Determination of Molecular Masses of Proteins in Solution; Implementation of an HPLC Size Exclusion Chromatography and Laser Light Scattering Service in a Core Laboratory
**Static and Dynamic LS**

Experimental Set-Up

Parameters derived

**Static LS**

- Theory
- SEC/LS “in-line” Set Up
- Results for Standards
- Sample Requirements
- Applications

**Dynamic LS**

- Theory
- Results for Standards
- Batch mode vs. SEC/LS “in-line” measurements

**Static vs. Dynamic LS Measurements**
Light Scattering Experiments

Monochromatic Laser Light

Sample cell

$I_o$ $\rightarrow$ $I$ $\theta$

$\theta$

$I_{\theta}$

detector

Computer
Light Scattering Experiments

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

• Dynamic (quasielastic)
  fluctuation of intensity of scattered light with time

Parameters derived:
• MW (weight-average)
• \((<r_g^2>^{1/2})\) root mean square radii for \((<r_g^2>^{1/2})\) \(\sim 30\) nm

Parameters derived:
• \(D_T\) translation diffusion coefficient
• \(R_h\) hydrodynamic radius (Stokes radius)
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
Static Light Scattering

- Theory
- SEC/LS “in-line” Set Up
- Results for Standards
- Sample Requirements
- Applications
Static Light Scattering Experiments

Debye-Zimm formalism for $R(\theta)$, the excess intensity of scattered light at an angle $\theta$

\[
\frac{K^* c}{R(\theta)} = \frac{1}{M_w P(\theta)} + 2A_2 c
\]

$c$ is the sample concentration (g/ml)

$M_w$ is the weight-average molecular weight (molar mass)

$A_2$ is the second virial coefficient (ml-mol/g^2)

$K^*$ is an optical parameter equal to $4\pi^2 n^2 (dn/dc)^2 / (\lambda_0^4 N_A)$

$n$ is the solvent refractive index and $dn/dc$ is the refractive index increment

$N_A$ is Avogadro’s number

$\lambda_0$ is the wavelength of the scattered light in vacuum (cm)

$P(\theta)$ is the form factor (describes angular dependence of scattered light)
Static Light Scattering Experiments

\[ \frac{K^* c}{R(\theta)} = \frac{1}{M_w} \left( 1 + \frac{16\pi^2}{3\lambda^2} \langle r_g^2 \rangle \sin^2 \left( \frac{\theta}{2} \right) \right) \]

Using a multi angle instrument
construct a plot of

\[ \frac{K^* c}{R(\theta)} \quad \text{against} \quad \sin^2 \left( \frac{\theta}{2} \right) \]

From intercept \( \rightarrow \) Derived MW

weight-average
**Zimm Plot**  
**Ovalbumin (43 kDa)**

\[
\frac{K^*c}{R(\theta)} = \frac{1}{M_w} \left(1 + f\left(\sin^2\left(\frac{\theta}{2}\right)\right)\right)
\]

Volume : 16.300 mL  
Conc. : (0.173 ± 0.000) mg/mL  

**Mw**  
(42.79 ± 0.03) x10³ g/mol  

Radius : 0.0 ± 0.0 nm  

90° & AUX detectors
Static Light Scattering

- Theory
- SEC/LS “in-line” Set Up
- Results for Standards
- Sample Requirements
- Applications
HPLC system

SEC column

Sample

UV detector

LS detector

RI detector

Waste

0.1 µm pre-filtered buffer

0.1 µm "in-line" filter

Computer

ASTRA software
Zimm Plot  Ovalbumin (43 kDa)

\[ \frac{K^*c}{R(\theta)} = \frac{1}{M_w} \left(1 + f(\sin^2(\theta/2))\right) \]

Volume : 16.300 mL
Conc.  : (0.173 ± 0.000) mg/mL
Mw      : (42.79 ± 0.03) x10^3 g/mol
Radius : 0.0 ± 0.0 nm
Molar Mass Distribution Plot

Ovalbumin 43 kDa

Molar Mass vs. Volume

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

- OVA0521A
Molar Mass Distribution Plot

BSA  66 kDa

Molar Mass vs. Volume

Molar Mass (g/mol)

Volume (mL)
Static Light Scattering

- Theory
- SEC/LS “in-line” Set Up
- Results for Standards
- Sample Requirements
- Applications
### Molecular Weights Determined from "in line" analyses; static LS with SEC in line

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oligomeric state</th>
<th># Runs</th>
<th>Pred. MW (kDa)</th>
<th>Average MW ± St. Dev. (kDa)</th>
<th>Average error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>monomer</td>
<td>2</td>
<td>6.5</td>
<td>6.8 ± 0.5</td>
<td>4.6</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>monomer</td>
<td>5</td>
<td>12.3</td>
<td>12.01 ± 0.57</td>
<td>2.4</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>monomer</td>
<td>2</td>
<td>14.2</td>
<td>14.32 ± 0.01</td>
<td>0.9</td>
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<tr>
<td>Myoglobin</td>
<td>monomer</td>
<td>3</td>
<td>17.0</td>
<td>14.19 ± 0.91</td>
<td>16</td>
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<tr>
<td>β-Lactoglobulin</td>
<td>monomer</td>
<td>2</td>
<td>18.3</td>
<td>20.06 ± 0.33</td>
<td>9.7</td>
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<td>Tripsin inhibitor</td>
<td>monomer</td>
<td>1</td>
<td>20.0</td>
<td>20.50</td>
<td>2.3</td>
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<tr>
<td>Carbonic anhydrase</td>
<td>monomer</td>
<td>4</td>
<td>29.0</td>
<td>29.22 ± 0.20</td>
<td>0.8</td>
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<tr>
<td>Ovalbumin</td>
<td>monomer</td>
<td>10</td>
<td>42.8</td>
<td>42.52 ± 0.68</td>
<td>1.4</td>
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<tr>
<td>BSA (monomer)</td>
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<td>5</td>
<td>66.4</td>
<td>66.41 ± 1.00</td>
<td>1.2</td>
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<tr>
<td>Transferrin</td>
<td>monomer</td>
<td>2</td>
<td>75.2</td>
<td>76.92 ± 0.98</td>
<td>2.3</td>
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<tr>
<td>Enolase (yeast)</td>
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<td>93.3</td>
<td>80.74 ± 1.18</td>
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<td>Enolase (rabbit)</td>
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<td>93.7</td>
<td>86.44 ± 1.90</td>
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<td>BSA (dimer)</td>
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<td>132.9</td>
<td>137.10 ± 3.93</td>
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<td>Alc. dehydrogenase</td>
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<td>147.4</td>
<td>144.02 ± 0.86</td>
<td>2.4</td>
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<td>Aldolase (rabbit)</td>
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<td>156.8</td>
<td>153.7 ± 1.91</td>
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<tr>
<td>Apo-ferritin</td>
<td>monomer</td>
<td>24</td>
<td>475.9</td>
<td>470.3 ± 2.62</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Median error:** 2.3

*Buffer: 20 mM HEPES, 150 mM KCl, 1 mM EDTA, pH=8.0; column: Superdex 200 or Superdex 75*
Correlation between the amount of protein analyzed and the accuracy of MW determination

<table>
<thead>
<tr>
<th></th>
<th>Amount loaded (µg)</th>
<th># Runs</th>
<th>Pred. MW (kDa)</th>
<th>Avrg. MW (kDa)</th>
<th>SD (kDa)</th>
<th>Avrg. error (%)</th>
<th>Range of accuracy (%)</th>
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</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>150</td>
<td>4</td>
<td>42.8</td>
<td>42.4</td>
<td>0.3</td>
<td>0.9</td>
<td>0.2 to 1.6</td>
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<tr>
<td></td>
<td>100</td>
<td>7</td>
<td>42.8</td>
<td>42.3</td>
<td>0.8</td>
<td>1.2</td>
<td>0.2 to 2.4</td>
</tr>
<tr>
<td></td>
<td>45-50</td>
<td>4</td>
<td>42.8</td>
<td>41.6</td>
<td>1</td>
<td>2.8</td>
<td>0.5 to 5.8</td>
</tr>
<tr>
<td></td>
<td>6-10</td>
<td>5</td>
<td>42.8</td>
<td>42.9</td>
<td>2</td>
<td>0.2</td>
<td>1.4 to 4.5</td>
</tr>
<tr>
<td>Transferrin</td>
<td>100</td>
<td>3</td>
<td>75.2</td>
<td>76.5</td>
<td>1</td>
<td>1.7</td>
<td>0.7 to 3.2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5</td>
<td>75.2</td>
<td>76.3</td>
<td>2</td>
<td>1.5</td>
<td>0.3 to 5.2</td>
</tr>
</tbody>
</table>

column: TSK GEL G3000<sub>SWXL</sub> [TosoHaas], buffer: 20 mM phosphate, 150 mM NaCl, pH=7.5
Static Light Scattering

• Theory
• SEC/LS “in-line” Set Up
• Results for Standards
• Sample Requirements
• Applications
## Sample requirements for proteins.

<table>
<thead>
<tr>
<th>Column</th>
<th>Optimal amount of protein</th>
<th>Total volume of the eluting peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>for expected MW &gt;40 kDa</td>
<td></td>
</tr>
<tr>
<td>Superose 6 (Pharmacia)</td>
<td>100 µg</td>
<td>~ 2mL</td>
</tr>
<tr>
<td>Superdex 200 (Pharmacia)</td>
<td>100 µg, 200 - 300 µg</td>
<td>~ 2mL</td>
</tr>
<tr>
<td>Superdex 75 (Pharmacia)</td>
<td>50 µg, 100 - 200 µg, 400 µg</td>
<td>~ 1mL</td>
</tr>
<tr>
<td></td>
<td>for expected MW 10 - 40 kDa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 - 300 µg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>for expected MW&lt;10 kDa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>~ 2mL</td>
<td></td>
</tr>
</tbody>
</table>
Static Light Scattering

- Theory
- SEC/LS “in-line” Set Up
- Results for Standards
- Sample Requirements
- Applications
SEC/LS Applications

- Unusual elution positions
- Mixtures of non-interacting proteins
- Mixtures of interacting protein - detection of protein complexes
- Determination of the oligomeric state of mutant vs. wild type protein
Please note the convention:

All the proteins are referred by MW of their monomeric forms.
Unusual elution positions

Example:

BSA monomer - 66 kDa protein
Yeast Enolase - 93 kDa dimer (2x46kDa)
**Enolase (Yeast) 46 kDa**

- **dimer 93 kDa**
- **BSA 66kDa**
- **OVA 43 kDa**

The diagram shows a plot of Molar Mass vs. Volume for different samples:

- **BSA0723A**: Represented by black diamonds.
- **ENY0723A**: Represented by blue dots.
- **OVA0723A**: Represented by green triangles.

The molar mass values range from 0.0 to 1.0x10^5 g/mol, and the volume values range from 7.0 to 11.0 mL.
Mixtures of non-interacting proteins

Example:

BSA monomer - 66 kDa protein
Yeast Enolase - 93 kDa dimer (2x46kDa)
Analysis of co-eluting protein mixture

**BSA** 66kDa  **BSA+ENY mixture**  **ENY dimer 93 kDa**

**Molar Mass vs. Volume**

**BSA**  
BSA 66kDa

**BSA+ENY mixture**

**ENY dimer 93 kDa**

---

**Molar Mass (g/mol)**

**Volume (mL)**

ENY + BSA
Analysis of interacting proteins

Example:

protein 27 kDa (protein exists as a mixture of monomer and dimer)

ligand 7 kDa

Ligand binding shifts the protein into dimeric form
Analysis of interacting proteins

![Graph showing Molar Mass vs. Volume](attachment:image.png)

- The graph depicts the relationship between Molar Mass (g/mol) and Volume (mL).
- The graph highlights a peak around 27 kDa, indicating a significant interaction or concentration at this Molar Mass.

Key points:
- The x-axis represents Volume in mL, ranging from 10.0 to 16.0.
- The y-axis represents Molar Mass in g/mol, with values ranging from 0.0 to 8.0x10^4.
- The data suggests a peak at approximately 27 kDa, which could be indicative of a significant interaction or concentration at this Molar Mass.
Molar Mass vs. Volume

- 27 kDa
- 7 kDa
Molar Mass vs. Volume

- 27 kDa
- 27 kDa
- 7 kDa
- 27 + 7 kDa
Molar Mass vs. Volume

- **27 kDa**
- **27 + 7 kDa**
Determination of the oligomeric state of mutant vs. wild type protein

Example:

protein  12 kDa  (WT protein exists as a trimer)

Three mutans and WT protein were analyzed.

There are significant differences in elution positions from SEC, however, all proteins were found to be trimeric forms- please note the abnormal elution position for each of the proteins.
Molar Mass vs. Volume

- MWT 12kDa
- P1S 12kDa
- PAM 12kDa
- P1G 12kDa

Molar Mass (g/mol)

Volume (mL)

43 kDa
20 kDa
12 kDa
Dynamic Light Scattering
Light Scattering Experiments

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

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

**Parameters derived:**

- **MW**
- \( (\langle r_g^2 \rangle^{1/2}) \) root mean square radii for \( (\langle r_g^2 \rangle^{1/2}) \) \( (\lambda/20) \) ~ 30 nm

- **Parameters derived:**
  - \( D_T \) translation diffusion coefficient
  - \( R_h \) hydrodynamic radius (Stokes radius)
Dynamic Light Scattering

- **Theory**
- Results for Standards
- *Batch mode vs. SEC/LS “in-line” measurements*
Dynamic Light Scattering Experiments

fluctuation of scattered light intensity with time

comparison of scattering intensity at various time intervals (µsec) with the initial (t=0 sec) intensity

autocorrelator

constructing an autocorrelation function $g^{(2)}(\tau) = f(\tau)$

**calculating the diffusion coefficient, $D$**

$$D_T = \frac{kT}{6\pi\eta R_H}$$

Stokes-Einstein equation

MODEL: dilute system of spherical molecules
Dynamic Light Scattering Experiments

Autocorrelation function

Ovalbumin  43 kDa
Dynamic Light Scattering Experiments

Ovalbumin 43 kDa

Size distribution

$R = 2.9 \pm 0.2 \text{ nm}$  \hspace{1cm} $MW(R) = 40 \text{ kDa}$

$MW$ calculated from the calibration curve
Dynamic Light Scattering Experiments

Carbonic Anhydrase  29 kDa

Size distribution

$R = 2.7 \pm 0.4 \text{ nm} \quad \text{MW}(R) = 33 \text{ kDa}$
Dynamic Light Scattering

- **Theory**
- **Results for Standards**
- *Batch mode vs. SEC/LS “in-line” measurements*
### Hydrodynamic Radiiuses and Molecular Weights

**Determined from DLS batch-mode analyses**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oligomeric State</th>
<th># Runs</th>
<th>Radius ± SD (nm)</th>
<th>Average MW (kDa)</th>
<th>Predicted MW (kDa)</th>
<th>Avrg. error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>monomer</td>
<td>15</td>
<td>1.64 ± 0.02</td>
<td>10.7</td>
<td>6.5</td>
<td>65</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>monomer</td>
<td>20</td>
<td>1.97 ± 0.05</td>
<td>16.6</td>
<td>12.3</td>
<td>35</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>monomer</td>
<td>25</td>
<td>2.09 ± 0.07</td>
<td>19.1</td>
<td>14.2</td>
<td>34</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>monomer</td>
<td>25</td>
<td>2.27 ± 0.04</td>
<td>23.0</td>
<td>17.0</td>
<td>35</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>monomer</td>
<td>20</td>
<td>2.85 ± 0.05</td>
<td>38.8</td>
<td>18.3</td>
<td>111</td>
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<tr>
<td>Trypsin inhibitor</td>
<td>monomer</td>
<td>20</td>
<td>2.53 ± 0.05</td>
<td>29.4</td>
<td>20.0</td>
<td>47</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>monomer</td>
<td>20</td>
<td>2.70 ± 0.03</td>
<td>34.7</td>
<td>29.0</td>
<td>19</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>monomer</td>
<td>30</td>
<td>3.21 ± 0.06</td>
<td>51.7</td>
<td>42.8</td>
<td>20</td>
</tr>
<tr>
<td>BSA (monomer)</td>
<td>monomer</td>
<td>20</td>
<td>3.97 ± 0.06</td>
<td>85.3</td>
<td>66.4</td>
<td>28</td>
</tr>
<tr>
<td>Transferrin</td>
<td>monomer</td>
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<td>4.04 ± 0.13</td>
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<td>75.2</td>
<td>18</td>
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<tr>
<td>Enolase (yeast)</td>
<td>dimer</td>
<td>25</td>
<td>3.78 ± 0.04</td>
<td>75.4</td>
<td>93.3</td>
<td>19</td>
</tr>
<tr>
<td>Alc. dehydrogenase</td>
<td>tetramer</td>
<td>20</td>
<td>4.52 ± 0.29</td>
<td>116.2</td>
<td>147.4</td>
<td>21</td>
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<tr>
<td>Aldolase (rabbit)</td>
<td>tetramer</td>
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<td>5.70 ± 0.69</td>
<td>217.9</td>
<td>156.8</td>
<td>39</td>
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<tr>
<td>Apo-ferritin</td>
<td>24 x monomer</td>
<td>25</td>
<td>7.86 ± 0.21</td>
<td>420.4</td>
<td>475.9</td>
<td>12</td>
</tr>
</tbody>
</table>

**Median:** 31
Results obtained in “batch-mode” for polydisperse samples

In “batch-mode” the DLS experiment is able to detect that the sample is POLYDISPERSE (i.e. the sample is not homogeneous in respect to oligomeric state); it cannot however discriminate what oligomeric form are present

Example:

BSA : mixture of monomer, dimers
Dynamic Light Scattering Experiments

BSA  66 kDa

Size distribution

\[ R = 4.0 \pm 0.6 \text{ nm} \quad \text{MW}(R) = 84 \text{ kDa} \]
Dynamic Light Scattering

• Theory
• Results for Standards

• Batch mode vs. SEC/LS “in-line” measurements
Results obtained in “SEC/LS” mode for polydisperse samples

In “SEC/LS” mode, the SEC serves as a fractionation step enabling determination of oligomeric state for each of the oligomeric forms that are present in the sample

Example:

BSA : mixture of monomer, dimers
Molar Mass Distribution Plot

BSA 66 kDa
Results obtained in “SEC/LS” mode for standard proteins; data are reported for the major eluting peak.
Hydrodynamic Radiiues and Molecular Weights Determined from “in-line” DLS analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oligomeric state</th>
<th># Runs</th>
<th>Radius ± SD (nm)</th>
<th>Average MW (kDa)</th>
<th>Predicted MW (kDa)</th>
<th>Average error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>monomer</td>
<td>3</td>
<td>1.35 ± .06</td>
<td>6.8</td>
<td>6.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>monomer</td>
<td>3</td>
<td>1.77 ± .12</td>
<td>12.8</td>
<td>12.3</td>
<td>4.3</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>monomer</td>
<td>3</td>
<td>1.91 ± .08</td>
<td>15.3</td>
<td>14.2</td>
<td>7.8</td>
</tr>
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<td>monomer</td>
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<tr>
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<td>2.47 ± .08</td>
<td>28.0</td>
<td>20.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>monomer</td>
<td>3</td>
<td>2.35 ± .16</td>
<td>25.0</td>
<td>29.0</td>
<td>14.0</td>
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<td>Ovalbumin</td>
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<td>2.98 ± .02</td>
<td>43.5</td>
<td>42.8</td>
<td>1.6</td>
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<td>BSA (monomer)</td>
<td>monomer</td>
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<td>3.56 ± .01</td>
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<td>66.4</td>
<td>0.9</td>
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<td>Transferrin</td>
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<td>4.02 ± .06</td>
<td>87.1</td>
<td>75.2</td>
<td>15.9</td>
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<td>3.57 ± .02</td>
<td>66.0</td>
<td>93.3</td>
<td>29.3</td>
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<tr>
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<td>dimer</td>
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<td>3.65 ± .10</td>
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<td>4.50 ± .10</td>
<td>113.8</td>
<td>147.4</td>
<td>22.8</td>
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<tr>
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<td>4.77 ± .06</td>
<td>130.5</td>
<td>156.8</td>
<td>16.8</td>
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</table>

Median: 20.0
Hydrodynamic Radiiuses and Molecular Weights Determined from “in-line” DLS analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oligomeric state</th>
<th># Runs</th>
<th>Radius ± SD (nm)</th>
<th>Average MW (kDa)</th>
<th>Predicted MW (kDa)</th>
<th>Average error (%)</th>
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<tbody>
<tr>
<td>Aprotinin</td>
<td>monomer</td>
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<td>1.35 ± .06</td>
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</tr>
</tbody>
</table>

Median: 10.9
Conclusions
**Static LS**

- fast and accurate determination of molecular weight (MW) of macromolecules in solution
- single SEC/LS measurement should be sufficient to estimate a MW with a precession of ± 5%
- SEC/LS suitable for characterization of non-interacting and interacting systems

**Dynamic LS**

- in batch mode, very fast evaluation of sample polydispersity
- fast and accurate determination of hydrodynamic radius in solution
- MW can be estimated (with a precession of ~10-20% for SEC/LS set-up)
Ken Williams
Director of HHMI Biopolymer & W.M. Keck Biotechnology Resource Laboratory

NIH

Thomas Mozdzer

Users of SEC/LS Service

Wyatt Technology        Protein Solutions