R_g/R_h \sim 0.8$

filled sphere
Case Study 3: Formulation stability

High-throughput aggregate screening

Colloidal stability: $k_D$ and $A_2$

Conformational stability: $T_m$ and $T_{agg}$
Benefits of batch DLS

Screening and characterization tool for formulation development

- High-throughput, low volume quality control for aggregates
- Perform studies as a function of time and temperature
- Screen small-molecule drugs: promiscuous inhibitors and binders
- Measure formulation viscosity
Measure interactions among molecules

**Dynamic light scattering:**
Diffusion Interaction Parameter, $k_D$

$$D_t = D_0 (1 + k_D c)$$

- $k_D < 0$ attraction
- $k_D > 0$ repulsion

**Static light scattering:**
Second virial coefficient, $A_2$

$$\frac{R}{K^*} = Mc[1 - 2A_2Mc]$$

- $A_2 < 0$ attraction
- $A_2 > 0$ repulsion
Why measure concentration dependence?

$k_D$ correlates with solution properties
- $k_D > 0$ correlates to low viscosity
- $k_D \lesssim 0$ correlates to high viscosity
- $k_D \lesssim 0$ correlates with particles formation (e.g., after agitation)

Sample formulation space and determining key stability attributes.
- Interactions as a function of pH, excipient, salts, etc.
- Observe correlations between $k_D$, $A_2$, and $T_{agg}$

Positive correlation between $k_D$ and many other stability-indicating parameters!

Quantify colloidal stability, $k_D$ and $A_2$

Formulation pH influences intermolecular interactions.

— Neutral pH causes undesirable attractive interactions
— Acidic and basic pH exhibit net repulsive interactions

<table>
<thead>
<tr>
<th></th>
<th>DLS</th>
<th>SLS</th>
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<tbody>
<tr>
<td></td>
<td>$R_h$ (nm)</td>
<td>$k_D$ (mL/g)</td>
</tr>
<tr>
<td>Neutral</td>
<td>1.9</td>
<td>-8.5</td>
</tr>
<tr>
<td>Acidic</td>
<td>2.0</td>
<td>+4.6</td>
</tr>
<tr>
<td>Basic</td>
<td>2.1</td>
<td>+3.7</td>
</tr>
</tbody>
</table>
Conformational stability from temperature

**Acidic pH**

Acidic pH provides conformational stability.

- Midpoint unfolding temperature $T_m = 69 \, ^\circ C$
- Confirm unfolding (not aggregation) via constant measured $M_w$

**Basic pH**

Basic pH shows aggregation at elevated temperature

- Onset of aggregation/unfolding happens at lower $T$ compared to acidic pH
- $T_{agg}$ varies with concentration, ranging from 48 °C to 60 °C
Conclusion

Light scattering is not just for protein molecules and aggregates!

— Assess wide range of biotherapeutics, protein conjugates, and higher order structures.
— Extend characterization and quantitation to viruses, gene therapy and drug delivery vectors.

Static and dynamic light scattering provide a wide range of solutions for formulation stability.

— Measure nonspecific interactions and propensity to aggregate with DLS ($k_D$) or SLS ($A_2$).
— Characterize conformational stability ($T_m$, $T_{agg}$, time to aggregation, etc.)

Combine with complementary information for complete characterization.
For More Information

Sample application notes, webinars, & more at www.wyatt.com/Library.

Search over 14,000 peer-reviewed publications that feature Wyatt instruments at www.wyatt.com/Bibliography.

For information about a particular topic:

— Light Scattering Solutions: www.wyatt.com/Solutions
— SEC-MALS: www.wyatt.com/SEC-MALS
— DLS: www.wyatt.com/DLS
— CG-MALS: www.wyatt.com/CG-MALS