Sample preparation and characterization around SAXS

Rob Meijers
EMBL Hamburg
Quality control

SDS Page

Mass spec

Gel filtration

DLS/SLS
A gel of a heterodimeric receptor
A size exclusion profile
A second look
Different gels

• SDS
• SDS non-reduced
• Native
• Iso-electric focussing

• Purity
• Disulphide bonds
• Post-translational modifications:
  – Phosphorylation
  – glycosylation
Glycans form a sizeable part of the protein

- Depends on expression system
- In pichia: > 40 % of MW can be glycans!
- Deglycosylate
- Change to HEK/COS/CHO-Lec
Another capricious sample

7XCYS ORIGINAL SEQUENCE, WITH HIS-TAG

SDS-PAGE 12%: Size Exclusion peaks 1 (P1) and 2 (P2) under reducing and non-reducing conditions.
Sizing profile

Tetramer

Dimer

Add DTT

Monomer
Cysteine point mutations
Sizing profile
Quality control

SDS Page

Gel filtration

DLS/SLS
Dynamic Light Scattering scheme
Dynamic light scattering
Information from the correlation curve

The time at which the correlation of the signal starts to decay gives information about the mean diameter.

The baseline gives information about the presence of large particles/aggregates.

The angle of decay gives information about the polydispersity of the distribution.
Stokes-Einstein relation

- $D =$ Diffusion coefficient
- $k =$ Boltzmann’s coefficient
- $T =$ Temperature
- $\eta =$ Viscosity
- $R =$ hydrodynamic radius

$$D = \frac{kT}{6\pi \eta R}$$
Static Light Scattering

Courtesy Bernd Tartsch, Malvern Instruments
Sizing profile, SLS twist

<table>
<thead>
<tr>
<th>Peak</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw (Daltons)</td>
<td>64.151</td>
</tr>
<tr>
<td>Mw/Mn</td>
<td>1.055</td>
</tr>
<tr>
<td>IV (dl/g)</td>
<td>0.0434</td>
</tr>
<tr>
<td>Rh (nm)</td>
<td>3.50</td>
</tr>
<tr>
<td>Wt Fr (Peak)</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ID</th>
<th>ctp11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>20mM HEPES, 150mM NaCl</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.5000</td>
</tr>
<tr>
<td>Conc (mg/ml)</td>
<td>2.0079</td>
</tr>
<tr>
<td>Inj VOl (ul)</td>
<td>100.0</td>
</tr>
</tbody>
</table>
BSA – Molecular Weight

- Trimer: 205 kDa
- Dimer: 134 kDa
- Monomer: 66 kDa
Angular Dependence

small molecules
radius < 15 nm
$P_\theta = 1$ for all $\theta$

large molecules
radius > 15 nm
$P_\theta = 1$ for $\theta = 0^\circ$
Intramolecular interference produces a disymmetry in the scattered light.

Size of molecule/particle must be significant compared to wavelength of light.
Angular Dependence

\[ P(\theta) = 2 \frac{e^{-x} - (1-x)}{x^2} \]

with

\[ x = \frac{8}{3} R g \frac{\pi n}{\lambda} \sin(\theta/2)^2 \]
Low Angle Light Scattering - LALS
## Relationship between Size and Molecular Weight

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Mol. Weight (D)</th>
<th>$R_h$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease</td>
<td>13,700</td>
<td>1.93</td>
</tr>
<tr>
<td>b-Lactoglobulin</td>
<td>35,000</td>
<td>2.67</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>65,000</td>
<td>3.38</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>68,000</td>
<td>3.40</td>
</tr>
<tr>
<td>IgG</td>
<td>150,000</td>
<td>5.02</td>
</tr>
<tr>
<td>Catalase</td>
<td>250,000</td>
<td>5.38</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>660,000</td>
<td>7.50</td>
</tr>
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### RALS

<table>
<thead>
<tr>
<th>Polysaccharides/PEG</th>
<th>MW</th>
<th>$R_h$ (nm)</th>
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<tr>
<td>12,000</td>
<td>2.62</td>
<td></td>
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<tr>
<td>80,000</td>
<td>7.27</td>
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<td>150,000</td>
<td>9.81</td>
<td></td>
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### LALS

| IgM                     | 900,000   | 11.7       |
| DNA                    | 3 Mio     | 70         |

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### LALS

| IgM                     | 900,000   | 11.7       |
| DNA                    | 3 Mio     | 70         |
Normalised LS signals show no angular dependence for proteins

- Molecular weight requires only RALS
- Can not measure size by light scattering alone
Addition of a Viscosity Detector gives hydrodynamic radius
Intrinsic Viscosity

Intrinsic viscosity is the concentration normalized viscosity of the sample in solution at infinite dilution.

\[ IV = \left[ \eta \right] = \frac{\eta_{sp}}{c} \bigg|_{c \to 0} \quad \eta_{sp} = \frac{\eta - \eta_0}{\eta_0} \]

\( \eta_{sp} \) is called the specific viscosity of the solution whose concentration is \( C \).
\( \eta_0 \) is the Solvent Viscosity.
\( \eta \) is the Solution Viscosity.

In GPC, concentrations of solutions are sufficiently dilute that the extrapolation to zero concentration is negligible.
Hydrodynamic Radius

\[ [\eta] \frac{M}{\rho} = \frac{2.5}{\rho} = \frac{2.5 \cdot V_h}{M} \]

\[ [\eta] M = 2.5 \cdot V_h = 2.5 \cdot \left( \frac{4/3 \pi R_h^3}{R_h} \right) = 10.47 \]

Intrinsic Viscosity is inverse proportional to the density

Intrinsic Viscosity x Molecular weight is proportional to the hydrodynamic radius of a sample

Intrinsic Viscosity: from Viscosity Detector
Molecular Weight: from Light Scattering Detector
Capillary Differential Viscosity Detector

\[ \eta_{sp} = \frac{4DP}{IP - 2DP} = C \times IV \]

Wheatstone bridge concept
M. Haney, 1983
Hydrodynamic Radius

BSA

Data File: 2005-08-04_13;42;17_BSA_02.vdt  Method: Proteins SEC-0004.vcm

6.2 nm
5.1 nm
3.8 nm
Online Quality control

- UV
- Refractive index
- Viscosity
- Low angle light scattering

Viscotek/Malvern
What are the detectors responding to?

Refractometer \[= K_{RI} \times dn/dc \times \text{Conc} \]

UV-Detector \[= K_{UV} \times dA/dc \times \text{Conc} \]

Viscometer \[= K_{Visc} \times \text{Intrinsic Viscosity} \times \text{Conc} \]

Light Scattering \[= K_{LS} \times M_w \times (dn/dc)^2 \times \text{Conc} \]
<table>
<thead>
<tr>
<th>Mw (D)</th>
<th>IVw (dl/g)</th>
<th>Rh (nm)</th>
<th>Weight Fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>66.430</td>
<td>0.056</td>
<td>3.88</td>
</tr>
<tr>
<td>Dimer</td>
<td>133.000</td>
<td>0.071</td>
<td>5.32</td>
</tr>
<tr>
<td>Trimer</td>
<td>201.000</td>
<td>0.095</td>
<td>6.69</td>
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**Light Scattering**

**Viscometer**

**Refractive Index**
Laser Light Scattering Detector, Refractive Index Detector and UV-Cell

GPC columns

LALS (7°) detector

UV detector

RI detector

RALS (90°)
Balanced Bridge 4-Capillary Viscometer Detector

Delay Volume

GPC Column(s)
Online purification & QC
Sample optimization

• Reduced Gel problematic: change purification protocol
• NR Gel problematic: check cysteines
• Protein aggregation, folding stability:
  – Size exclusion, light scattering, CD, NMR, thermofluor
Thermal stability

• Thermofluor
• Modified real-time PCR machine
  – Add hydrophobic fluorescent probe
  – When protein unfolds...
  – Fluorescence increases
Thermal stability

- Check protein stability
- Additive/ligand screen

Sample optimization

- Reduced Gel problematic: change purification protocol
- NR Gel problematic: check cysteines
- Protein aggregation, folding stability:
  - Size exclusion, light scattering, CD, NMR, thermofluor
- Modify buffers, additives
- If nothing works: change construct
Additives?

- **DTT**
- **Glycerol**
- **Detergents at less than 2xCMC**
  - 0.1% 1-s-Nonyl-β-D-thiogluicoside
  - 0.2% n-Decanoylsucrose
  - 0.3% n-Nonyl-β-D-maltoside
  - 0.4% DDAO
  - 0.5% C8E5
  - 0.8% FOS-Choline®-10
  - 1.1% FOS-Choline®-9
Mass spectrometry

• Traditional MS:
  – Confirm mass
  – Confirm sequence
  – Check folding state

• MS/MS + Ion mobility:
  – Detailed folding state
  – Protein-protein interactions
  – Whole protein size...
Ion mobility derived particle size


in combination with SAXS...
Conclusions

- QC at home is crucial
- But soon we hope to do it for you at EMBL@PETRA3
- Some quality control methods can provide useful complementary data
- This all can filter the garbage “in” bit
But the garbage out?
Acknowledgements

• Malvern Instruments
  Bernd Tartsch