Application of Light Scattering for Analysis of Protein-Protein Interaction and Aggregation

Ewa Folta-Stogniew
Yale University
• Light Scattering Technologies
  – Static and dynamic light scattering
  – Parameters derived from SLS and DLS measurements

• Flow Mode Light Scattering Applications
  – Molar mass distributions and differences in populations
  – Determination of an oligomeric state of modified proteins from SEC-LS/UV/RI measurement
  – Determination of dimerization constant from SEC-LS measurements

• Capabilities and limitation of LS measurements
Light Scattering Experiments

Monochromatic Laser Light

sample cell

detector at angle $\Theta_2$

Computer

$I_o$ $I$
Light Scattering Experiments

- **Static (classical)**
  - time-averaged intensity of scattered light

- **Dynamic (quasielastic)**
  - fluctuation of intensity of scattered light with time

**Measurements:**
- batch mode
- “in-line” mode combined with a fractionation step,
  - i.e. chromatography, mainly Size Exclusion Chromatography, Flow Field Fractionation
Typical SEC-MALLS system

- Sample
- SEC column
- HPLC system
- UV detector
- LS detector (DLS+SLS)
- RI detector
- 0.1 μm pre-filtered buffer
- 0.1 μm “in-line” filter
- Computer
- Waste or collection
Three Detector monitoring

Peak ID - Ova_071305a_01_P_N

Volume (mL)

LS #11, AUX1, AUX2

UV at 280 nm  RI  LS at 90°
Molar mass distribution for multiple analyses

Ovalbumin 43 kDa automated template processing of five data sets
Determination of Weight Fractions

Cumulative Molar Mass

<table>
<thead>
<tr>
<th>Oligomeric state</th>
<th>Average Mw ± SD [kDa] (3 analyses)</th>
<th>Fraction of Mass [% of total] (3 analyses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono (20-50 kDa)</td>
<td>42.80 ± 0.02</td>
<td>85.23 ± 0.06</td>
</tr>
<tr>
<td>Di (50-96 kDa)</td>
<td>84.1 ± 0.2</td>
<td>9.4 ± 0.0</td>
</tr>
<tr>
<td>Tri (96-130 kDa)</td>
<td>121.8 ± 0.7</td>
<td>1.9 ± 0.0</td>
</tr>
<tr>
<td>Agg. (0.13 –1 MDa)</td>
<td>284 ± 2</td>
<td>2.87 ± 0.06</td>
</tr>
<tr>
<td>Agg. (1 –100 MDa)</td>
<td>10.9±0.4 x10^3</td>
<td>0.6 ± 0.0</td>
</tr>
</tbody>
</table>
Differences in population based on molar mass distribution

Ovalbumin (5 runs)
Mw = 108 ± 17 kDa
Polydispersity Mw/Mn
2.3 ± 0.4

Ovalbumin (3 runs)
Mw = 141 ± 3 kDa
Polydispersity Mw/Mn
2.92 ± 0.06
Differences in population based on molar mass distribution

Ovalbumin 43 kDa

<table>
<thead>
<tr>
<th>Oligomeric state</th>
<th>Average Mw ± SD [kDa] (5 analyses)</th>
<th>Average Mw ± SD [kDa] (3 analyses)</th>
<th>Fraction of Mass [% of total] (5 analyses)</th>
<th>Fraction of Mass [% of total] (3 analyses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono (20-50 kDa)</td>
<td>43.0 ± 0.1</td>
<td>42.80 ± 0.02</td>
<td>88.1 ± 0.1</td>
<td>85.23 ± 0.06</td>
</tr>
<tr>
<td>Di (50-96 kDa)</td>
<td>82.7 ± 0.4</td>
<td>84.1 ± 0.2</td>
<td>7.68 ± 0.04</td>
<td>9.4 ± 0.0</td>
</tr>
<tr>
<td>Tri (96-130 kDa)</td>
<td>114 ± 4</td>
<td>121.8 ± 0.7</td>
<td>1.54 ± 0.05</td>
<td>1.9 ± 0.0</td>
</tr>
<tr>
<td>Agg. (0.13 –1 MDa)</td>
<td>270 ±10</td>
<td>284 ± 2</td>
<td>2.18 ± 0.08</td>
<td>2.87± 0.06</td>
</tr>
<tr>
<td>Agg. (1 –100 MDa)</td>
<td>10±1 x10³</td>
<td>10.9±0.4 x10³</td>
<td>0.4 ± 0.0</td>
<td>0.6 ± 0.0</td>
</tr>
</tbody>
</table>

Mw = 108 ± 17

Mw = 141 ± 3

Mw = 108 ± 17

Mw = 141 ± 3
Determination of the oligomeric state of modified proteins from SEC-LS/UV/RI analysis

1. Glycosylated proteins
2. Proteins conjugated with polyethylene glycol
3. Membrane protein present as a complex with lipids and detergents

Input:
- Polypeptide sequence
- *Chemical nature of the modifier*

Results:
- Oligomeric state of the polypeptide
- Extend of modification (grams of modifier / gram of polypeptide)

“three detector method”

Three Detector Method

\[ MW_p = \frac{k^* (LS)(UV)}{\varepsilon(RI)^2} \]

- \( MW_p \): Molecular Weight (polypeptide)
- \( \varepsilon \): extinction coefficient
- \( LS \): light scattering intensity
- \( UV \): absorbance (\( \varepsilon \))
- \( RI \): refractive index change
- \( k \): calibration constant

PEG-ylated protein: 75 kDa

36 kDa polypeptide + 39 kDa PEG

Polypeptide: 146 kDa
(tetramer: 144 kDa)

Full protein: 291 kDa
(tetramer: 300 kDa)

Modified proteins: PEG-ylated and Glycoproteins
PEG-ylated protein: 75 kDa

36 kDa polypeptide + 39 kDa PEG

Polypeptide: 146 kDa
(tetramer: 144 kDa)

Full protein: 291 kDa
(tetramer: 300 kDa)
**Glycoprotein**  
44.1 kDa polypeptide; unknown level of glycosylation

<table>
<thead>
<tr>
<th>Peak</th>
<th>UV/RI</th>
<th>$M_{\text{pp}}$ (kDa)</th>
<th>Grams of sugar/gram of polypeptide</th>
<th>Full Glycoprotein (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.54</td>
<td>90</td>
<td>0.4</td>
<td>122</td>
</tr>
<tr>
<td>2</td>
<td>0.52</td>
<td>45</td>
<td>0.4</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>0.37</td>
<td>23</td>
<td>1.1</td>
<td>48</td>
</tr>
</tbody>
</table>

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Full glycoprotein

Polypeptide
Glycosylated peptide: 9.13 kDa; 350 μg

Alfa-lactalbumin 14 kDa; 20 μg
Determination of the oligomeric state of a complex of glycosylated protein+peptide

**protein**  58 kDa   extracellular ANP-binding domain (ECD) of cell-surface receptor   16% of mass is sugar

\[ \frac{dn}{dc_t} = 0.179 \text{ g/mL} \]

48 kDa   polypeptide portion

**ligand**  2.7 kDa   atrial natriuretic peptide (ANP)
Injected sample complex (ECD : ANP) 2:1

ECD_{dimer} = 2 \times 58 = 116 \text{ kDa} \quad (\text{polypeptide 96 kDa})

ANP = 2.7 \text{ kDa}
Injected sample complex (ECD : ANP) 2:1

ECD_{dimer} = 2 \times 58 = 116 \text{ kDa} \quad (\text{polypeptide } 96 \text{ kDa})

ANP = 2.7 \text{ kDa}
ECD-ANP complex; ~3 μg

MW_{glycoprotein} = 103 ± 10 kDa  
MW_{polypeptide} = 96 ± 7 kDa

ECD; ~2 μg

MW_{glycoprotein} = 54 ± 6 kDa  
MW_{polypeptide} = 44 ± 5 kDa

\[ \text{ECD dimer} = 2 \times 58 = 116 \text{ kDa (polypeptide } 96 \text{ kDa)} \]

\[ \text{ANP} = 2.7 \text{ kDa} \]
Hydrophobic proteins

Determination of the oligomeric state of detergent solubilized proteins:

polypeptide+lipids+detergent complexes of unknown detergent+lipids content

Proteins:

<table>
<thead>
<tr>
<th>Protein</th>
<th>M.W.</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>47 kDa porin LamB trimer</td>
<td>141 kDa</td>
<td></td>
</tr>
<tr>
<td>33 kDa hemolysin α-HL heptamer</td>
<td>231 kDa</td>
<td></td>
</tr>
</tbody>
</table>

Detergent:

dodecyl maltoside (C12M) [MW = 511 g/mol]

0.5g/L i.e. 0.05%

CMC = 0.008% micelle size 50-70 kDa
Proteins:  

47 kDa porin LamB trimer = 141±3 kDa (141 kDa)  
33 kDa hemolysin α-HL heptamer = 215±20 kDa (231 kDa)
Three Detector Method

Yutaro Hayashi, Hideo Matsui and Toshio Takagi


allows determination of mass of detergent/lipids bound to a polypeptide

\[
\left( \frac{dn}{dc} \right)_{app} = k_2 A \frac{(RI)}{(UV)}
\]

\[
\left( \frac{dn}{dc} \right)_{app} = \left( \frac{dn}{dc} \right)_{pp} + \delta \left( \frac{dn}{dc} \right)_{d+l} = K' \frac{(RI)}{\varepsilon(UV)}
\]

\( \delta \) is mass of detergent and/or lipids per 1 gram of polypeptide

Assumption: detergent does not produce any signal in UV
\[ MW_{\text{complex}} = 285 \text{ kDa} \]
\[ MW_{\text{polypeptide}} = 141 \text{ kDa} \]
\[ \delta = 1.02 \text{ lipids per 1 gram of polypeptide} \]

\[ MW_{\text{complex}} = 271 \text{ kDa} \]
\[ MW_{\text{polypeptide}} = 215 \text{ kDa} \]
\[ \delta = 0.26 \text{ lipids per 1 gram of polypeptide} \]

Determination of dimerization constant from SEC-LS measurements

SecA protein

WT                               monomer =   102 kDa
DS8  deletion mutant  monomer =   101 kDa
D11  deletion mutant  monomer =   100 kDa
SecA protein

WT 102 kDa
DS8 deletion mutant 101 kDa
D11 deletion mutant 100 kDa

Low salt buffer:
10 mM Tris pH 7.5, 5 mM Mg2+, 100 mM KCl

High salt buffer:
10 mM Tris pH 7.5, 5 mM Mg2+, 300 mM KCl
D11 deletion mutant  mono= 101 kDa

High salt buffer:
10 mM Tris pH 7.5, 5 mM Mg2+, 300 mM KCl,
**D11 deletion mutant  mono = 101 kDa**

**Low salt buffer:**

10 mM Tris pH 7.5, 5 mM Mg2+, 100 mM KCl,

\[
M_w = f_m M_m + f_d M_d = M_m (2 - f_m)
\]

\[2M = D\]

\[K_a = \frac{[D]}{[M]^2} = \frac{(1 - f_m)}{2(f_m)^2 c_t}\]

\[f_m = \frac{-1 + \sqrt{1 + 8K_a c_t}}{4K_a c_t}\]
WT  monomer = 102 kDa
DS8 deletion mutant  monomer = 101 kDa
D11 deletion mutant  monomer = 100 kDa

Low salt buffer: 100 mM KCl

High salt buffer: 300 mM KCl

WT  Kd= <1e-9
DS8  Kd= 7±1e-8 M
D11  Kd= 3.5±0.2e-6 M

WT  Kd= 2.2±0.2e-6 M
DS8  Kd= 2.41±0.05e-5 M
D11  Kd> 2.4e-4 M
Thermodynamic linkage for SecA dimerization

<table>
<thead>
<tr>
<th>Protein</th>
<th>Low Salt 100 mM KCl</th>
<th>High Salt 300 mM KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>&lt;1x10⁻⁹</td>
<td>-12.3</td>
</tr>
<tr>
<td><strong>DS8</strong></td>
<td>7±1x10⁻⁸</td>
<td>-9.7</td>
</tr>
<tr>
<td><strong>D11</strong></td>
<td>3.5±0.2x10⁻⁶</td>
<td>-7.4</td>
</tr>
</tbody>
</table>

**Diagrams**:
- **WT**<sub>high</sub> ΔG = -7.7 kcal/mol
- **D11**<sub>high</sub> ΔG = -4.9 kcal/mol
- **D11**<sub>low</sub> ΔG = -7.4 kcal/mol
- **WT**<sub>low</sub> ΔG = -12.3 kcal/mol
- **DS8**<sub>high</sub> ΔG = -6.3 kcal/mol
- **DS8**<sub>low</sub> ΔG = -9.7 kcal/mol
- **WT**<sub>low</sub> ΔG = -12.3 kcal/mol
Capabilities

Static LS

- fast and accurate determination of molar masses (weight average)
  - glycosylated protein, conjugated with PEG, protein-lipids-detergent complexes, protein-nucleic acid complexes

- accuracy of ± 5% in molar mass determination

- easy to implement, fully automated (data collection and data analysis)

- highly reproducible (no operator’s bias)

- SEC/MALS excellent in detecting and quantifying population with various oligomeric state in protein

- excellent approach for determination of oligomeric state of modified proteins and peptides

- can be used to determine association constant (concentration gradient measurements)
Limitations

Static LS

- measures *weight average* molar mass – needs fractionation to resolve different oligomeric states
- possible losses of sample during filtration and fractionation
- limitation on solvent choices (related to a fractionation step)
- SEC/MALS dilution during experiment
Ken Williams  
Director of W.M. Keck Biotechnology Resource Laboratory at Yale University School of Medicine

NIH

Users of SEC/LS Service

http://info.med.yale.edu/wmkeck/biophysics
Ewa.Folta-Stogniew@yale.edu
http://info.med.yale.edu/wmkeck/biophysics/publications_biophysics_resource.pdf
Ovalbumin 43 kDa

Aggregates
angular dependence of scattered light

Lower order oligomers
no angular dependence of scattered light
Morphology of aggregates from angular dependence of LS signal;

size determination- $R_g$

Determination of radius of gyration, $R_g$, (root mean square radius, R.M.S.,) from angular dependence of scattered light

$$\frac{K^*c}{R(\theta)} = \frac{1}{M_w} \left( 1 + \left( 16\pi^2 / 3 \lambda^2 \right) R_g^2 \right) \frac{\sin^2(\theta/2)}{2}$$

Zimm Plot

Peak, Slice : 1, 944
Volume : 7.667 mL
Fit degree : 1
Conc. : $(1.915 \pm 0.020)e-6$ g/mL
Mw : $(2.277 \pm 0.024)e+7$ g/mol

Radius: 46.8±0.2 nm
Inferring conformational information from the relationship between molecular size (Rg) and molecular weight (Molar Mass)

\[ R_g \sim M^\nu \]

log(Rg) versus log(MM)

Slope = \nu

<table>
<thead>
<tr>
<th>For</th>
<th>\nu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere</td>
<td>0.33</td>
</tr>
<tr>
<td>Coil</td>
<td>0.5</td>
</tr>
<tr>
<td>Rod</td>
<td>1</td>
</tr>
</tbody>
</table>

Shape analysis: $\log(R_g)$ versus $\log(MM)$

Aggregates of Ovalbumin vs. “amyloid-type” fibers

<table>
<thead>
<tr>
<th>For</th>
<th>$\nu$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere</td>
<td>0.33</td>
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<tr>
<td>Coil</td>
<td>0.5</td>
</tr>
<tr>
<td>Rod</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ova_aggr</th>
<th>$\nu = 0.4$</th>
<th>Sphere/Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloids</td>
<td>$\nu = 0.8$</td>
<td>Coil/Rod</td>
</tr>
</tbody>
</table>
Shape analysis: **shape factor** $\rho = \frac{R_g}{R_h}$

Aggregates of Ovalbumin vs. amyloid fibers

Shape factor: $\rho = \frac{R_g}{R_h}$

Combination of MALS ($R_g$) and DLS ($R_h$)

- **Ovalbumin**
  - $R_g/R_h = 0.91$

- **Amyloids**
  - $R_g/R_h = 1.84$

---

Shape analysis: shape factor $\rho = \frac{R_g}{R_h}$

Aggregates of Ovalbumin vs. amyloid fibers

Shape factor: $\rho = \frac{R_g}{R_h}$

Combination of MALS ($R_g$) and DLS ($R_h$)

For $\rho = \frac{R_g}{R_h}$

<table>
<thead>
<tr>
<th>Shape</th>
<th>$\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere</td>
<td>0.774</td>
</tr>
<tr>
<td>Coil</td>
<td>0.816</td>
</tr>
<tr>
<td>Rod</td>
<td>1.732</td>
</tr>
</tbody>
</table>

Ovalbumin

$R_g/R_h = 0.91$  Coil

Amyloids

$R_g/R_h = 1.84$  Rod

Shape analysis:

\[ R_g \sim M^\nu \]

\[ \rho = \frac{R_g}{R_h} \]

<table>
<thead>
<tr>
<th>Shape</th>
<th>( \nu )</th>
<th>( \rho = \frac{R_g}{R_h} )</th>
</tr>
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<tr>
<td>Sphere</td>
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<td>1.732</td>
</tr>
<tr>
<td>Ova_aggr</td>
<td>( \nu = 0.4 )</td>
<td>Sphere/Coil</td>
</tr>
<tr>
<td>Amyloids</td>
<td>( \nu = 0.8 )</td>
<td>Coil/Rod</td>
</tr>
</tbody>
</table>

For Rod

\( \nu = 0.8 \)

\( \rho = \frac{R_g}{R_h} = 1.84 \)

Ovalbumin

\( \rho = \frac{R_g}{R_h} = 0.91 \)

Coil
Shape analysis:

shape factor $\rho = \frac{R_g}{R_h}$

$log(R_g)$ versus $log(MM)$  Slope = $\nu$

$\rho = \frac{R_g}{R_h} = 1.84$ Rod

Amyloids  $\nu = 0.8$  Coil/Rod

<table>
<thead>
<tr>
<th></th>
<th>$\gamma_c$</th>
<th>$\eta_c$</th>
<th>Avg $\rho$-ratio</th>
<th>$\gamma_{m\cdot(v)}$</th>
<th>$\eta_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCgn (α-chymotrypsinogen A)</td>
<td>$-0.3 \pm 0.1$</td>
<td>$-0.27 \pm 0.07$</td>
<td>$1.65 \pm 0.1$</td>
<td>$0.74 \pm 0.16$</td>
<td>$0.64 \pm 0.12$</td>
</tr>
<tr>
<td>bG-CSF (bovine granulocyte-colony stimulating factor)</td>
<td>$-1.13 \pm 0.34$</td>
<td>$-1.25 \pm 0.34$</td>
<td>$1.76 \pm 0.13$</td>
<td>$0.74 \pm 0.15$</td>
<td>$0.8 \pm 0.4$</td>
</tr>
</tbody>
</table>


Cryo-TEM micrograph of aCgn samples ($c_0 = 1$ mg/mL) at $m = 0.05$

Molar Mass Distribution Plot

BSA 66 kDa
Shape information from light scattering measurements

Protein “F”

Molar Mass (g/mol) vs. Volume (mL)

- 40.3 kDa
- 156 kDa
- 43 kDa

Molar Mass (g/mol)

Volume (mL)
Protein “F” frictional ratio $R_h/R_s = 1.85$ non-spherical shape

4 fold difference in mass – same hydrodynamic size

Shape Effects
• **Batch Mode Light Scattering Applications**
  – Detection of aggregates in DLS and SLS measurement
Batch Mode Static MALLS experiment

Monomer 14 kDa

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight Average MM, Mw ± SD* [kDa]</th>
<th>RMS [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>126 ± 8</td>
<td>56 ± 10</td>
</tr>
</tbody>
</table>

Angular dependence of scattered light clearly indicates presence of aggregates.
Determination of hydrodynamic radius, $R_h$, from a Dynamic LS experiment

Ovalbumin; monomer: 43 kDa; $R_h=3.0$ nm

$R_h = 8\pm 7$ nm from **Cumulant Fit** (Polydispersity 93%)

**Regularization Fit:**

<table>
<thead>
<tr>
<th>Peak</th>
<th>$R_h$ (nm)</th>
<th>Polydispersity (%)</th>
<th>MW (R) kDa</th>
<th>% Intensity</th>
<th>% Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.1</td>
<td>12.8</td>
<td>46</td>
<td>54</td>
<td>99.9</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>17.8</td>
<td>&gt;1MDa</td>
<td>23</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>86</td>
<td>13.4</td>
<td>&gt;1MDa</td>
<td>23</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>
Dissociation of aggregates upon dilution; time course

Protein H 23 kDa; Rh=2.3 nm

Rh=94 nm

Rh=23 nm

Rh=2.8 nm

Rh=2.3 nm; Pd=42%
Ovalbumin 43 kDa

- 88% monomer
- 8% dimer
- 1.5% trimer
- 3% aggregates < 1MDa
- 0.4% 1-100 MDa

UV at 280 nm

LS at 90 deg
Intensity of scattered light $\sim M_w^*c$
due to their high $M_w$ aggregates scatter very strongly

A monomeric protein 43 kDa and aggregates 10 MDa at 2 mg/mL:
Why Light Scattering?

• LS measurements are non-invasive and non-destructive
  • small sample volumes
  • great dynamic range for sizing: hydrodynamic radii ~ 2nm to 500 nm
  • great dynamic range for Mw determination: < 1kDa to >10 MDa
  • wide range of concentrations (non-ideality can be addressed through the determination of second virial coefficient)
  • perfectly suited for determination of oligomeric state of modified proteins without prior knowledge of extend of modification (glycosylated proteins, proteins modified by polyethylene glycol, or membrane proteins present as complexes with lipids and detergents)

• LS measurements are perfect tools for detection and characterization of aggregates
  • Scattering Intensity, \( R(\Theta) \sim M_w c \)
    
    because of their big Mw, aggregates scatter strongly even when present at low concentrations; easily detectable

• Angular variation of the scattered light is related to the size and shape of the molecule

  the light scattering signal from aggregates will show angular dependence, while LS signal produces by lower order oligomers like dimers, trimers, tetramers, et c. will not
Various uses of Light Scattering for assessing protein aggregates

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Detects Aggregates</th>
<th>Information about population (distribution)</th>
<th>Challenge in use</th>
<th>Sample dilution</th>
<th>Speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS</td>
<td>Yes</td>
<td>No</td>
<td>Low</td>
<td>No</td>
<td>Fast</td>
</tr>
<tr>
<td>Micro-batch MALS</td>
<td>Yes</td>
<td>No</td>
<td>High</td>
<td>No</td>
<td>Medium</td>
</tr>
<tr>
<td>SEC/MALLS/DLS</td>
<td>Yes</td>
<td>Yes</td>
<td>Medium</td>
<td>Yes</td>
<td>Medium</td>
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</tbody>
</table>
Determination of hydrodynamic radius, $R_h$, from a Dynamic LS experiment

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$R_h = 8\pm7$ nm from **Cumulant Fit** (Polydispersity 93%)

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<td>17.8</td>
<td>&gt;1MDa</td>
<td>23</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>86</td>
<td>13.4</td>
<td>&gt;1MDa</td>
<td>23</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Regularization Fit:

- **Peak 1**
  - $R_h = 3.1$ nm
  - Polydispersity = 12.8%
  - MW (R) = 46 kDa
  - % Intensity = 54%
  - % Mass = 99.9%

- **Peak 2**
  - $R_h = 24$ nm
  - Polydispersity = 17.8%
  - MW (R) = >1MDa
  - % Intensity = 23%
  - % Mass = 0.1%

- **Peak 3**
  - $R_h = 86$ nm
  - Polydispersity = 13.4%
  - MW (R) = >1MDa
  - % Intensity = 23%
  - % Mass = <0.1%
Results from a batch mode Dynamic LS experiment:

Ovalbumin 43 kDa; Rh=3.0 nm

Rh = 3.2±0.6 nm from Cumulant Fit (Polydispersity 19%)

Regularization Fit:

<table>
<thead>
<tr>
<th>Peak</th>
<th>Rh (nm)</th>
<th>Polydispersity (%)</th>
<th>MW (R) kDa</th>
<th>% Intensity</th>
<th>% Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.1</td>
<td>12.9</td>
<td>46</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>0</td>
<td>&gt;1MDa</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2423</td>
<td>0</td>
<td>&gt;1MDa</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
\[ MW_p = \frac{k^*(LS)(UV)}{\varepsilon(RI)^2} \]

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ova</td>
<td>43</td>
</tr>
<tr>
<td>BSA(1)</td>
<td>66</td>
</tr>
<tr>
<td>BSA(2)</td>
<td>132</td>
</tr>
<tr>
<td>Ald</td>
<td>156</td>
</tr>
<tr>
<td>Apo-Fer</td>
<td>475</td>
</tr>
</tbody>
</table>

Three-detector calibration
10-17-01

\[ y = 92.383x - 3.4044 \]

\[ R^2 = 0.9996 \]
Flow Mode Light Scattering Applications

- Molar mass distributions and differences in populations
- Determination of an oligomeric state of modified proteins and oligos from SEC-LS/UV/RI measurement
- Determination of dimerization constant from SEC-LS measurements
AUX, 90° Detector

Volume (mL)

Peak ID - PRNC__DC

LS @ 90 degree
RI
UV @ 280 nm

Volume (mL)
Protein “F” frictional ratio $P = \frac{R_h}{R_s} = 1.85$ non-spherical shape

Axial ratio $a/b$ (prolate) = 16.6 (oblate) = 22.9

40.3 kDa $R_h = 4.2$ nm
156 kDa $R_h = 4.2$ nm
43 kDa $R_h = 2.9$ nm

4 fold difference in mass – same hydrodynamic size

BSA solutions at concentrations: 0.22, 0.44, 0.75 and 1.1 mg/mL and the data were analyzed using Zimm formalism.

Determining $M_w$ and the second virial coefficient from Zimm plot analysis of light scattering data.

- $M_w = 65$
- $B = (5.226 \pm 0.316) \times 10^{-4} \text{ mol mL/g}^2$
• Batch Mode Light Scattering Applications
  – Detection of aggregates in DLS and SLS measurement
Determination of Molar Mass and second virial coefficient from a batch static LS experiment

BSA 66 kDa

Zimm plot analysis of static light scattering data

\[ \frac{K \cdot c}{R(\theta)} = \frac{1}{M_w P(\theta)} + 2A_2c \]

and Rh from DLS

\[ K \cdot c \]

\[ R(\theta) \]

Temperature: 27.831 °C
Rh: 3.4 nm

Batch Mode Static MALLS experiment

Monomer 14 kDa

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight Average MM, Mw ± SD* [kDa]</th>
<th>RMS [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>126 ± 8</td>
<td>56 ± 10</td>
</tr>
</tbody>
</table>

Angular dependence of scattered light clearly indicates presence of aggregates
Determination of hydrodynamic radius, $R_h$, from a Dynamic LS experiment

Ovalbumin; monomer: 43 kDa; $R_h=3.0$ nm

$R_h = 8\pm7$ nm from Cumulant Fit (Polydispersity 93%)

Regularization Fit:

<table>
<thead>
<tr>
<th>Peak</th>
<th>Rh (nm)</th>
<th>Polydispersity (%)</th>
<th>MW (R) kDa</th>
<th>% Intensity</th>
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</tbody>
</table>
Dissociation of aggregates upon dilution; time course

Protein H 23 kDa; Rh=2.3 nm
Feature detected in a batch mode LS measurements for sample containing aggregates

- Static (classical)
  Aggregates present:
  - elevated weight average Molar Mass ($M_w$ weight average)
  - angular dependence in scattered light
- Dynamic (quasielastic)
  Aggregates present:
  - autocorrelation function cannot be described by single exponential (cumulant fit)
  - polydispersity from cumulant fit >15%

Missing information: how much and what size?

Solutions
- Sample fractionation followed by batch measurements
- **Column separation with simultaneous LS characterization**
**PEG-ylated oligo**

**PEG (40K)**  
MM = 41.0 kDa  
(80 μg total)

Polydispersity = 1.001

40K PEG + 12.9 kDa oligo  
(73 μg total)

PEG-oligo  
MM = 52.1 kDa

**BSA**  
66 kDa

**PEG (40K)**  
MM = 41.0 kDa  
(40 μg total)

Polydispersity = 1.001

40K PEG + 8.3 kDa oligo  
(70 μg total)

PEG-oligo  
MM = 48.5 kDa