Light Scattering for Analysis of Oligomerization and Protein Interactions

Ewa Folta-Stogniew

Biophysics Resource; Keck Laboratory
Yale School of Medicine
Mission: quantitative characterization of interactions between biomolecules using in solution biophysical methods

Common questions:

• how tight is the binding? (binding affinity: $K_d$, $K_a$)
• how many of each molecule are in the complex (stoichiometry)
• how fast does the complex form? (kinetics)
• is the binding event enthalpy or entropy-driven? (thermodynamics)

List of technologies:

• Size Exclusion Chromatography coupled with Light Scattering (SEC/LS)
• Dynamic Light Scattering (DLS)
• Isothermal MicroCalorimeter (ITC)
• Spectrofluorometer
• Stopped-Flow Spectrofluorometer
• Surface Plasmon Resonance (SPR) Sensor [BiaCore Biosensor; T100]
• Composition Gradient Static Light Scattering (CGSLS)
• Asymmetric flow Field-Flow Fractionation (AFFF)

http://info.med.yale.edu/wmkeck/biophysics/
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Typical SEC(AFFF); MALLS system

- Sample or AFFF
- SEC column
- HPLC system
  - UV/Vis detector
  - DLS+SLS detector
  - RI detector
  - FL detector
- 0.1 µm pre-filtered buffer
- 0.1 µm "in-line" filter
- Waste or collection
- Computer
SEC/LS results: Molar Mass Distribution Plot

BSA
Monomer: 66 kDa

Weight-average molar mass
Measured every 2 µl

UV trace; $A_{280\text{nm}}$
(RI trace)
Dimerization of FIR

FIR: human *c-myc* FarUpStream Element (FUSE) Binding Protein (FBP) Interacting Repressor (FIR)

FIR protein fragment: first two RRM domains

FIR: 23.4 kDa monomer; seen as a dimer in the X-ray structure

Dimerization of FIR depends on DNA binding event

FIR protein: 23 kDa monomer

ssDNA fragment upstream of the P1 promoter, known as FUSE; 8 kDa

**FIR+DNA complex**; task: determine stoichiometry of the FIR+DNA complex in solution

<table>
<thead>
<tr>
<th>FIR-DNA complexes</th>
<th>MW (kDa)</th>
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<tbody>
<tr>
<td>FIR+DNA (2:1) complex</td>
<td>54.7</td>
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<td>FIR+DNA (2:2) complex</td>
<td>62.8</td>
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<tr>
<td>Observed MW</td>
<td>57.7</td>
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Concentration dependent measurements reveal that in solution the dimerization is driven by DNA binding.

Dimerization of FIR depends on DNA binding event

FIR protein: 23 kDa monomer; seen as a dimer in X-ray structure
ssDNA fragment upstream of the P1 promoter, known as FUSE; 8 kDa

**FIR+DNA complex;** task: determine stoichiometry of the FIR+DNA complex in solution

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Multiple oligomeric states for reconstituted KtrAB K⁺ Transporter

KtrAB ion transporter:
complex of KtrB membrane protein and KtrA RCK domain (regulating and conductance of K⁺)

KtrB: integral membrane protein isolated in the presence of detergent (DDM) as a polypeptide:detergent(lipid) complex


<table>
<thead>
<tr>
<th>Protein</th>
<th>Polypeptide [kDa]</th>
<th>Oligomeric state</th>
<th>Full complex [kDa]</th>
<th>Grams of detergent/lipids per gram of polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>KtrB (monomer 49kDa)</td>
<td>98</td>
<td>dimer</td>
<td>238</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Multiple oligomeric states for reconstituted KtrAB K+ Transporter

KtrAB ion transporter:
complex of KtrB membrane protein and KtrA RCK domain (regulating and conductance of K+)

KtrA RCK domain: basic assembly dimer, higher order oligomers: tetramer or octamer

Multiple oligomeric states for reconstituted KtrAB K+ Transporter

KtrAB ion transporter

complex : KtrAB octameric KtrA + dimeric KtrB (8:2) model polypeptide = 228 kDa
octameric KtrA + 2x dimeric KtrB (8:4) model polypeptide = 325 kDa

Buffer: 25 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM NADH, 1 mM DDM
Multiple oligomeric states for reconstituted KtrAB K⁺ Transporter

KtrAB ion transporter

(8:2) model  polypeptide = 228 kDa
(8:4) model  polypeptide = 325 kDa

<table>
<thead>
<tr>
<th>dimer:octamer KtrB:KtrA</th>
<th>Excess KtrB dimer?</th>
<th>Elution volume (ml)</th>
<th>8:2 model (228 kDa)</th>
<th>correct model?</th>
<th>8:4 model (325 kDa)</th>
<th>correct model?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>computed MW for complex (kDa)</td>
<td>difference from model (kDa)</td>
<td>computed MW for complex (kDa)</td>
<td>difference from model (kDa)</td>
</tr>
<tr>
<td>0.4</td>
<td></td>
<td>14.23</td>
<td>228</td>
<td>0</td>
<td>Yes</td>
<td>250</td>
</tr>
<tr>
<td>0.9</td>
<td></td>
<td>14.05</td>
<td>240</td>
<td>12</td>
<td>Yes</td>
<td>264</td>
</tr>
<tr>
<td>2.2</td>
<td>Yes</td>
<td>13.99</td>
<td>274</td>
<td>46</td>
<td></td>
<td>302</td>
</tr>
<tr>
<td>3.7</td>
<td>Yes</td>
<td>13.91</td>
<td>271</td>
<td>43</td>
<td></td>
<td>299</td>
</tr>
</tbody>
</table>
Determination of dimerization constant from SEC-LS measurements

**SecA protein**  (nanomotor promotes protein translocation in eubacteria)

conflicting reports about whether SecA functions as a monomer or dimer

<table>
<thead>
<tr>
<th>WT</th>
<th>monomer = 102 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS8 deletion mutant</td>
<td>monomer = 101 kDa</td>
</tr>
<tr>
<td>D11 deletion mutant</td>
<td>monomer = 100 kDa</td>
</tr>
</tbody>
</table>

The two subunits in the crystal structure of *B. subtilis* SecA
The first nine residues of each subunit are shown in *yellow* and *blue*.

SecA protein

WT monomer = 102 kDa

DS8 deletion mutant monomer = 101 kDa

D11 deletion mutant monomer = 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

Molar Mass vs. Volume

Molar Mass (g/mol)

Volume (mL)
**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

---

**Molar Mass vs. Volume**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Molar Mass</th>
<th>Mw</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.69 mg/ml</td>
<td>6.8 μM</td>
<td>163 kDa</td>
</tr>
<tr>
<td>0.17 mg/ml</td>
<td>1.7 μM</td>
<td>137 kDa</td>
</tr>
<tr>
<td>0.083 mg/ml</td>
<td>0.81 μM</td>
<td>129 kDa</td>
</tr>
<tr>
<td>0.037 mg/ml</td>
<td>0.36 μM</td>
<td>115 kDa</td>
</tr>
<tr>
<td>0.014 mg/ml</td>
<td>0.14 μM</td>
<td>105 kDa</td>
</tr>
</tbody>
</table>

**MW changes with concentration**

- D11 low salt
- D11 low salt
- D11 low salt
- D11 low salt

### Equations

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

\[ 2M = D \]

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

\[ f_m = \frac{-1 + \sqrt{1 + 8K_a c_t}}{4K_a c_t} \]
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></td>
<td>K&lt;sub&gt;d&lt;/sub&gt; [M]</td>
<td>ΔG dimer (kcal/mol)</td>
</tr>
<tr>
<td>WT</td>
<td>&lt;1x10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>-12.3</td>
</tr>
<tr>
<td>DS8</td>
<td>7±1x10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>-9.7</td>
</tr>
<tr>
<td>D11</td>
<td>3.5±0.2x10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>-7.4</td>
</tr>
</tbody>
</table>


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Met Leu Ile Lys Leu Leu Thr Lys Val Phe Gly
Monomer  52 kDa

Concentration range tested  <1 μg/ml  to  1 mg/ml
Concentration dependence of Mw from SEC/LS measurements

\[ K_d = 0.26 \mu M \pm 12\% \]
Concentration range accessible on an analytical SEC/LS system

~1 μg/ml to ~10 mg/ml

Concentration range: ~4 orders of magnitude
**SEC/LS**

- fast and accurate determination of molar masses (weight average) in solution

- applicable for analysis of:
  - protein-nucleic acid complexes
  - protein-lipids-detergent complexes
  - *glycosylated protein*
  - *proteins conjugated with PEG*

- can be used at wide range of protein concentrations
  from ~ 1μg/ml to >10mg/ml (correction for non-ideality)

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

Director of W.M. Keck Biotechnology Resource Laboratory at Yale University School of Medicine

NIH

Users of SEC/LS Service

Services provided by the Biophysics Resource contributed to at least **53 publications** (32 from Yale)

**Light Scattering Services** contributed to 37 publications

Full list at: [http://info.med.yale.edu/wmkeck/biophysics/publications_biophysics_resource.pdf](http://info.med.yale.edu/wmkeck/biophysics/publications_biophysics_resource.pdf)

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Ewa.Folta-Stogniew@yale.edu
Concentration range accessible on an analytical SEC/LS system

~1 μg/ml to ~10 mg/ml

Concentration at the apex of the eluting peak; ~ injected total mass

UV (280nm)
RI
LS (90 degree angle)
Concentration range accessible on an analytical SEC/LS system

\(~1 \mu g/ml\) to \(~10 \text{ mg/ml}\)

Concentration at the apex of the eluting peak; \(~\) injected total mass

\[
\begin{array}{cccccc}
1 \text{ mg/ml} & 0.1 \text{ mg/ml} & 10 \mu g/ml & 1.4 \mu g/ml & 0.7 \mu g/ml
\end{array}
\]
The streptococcal C1 bacteriophage lysin, PlyC, Holoenzyme is a multimeric protein:

50.3 kDa, “catalytic” subunit  
Ext. coeff. $A_{0.1\%}^{280} = 2.2$

8.0 kDa, “binding” subunit  
Ext. coeff. $A_{0.1\%}^{280} = 0.3$

SEC/LS  
MW = 114.0±0.4 kDa

PlyC 1 big+8 small  
predicted MW = 114.3 kDa

SEC/LS accuracy ~3 %, i.e. ~3kDa for PlyC

PlyC  
MW = 114.3 kDa  
Ext. coeff. $A_{0.1\%}^{280} = 1.2$

PlyC_bis  
2 big+2 small  
MW = 116.6 kDa  
Ext. coeff. $A_{0.1\%}^{280} = 2.0$

on-line” determination of extinction coefficient \(^a\) from UV/RI ratio

Evaluated models:

- 1 big + 8 small \(\text{MW=}\) PlyC model (1+8)
- 2 big + 2 small \(\text{MW=}\) PlyC\(_{\text{bis}}\) model (2+2)

Octameric PlyCB. The eight PlyCB subunits arranged in a ring as observed in the crystal structure of PlyC.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ext. coeff. Est.</th>
<th>UV/RI ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>observed</td>
<td>computed</td>
</tr>
<tr>
<td>Apo</td>
<td>1.026</td>
<td>1.279</td>
</tr>
<tr>
<td>BAM</td>
<td>1.788</td>
<td>2.147</td>
</tr>
<tr>
<td>BSA</td>
<td>0.700</td>
<td>0.821</td>
</tr>
<tr>
<td>CA</td>
<td>1.737</td>
<td>2.273</td>
</tr>
<tr>
<td>OVA</td>
<td>0.730</td>
<td>0.919</td>
</tr>
<tr>
<td>Ti</td>
<td>0.928</td>
<td>1.070</td>
</tr>
<tr>
<td>PlyC(_{(1+8)})</td>
<td>1.204</td>
<td>1.600</td>
</tr>
<tr>
<td>PlyC(<em>{\text{bis}})(</em>{(2+2)})</td>
<td>2.000</td>
<td>1.600</td>
</tr>
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SecAΔ11 25 mM KCl  \( K_d = 2.6 \times 10^{-7} \) M
SecAΔ11 100 mM KCl  \( K_d = 3.5 \times 10^{-6} \) M
SecAΔ11 300 mM KCl  \( K_d > 2.3 \times 10^{-4} \) M
SEC/LS results: Stokes radius Distribution Plot

BSA
Monomer: 66 kDa
$R_h = 3.4 \text{ nm}$

Stokes radius ($R_h$)
Measured every 5 μl

UV trace; $A_{280\text{nm}}$

RI trace
Effects of detergent on oligomeric state of KtrA RCK domain

KtrA RCK domain no detergent
(octamer)

KtrA RCK domain plus detergent
(tetramer and monomer) + micelle