Light Scattering

STATIC *Light Scattering*

Also known as *Rayleigh* *or* *Classical* Light Scattering

Measures avg. intensity of scattered light for

Absolute Molecular Weight

Dynamic *Light Scattering (**DLS**)*

Also known as *Quasi-elastic* Light Scattering *(QUELS)*
or Photon Correlation Spectroscopy (PCS)

Measures microsecond fluctuations of single photons

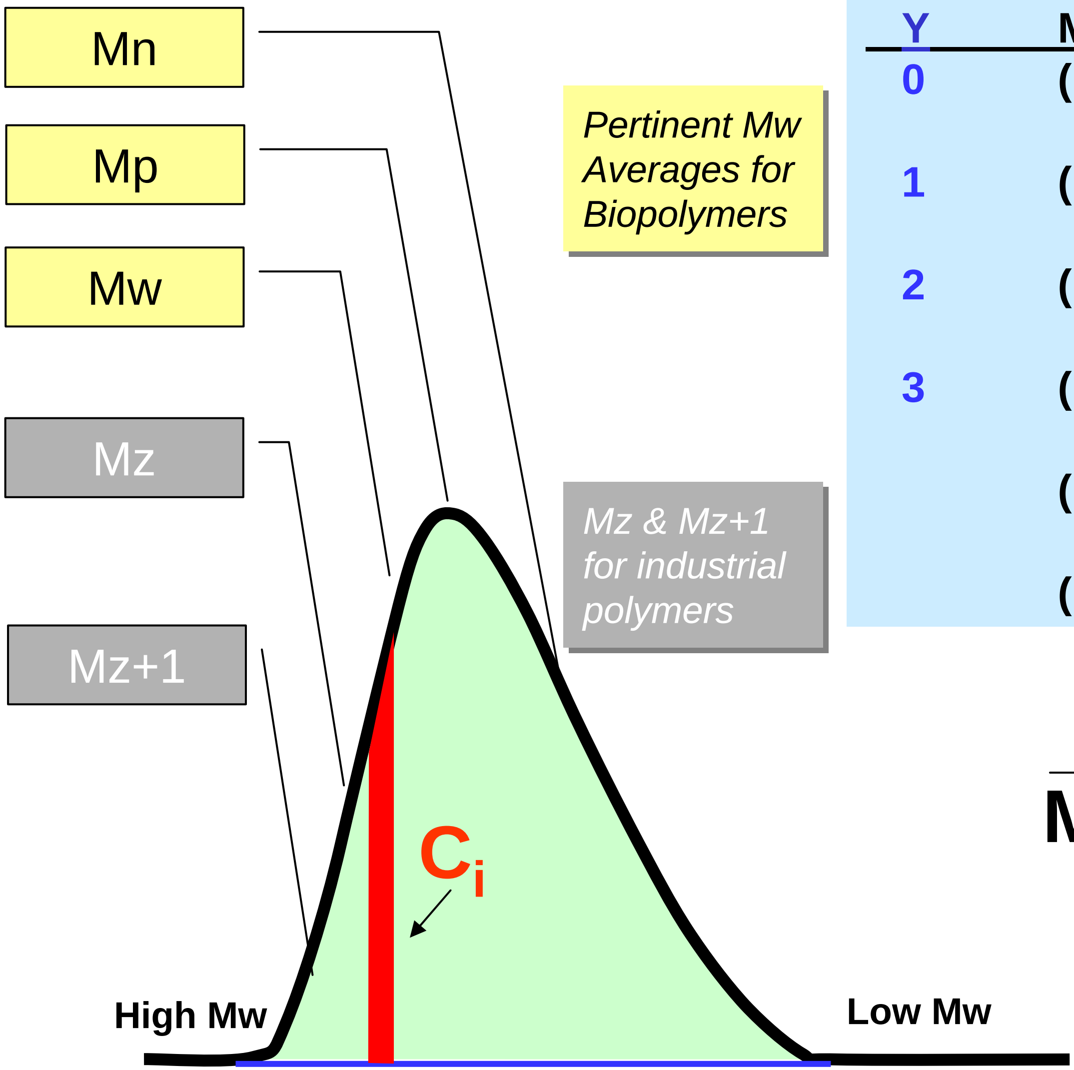
Hydrodynamic Radius (Size)

M.L.Hackert (with figures from Precesion Detectors and Wyatt Tech.)

What Do We Mean By ABSOLUTE?

- There are **4 Absolute Methods of Measuring MW**
- 1) Membrane **Osmometry** (Number Average MW)
- 2) Light Scattering (Weight Average MW)
- 3) Sedimentation Equilibrium (Ultracentrifugation) (z-average MW)
- 4) **Mass spectroscopy**
- ***NO*** Reference to standards of mass
- ***NO*** assumptions of molecular model/conformation
- ***ALL*** parameters measured directly from 1st principles
- ***Refractive indices***
- ***geometries of cell and detector***
- ***wavelength***
- ***concentrations***
- ***detector response***
- ***temperature***
- ***dn/dc***

Calculation of Mw Averages



Retention Volume (or Time)

Y	Mx	Name
0	(Mn)	Avg. Molecular Number
1	(Mw)	Avg. Molecular Weight
2	(Mz)	Avg. Z (Velocity)
3	(Mz+1)	Avg. Z+1
	(Mw/Mn)	Polydispersity
	(Mp)	Peak Molecular Weight

$$\overline{M}_x = \frac{\sum C_i M_i^{y}}{\sum C_i M_i^{y-1}}$$

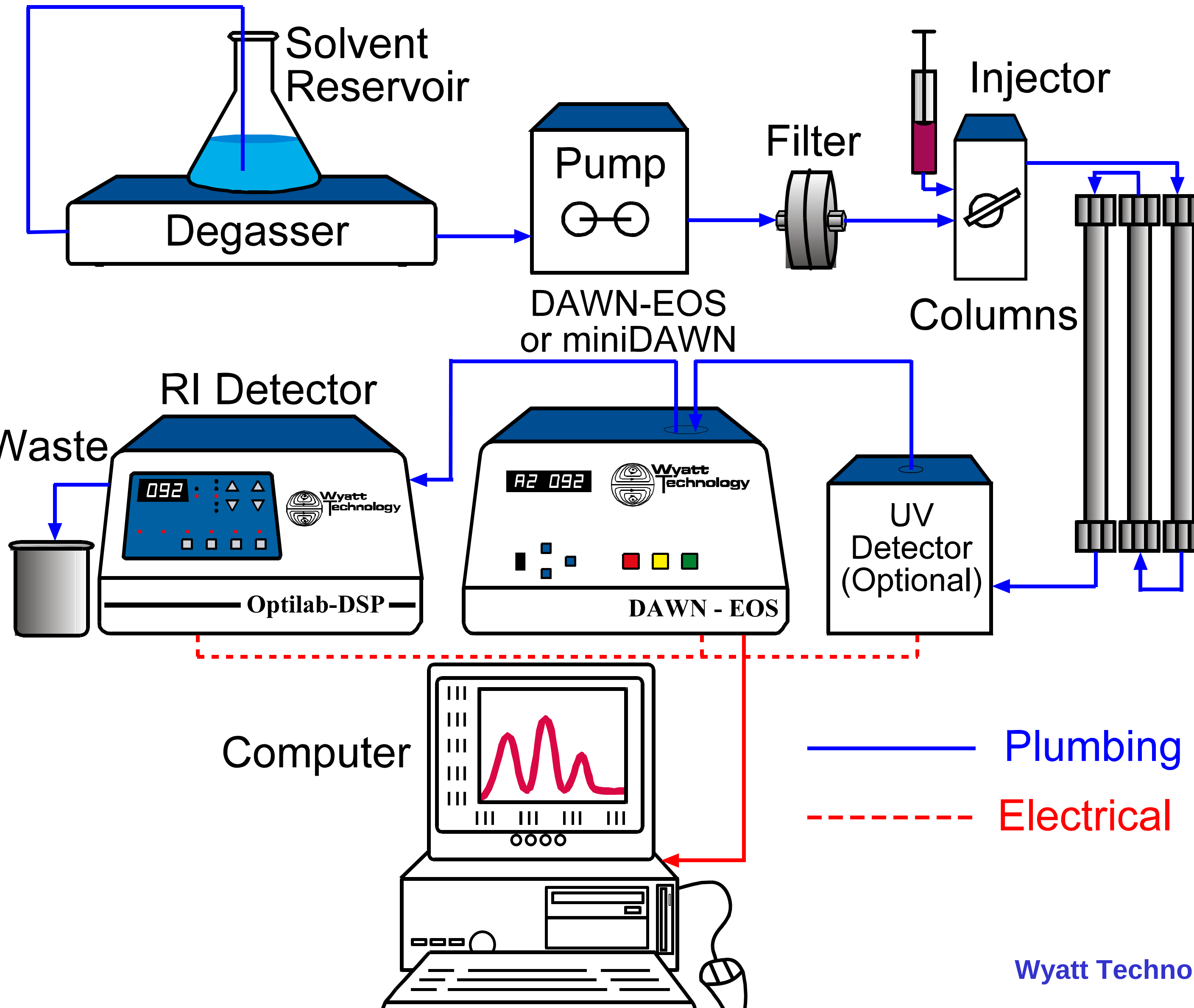
Abstract

Precision Detectors

Recent **advances in genomics and proteomics have produced a proliferation of new proteins requiring characterization**. **Mass spectrometry** is ideally suited for identification and **primary structural** purposes but is **not well suited for determining conformational structures in solution**. As these molecules are expressed in cell culture, purified and then formulated, rigorous production processes must be carefully evaluated to minimize impact on the protein structure and its long-term shelf life. **Obtaining a conformational stability profile of protein or antibodies can help weed out bad drug candidates from good ones as environmental factors can change their tertiary and quaternary structure. Environmental factors include pH, ionic strength, temperature, and excipient composition.**

HPLC is used in flow injection mode with a detector array composed of laser light scattering (static and dynamic modes) and a concentration source detector (RI or UV). This configuration determines the average molecular weight and average hydrodynamic radius with run times as short as 1 minute. Alternatively, a SEC guard column can be used isolate analyte from excipients (eliminating blank runs) with run times under 3 minutes.

Chromatography with LS Set-up

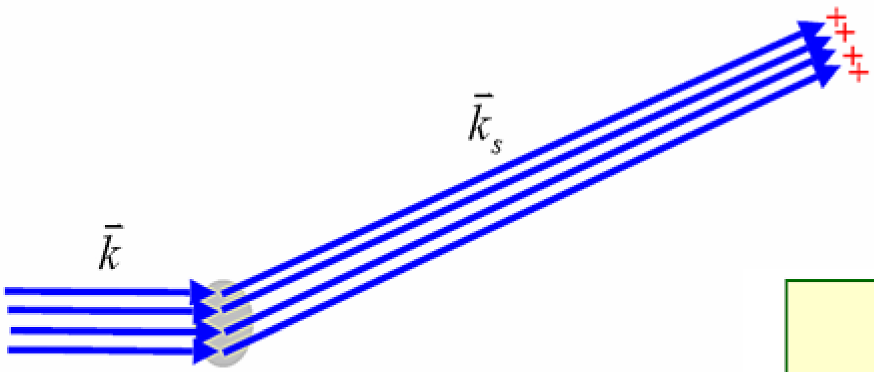


Wyatt Technologies


OPTILAB DSP

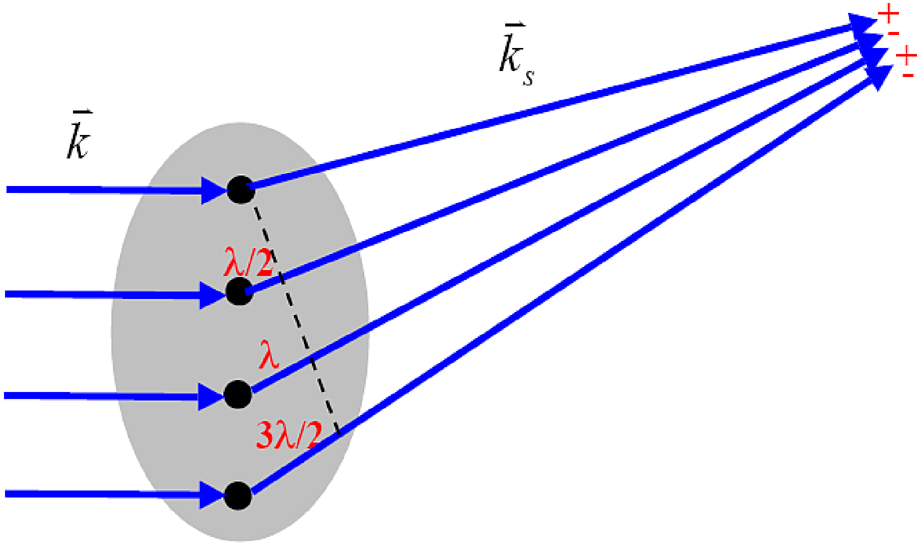

OPTILAB DSP

Scattering from molecules much smaller than λ



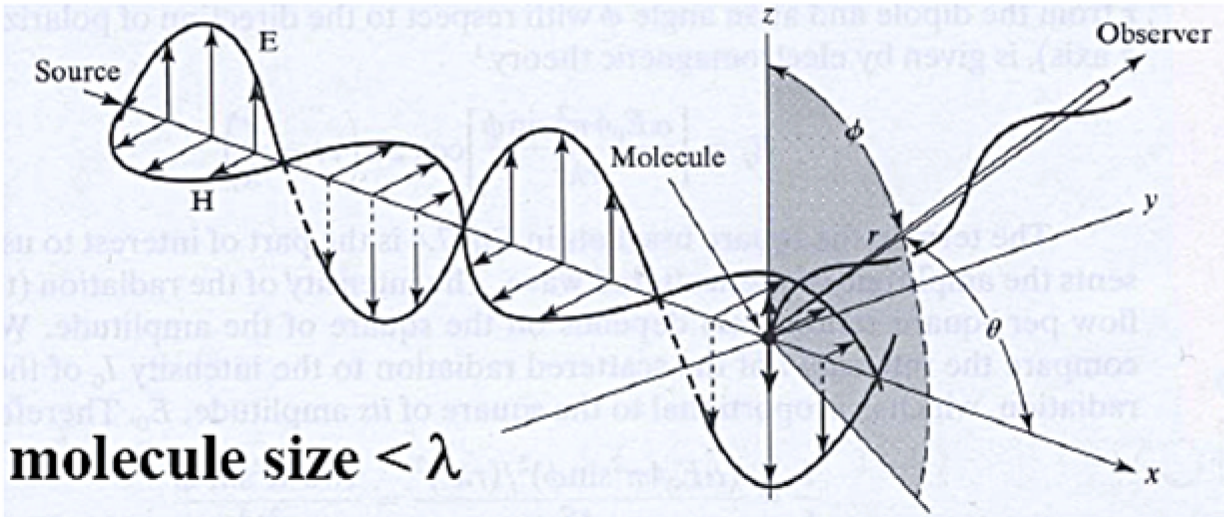
Do not need to consider interference effect.

Scattering from molecules comparable to or larger than λ



Need to consider interference effect.

EM wave scattered by a small molecule



molecule size $< \lambda$

Dipole induced in the molecule at the origin

$$\vec{p} = \alpha \vec{E} = \alpha \vec{E}_0 e^{i\omega t} \qquad E_0\text{: incident field}$$

Electromagnetic wave emitted by the oscillating dipole

$$E = \frac{4\pi^2 \alpha E_0 \sin\phi}{\lambda^2 r} e^{i(\omega t - \vec{k}_s \cdot \vec{x})}$$

EM wave scattered by a molecule

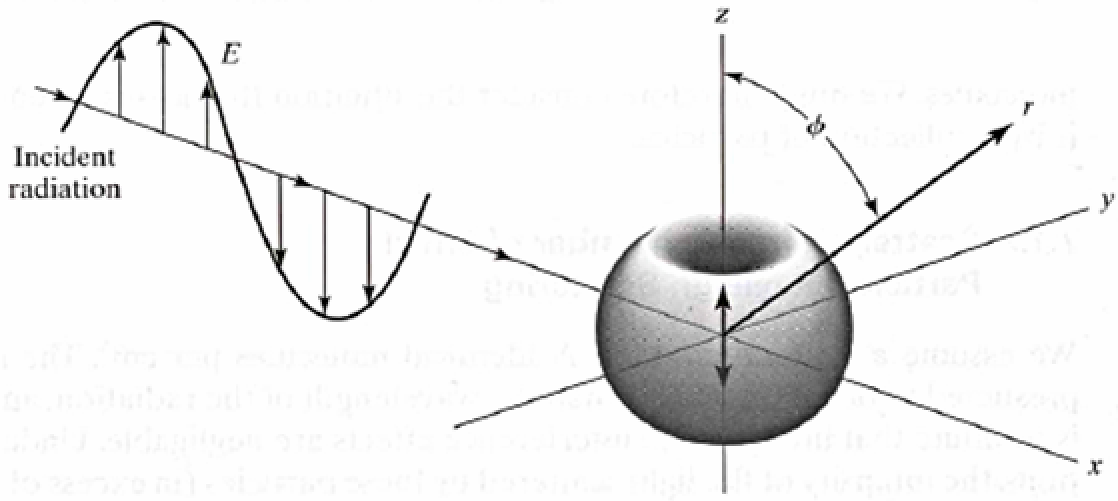
Electromagnetic wave emitted by the oscillating dipole

$$E = \frac{4\pi^2 \alpha E_0 \sin\phi}{\lambda^2 r} e^{i(\omega t - \vec{k}_s \cdot \vec{x})}$$

Scattering intensity to the incident intensity

$$\frac{I}{I_0} = \frac{16\pi^4 \alpha^2 \sin^2\phi}{\lambda^4 r^2}$$

for polarized incident light of intensity I_0



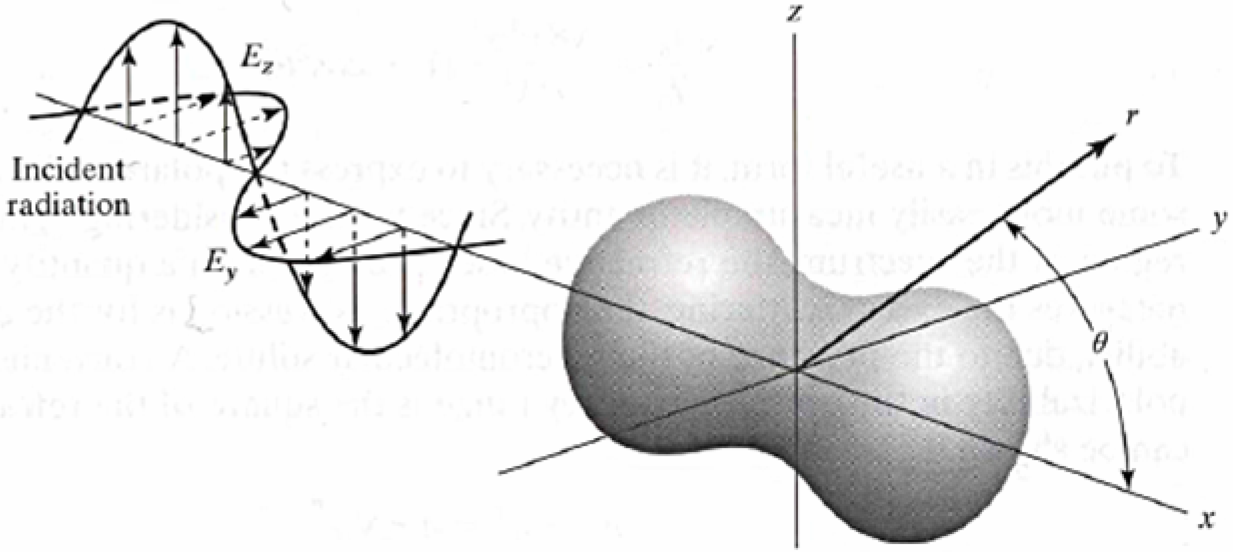
EM wave scattered by a molecule

Scattering intensity to the incident intensity

$$\frac{I}{I_0} = \frac{8\pi^4\alpha^2(1+\cos^2\theta)}{\lambda^4 r^2}$$

for unpolarized incident light

$I \propto 1/r^2$; $I \propto 1/\lambda^4$; I depends on scattering angle



Scattering from molecules much smaller than λ

molecule size << λ

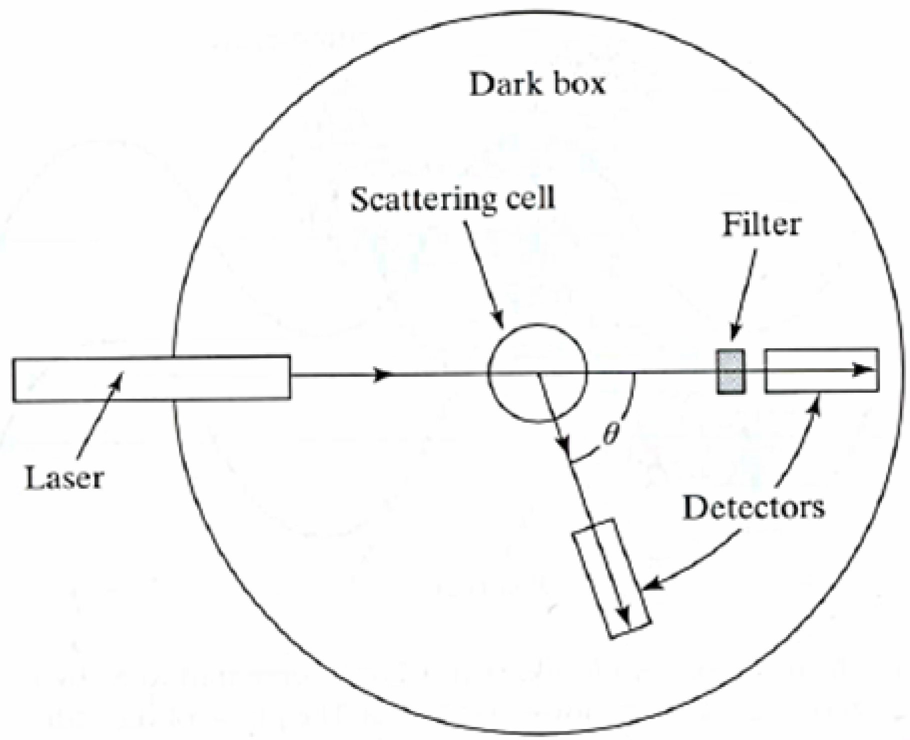
$$\frac{I(\theta)}{I_0} = \frac{2\pi^2 n_0^2}{A\lambda^4 r^2}\left(\frac{dn}{dC}\right)^2 CM(1+\cos^2\theta)$$

Light scattering can be used to determine the molecular weight.

Define **"Raleigh Ratio" R_θ**

$$R_\theta = \frac{I(\theta)}{I_0}\,\frac{r^2}{(1+\cos^2\theta)}$$

$$R_\theta = KCM \quad \textit{or} \quad \frac{K^*c}{R(\theta)} = \frac{1}{M}$$



Basic Light Scattering Principles

- The amount of **light scattered** is directly proportional to the product of the **molar mass** and the **molecular concentration**

$$I_{LS} = \boxed{C\ M_w} (dn/dc)^2\ P_\theta\ K_\theta$$

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

$$\frac{K^*c}{R(\theta)} = \frac{1}{M}\left[1 + \frac{16\pi^2}{3\lambda^2}\langle r_g^2\rangle \sin^2(\theta/2) + \cdots\right] + 2A_2c$$

Static *Light Scattering Detection*

Determines

- **Absolute Molecular Weight**

  Independent of Column Calibration

- **Radius of Gyration (R_g)**

  > 10 nm to 150 nm

$$I_{LS} = C\ M_w\ (dn/dc)^2\ P_\theta\ K_\theta$$

- **Form Factor** (P_θ)
- **Detector Constants** (K_θ)
- **Specific Refractive Index Increment** (dn/dc)
- **Molecular Weight** (M_w)
- **Concentration of Solute** (C)
- **Excess Light Scattering Signal** (I_{LS})

Rayleigh Equation

Basic Light Scattering Equation

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

Where:

$K^* = 4\pi^2 (dn/dc)^2 n_0^2 / (N_A \lambda_0^4)$

n_0 is the refractive index of the solvent.

c is the concentration of the solute molecules (g/ml).

$R(\theta)$ is the fraction of light scattered per unit solid angle, in excess of the light scattered by the solvent, divided by the incident intensity.

N_A is Avogodro's number.

λ_0 is the vacuum wavelength of the incident light.

dn/dc is the refractive index increment, which tells how much the refractive index of the solution varies with solute concentration.

M_w is the weight-average molar mass.

Accuracy of Molecular Masses of Test Proteins Determined by Light Scattering

Protein	Mass From Structure	Light Scattering*	Apparent Error
	[Da]	[Da]	[%]
Carbonic anhydrase	29,023	29,800	+2.7
Alcohol dehydrogenase	145,980	149,000	+1.4
β-Amylase	224,340	228,000	+1.6
Apoferritin	476,316	484,400	+1.7
Thyroglobulin	669,000	679,000	+1.5
Ornithine decarboxylase	990,684	978,000	-1.3
Octopus Hemocyanin	3,440,000	3,450,000	+0.3

*DAWN detector model-F, 0.19 was used as dn/dc value for all the proteins
Adapted from "Assembly of the Gigantic Hemoglobin of the Earthworm *Lumbricus terrestris* by A. Riggs *et.al.* In *J. Bio. Chem.*, Vol. 271, No. 47, pp 30007-30021, 1996.

RI & Light Scattering Combined for Mw

$$R_\theta = C\ M\ (dn/dc)^2\ P(\theta)\ K(\theta)$$ Rayleigh Equation

$$RI_{signal} = K_{RI}\ (dn/dc)\ C$$ RI Equation

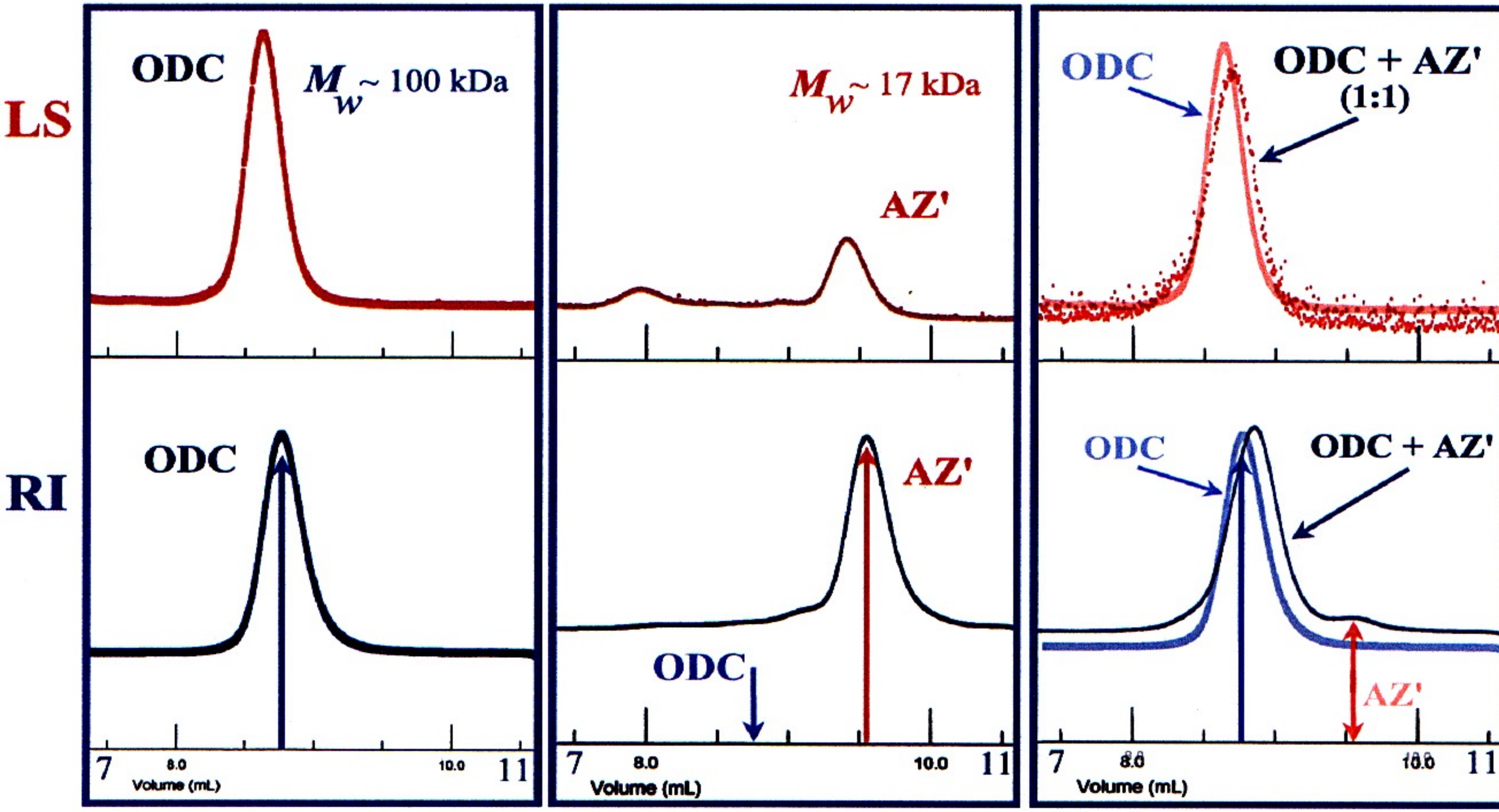
$$\frac{R_\theta}{RI_{signal}} = \frac{K(\theta)\ M_w\ (dn/dc)\ P(\theta)}{K_{RI}}$$

$$\frac{R_\theta}{RI_{signal}} \sim M_w$$

For a truly effective measurement of molecular weight the static light scattering detector must be combined with a well matched refractometer

- **The light scattering signal is directly proportional to the Mw .**
- **The concentration source signal (e.g. RI) is indirectly proportional to the Mw.**

Precision Detectors



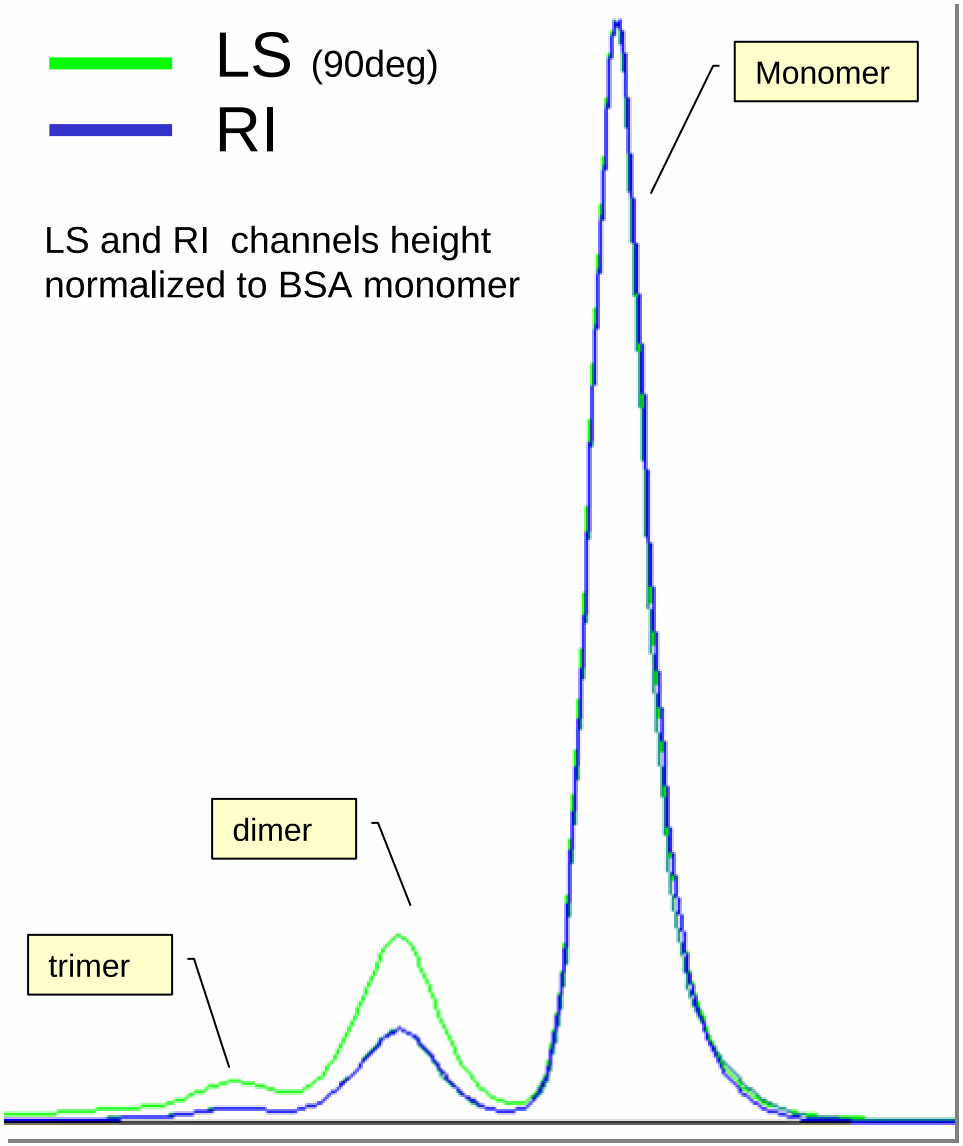
Light Scattering (LS) and Refractive Increment (RI) Results of ODC and AZ'. *ODC, AZ' and ODC:AZ' complex were injected onto an HPLC sizing column, separated and analyzed by LS (DAWN EOS) and RI (OptiLab DSP interfermetric refractometer). The results shown are for 7 to 11 minutes of elution volume. Frame 1 is for ODC, frame 2 for AZ', and frame 3 for the ODC:AZ' mixture at a 1:1 subunit ratio with the ODC trace from frame 1 superimposed for reference.*

Static LS Equation for Proteins

$$M_w = \frac{I_{LS}}{RI} K_{total}$$

1. **Mw is directly proportional to the LS signal**
2. **Mw is indirectly proportional to the conc. source (RI)**
3. **The dn/dc is constant for the protein and it's aggregates (0.186 mL/g)**
4. **Degree of aggregation can be approximated from visual inspection**

Visualizing Aggregation State



$$M_w = \frac{I_{LS}}{RI} K_{total}$$

Using visual inspection

- Monomer (LS is equal to RI)
- Dimer (LS is 2X as large as RI)
- Trimer (LS is 3X as large as RI)

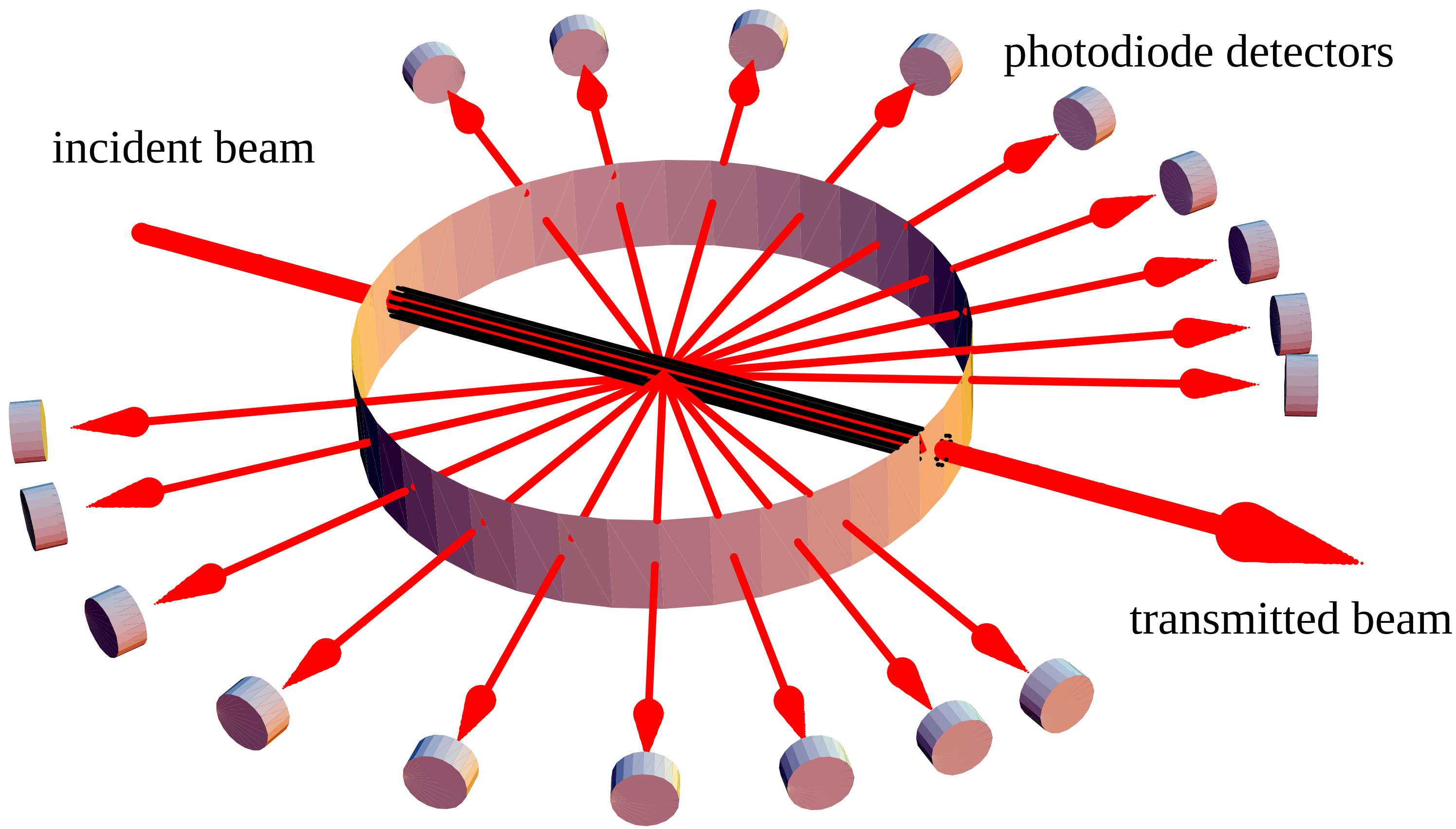
Precision Detectors

Why Multi-Angle Detection?

Light Scattering Intensity of Particles Shows an Angular Dependence on Size

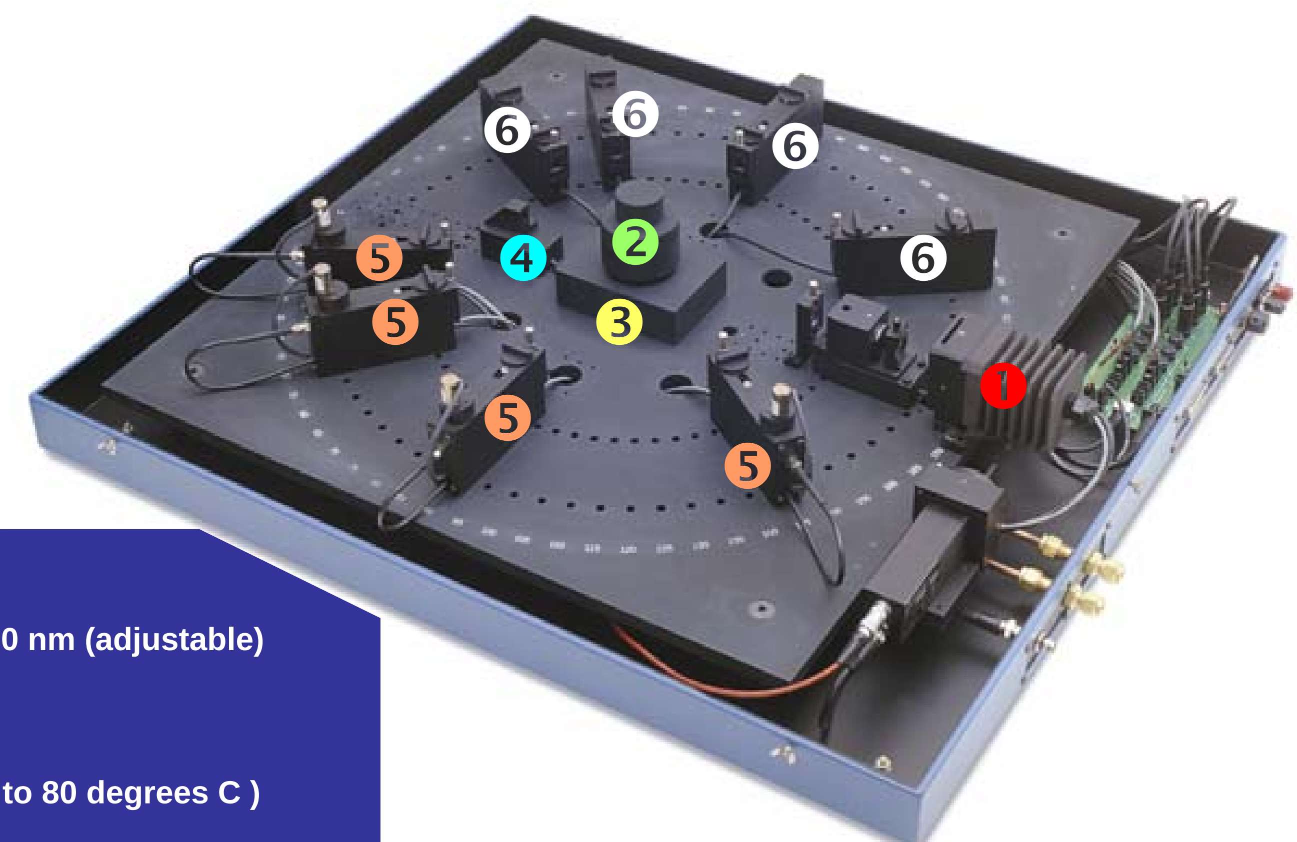
- **Low angles sensitive to large particles**
- **90 degree angle more sensitive to smaller particles**
- **High angles less sensitive to larger particles**
- **Back angles better suited for opaque matrices**

Flow Cell & Detector Geometry

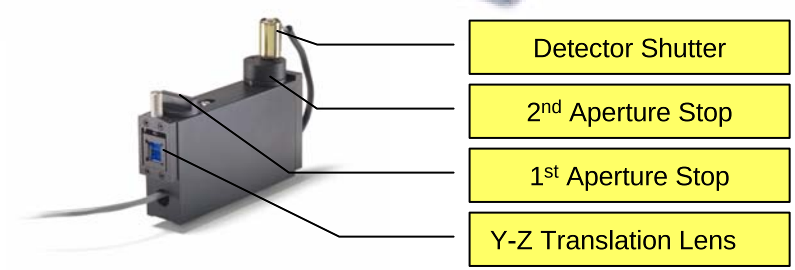


Wyatt Technologies

ALS4000 Optical Platform Features



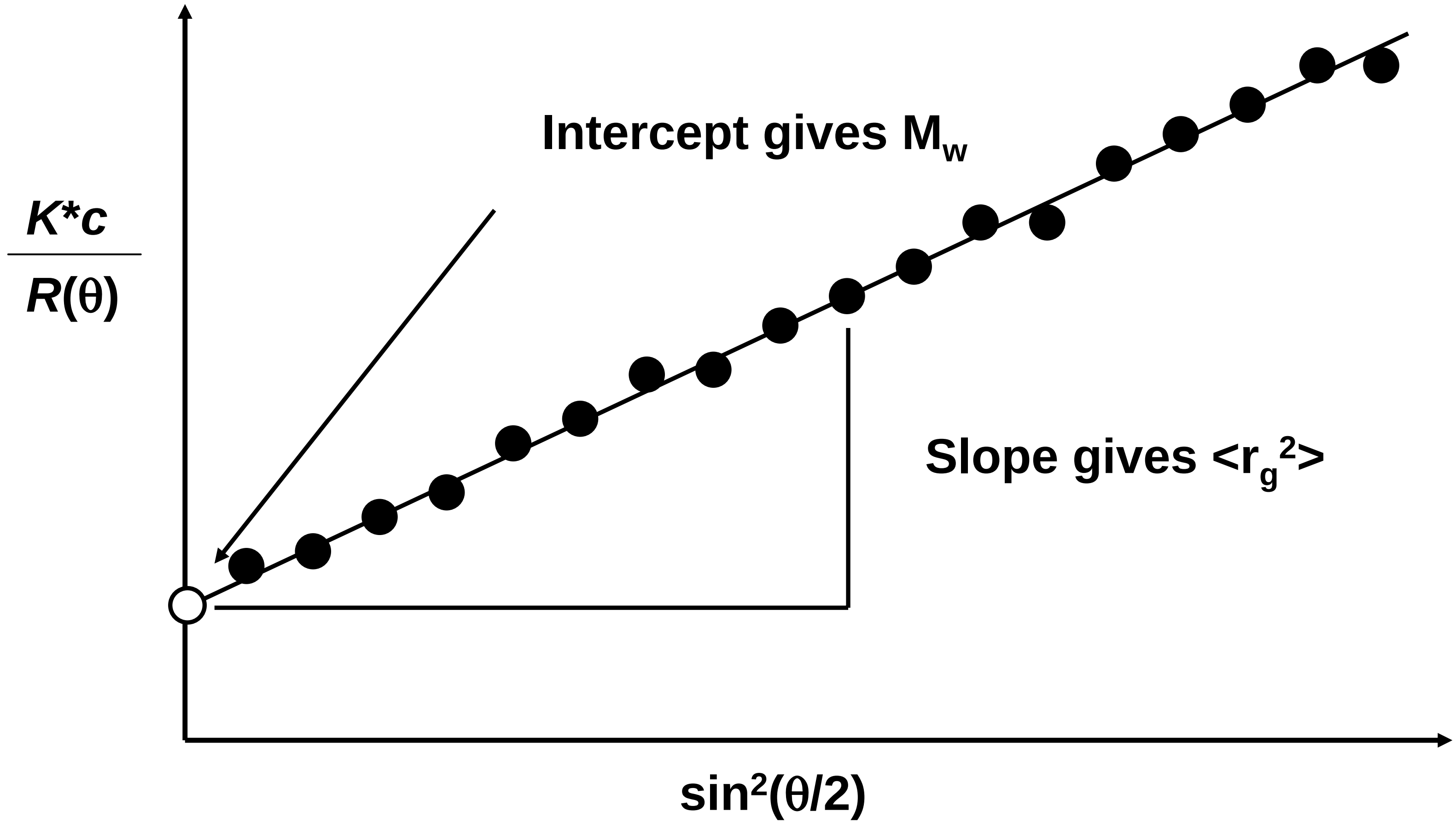
1. **Diode laser: 230 mW @ 830 nm (adjustable)**
2. **Flow Cell (30 uL)**
3. **Peltier cooling/heating (0 to 80 degrees C)**
4. **Laser Beam Stop**
5. **DLS Detectors (Up to 4)**
6. **Static LS Detectors (Up to 8)**



DLS Detector

Precision Detectors

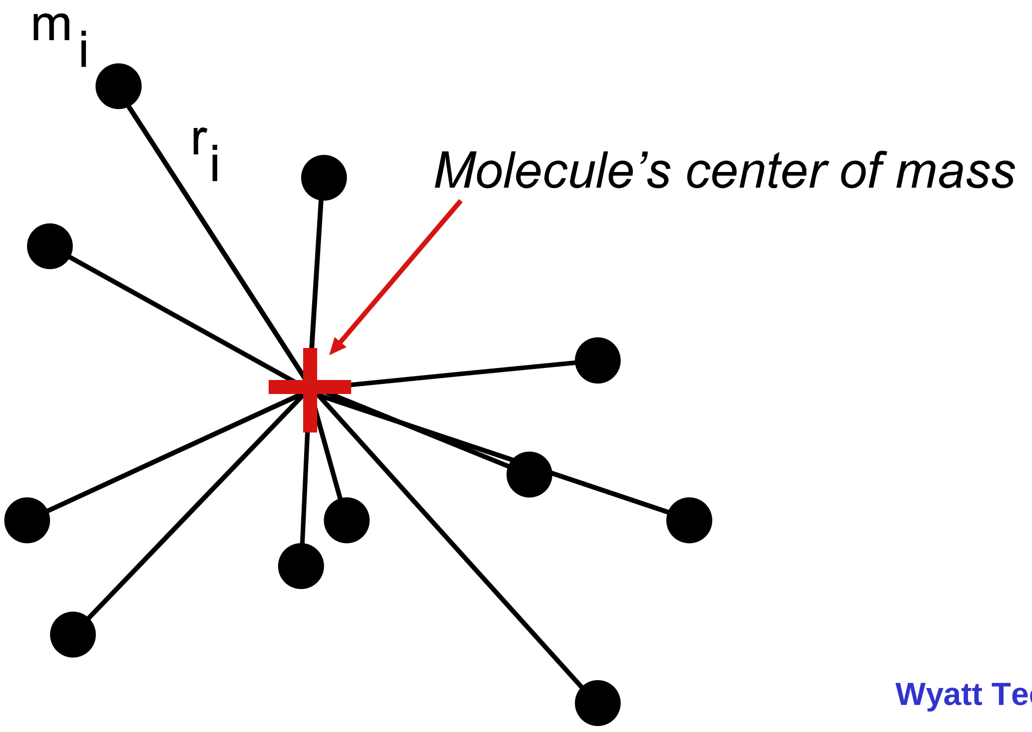
M_w & $\langle r_g^2 \rangle$ determined by MALS



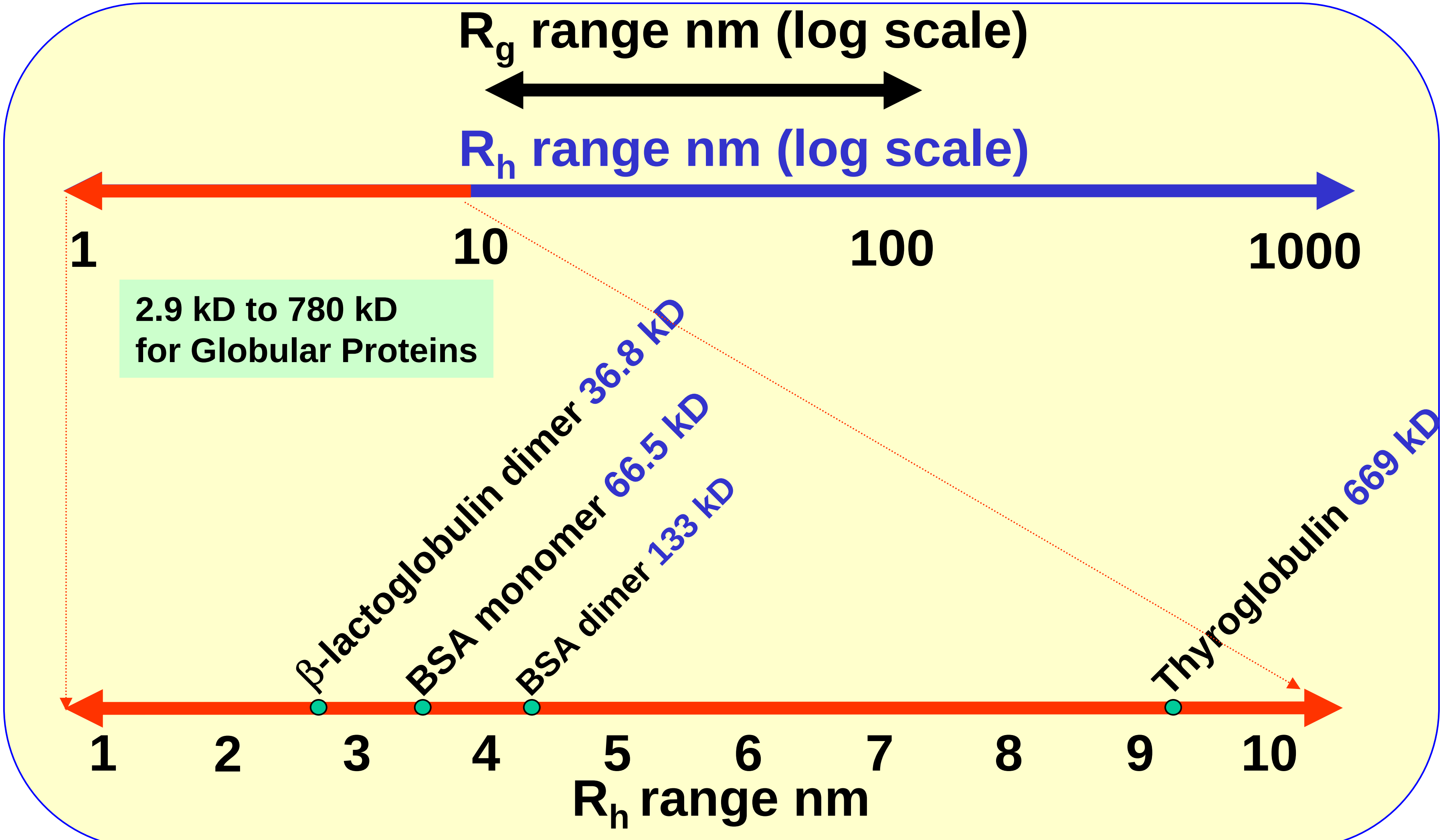
$$\frac{K^*c}{R(\theta)} = \frac{1}{M}\left[1 + \frac{16\pi^2}{3\lambda^2}\langle r_g^2\rangle \sin^2(\theta/2) + \cdots\right] + 2A_2c$$

$$\langle r_g^2 \rangle = \frac{\sum r_i^2 m_i}{M}$$

- $\langle r_g^2 \rangle$ is the mean square radius, relating to the distribution of mass within the molecule, given by



Why use R_h instead of R_g for Biomolecules?



Precision Detectors

Dynamic Light Scattering Detection

Determines

- **Molecular or Particle Size**

  As Hydrodynamic Radius (Rh)

- **Size Range**

  1 to 1000 nm

$$D_0 = \kappa \, T (6\pi \, \eta_0 \, R_h)^{-1}$$

Diffusion Coefficient

Boltzmann Constant

Temperature

Constants

Solvent Viscosity

Hydrodynamic Radius

Stokes-Einstein Equation

Precision Detectors

Hydrodynamic Radius Determination

From $g_1(\tau)$ the diffusion coefficient (D) for the scattering particles can be determined. From the diffusion coefficient, the hydrodynamic radius can be calculated.

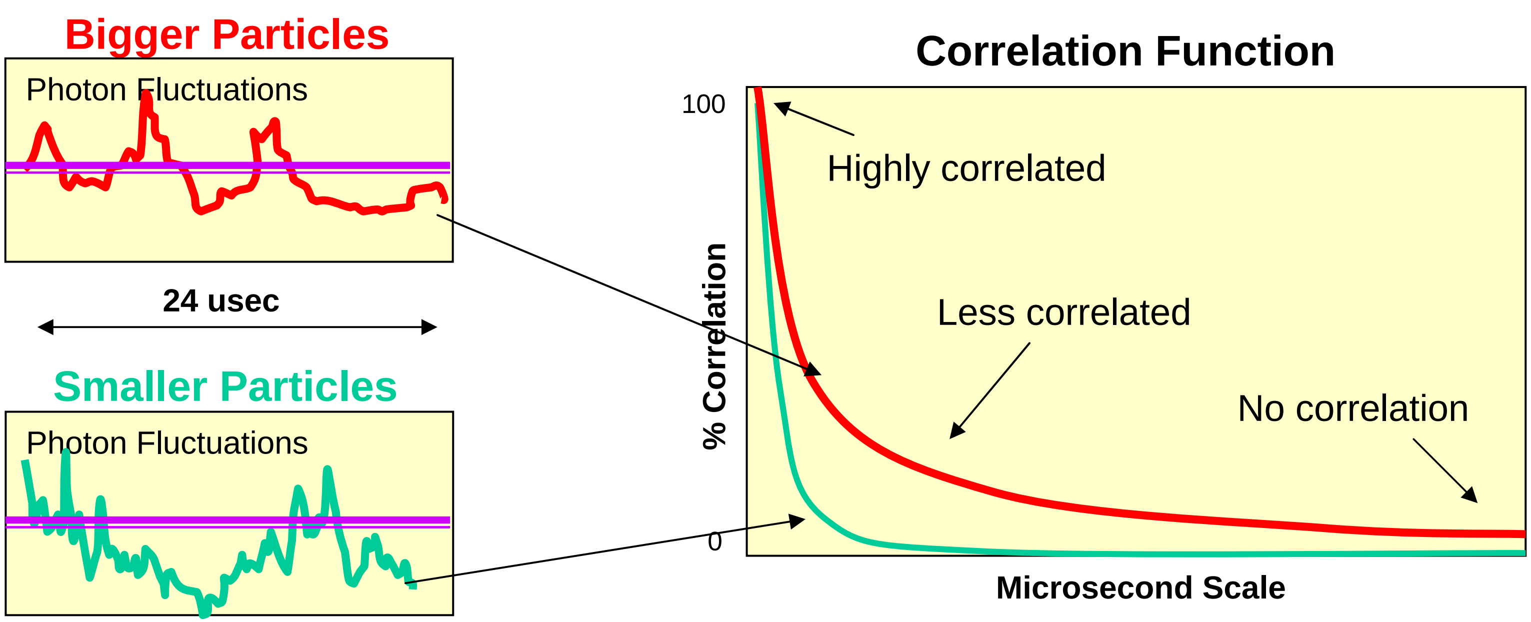
Diffusion Coefficient, Boltzmann Constant, Temperature (Kelvin), Solvent Viscosity (Poise), Hydrodynamic Radius (nm)

$$D_0 = \kappa T (6\pi \eta_0 R_h)^{-1}$$

Stokes-Einstein Equation

Applicable DLS Size Range

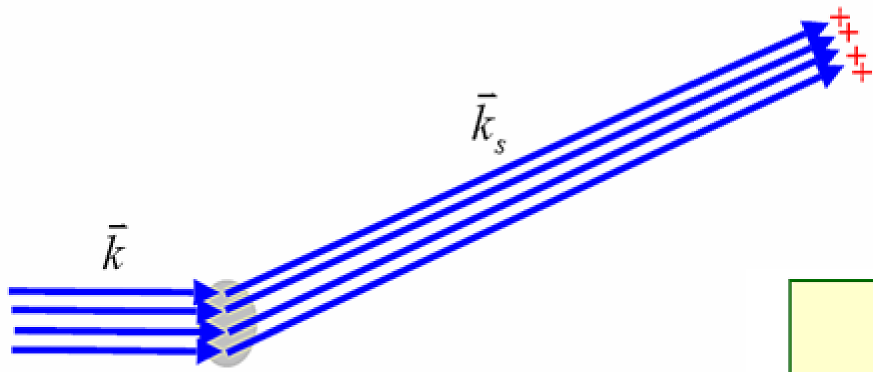
1.5 to 1000 nm Radius



Conclusions on Static and Dynamic LS

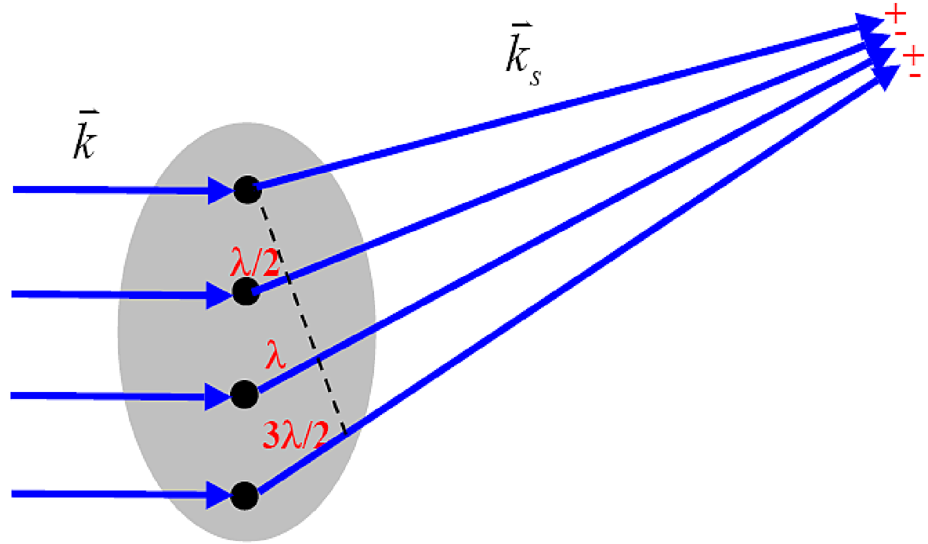
- Measures **Hydrodynamic Radius, Radius of Gyration, Molecular Weight. Particle Size Distribution**.
- Instrument Measures
  - **Rh size** from 1.7 nm to 1000 nm.
  - **Rg size** from 9 nm to 150 nm.
  - **Molecular Weight 200 to 20,000,000 daltons**
- Detects branching, aggregates and calculates Mw.
- **DLS**, does not require conc or dn/dc measurement for size.
- Batch Mode for non-flow system accessories.

Scattering from molecules much smaller than λ



Do not need to consider interference effect.

Scattering from molecules comparable to or larger than λ



Need to consider interference effect.

Scattering from molecules comparable to or larger than λ

$$P(\theta) = \frac{\langle I(\theta)\rangle}{\langle I(0)\rangle} = \exp\left(-\frac{16\pi^2}{3}\cdot\frac{R_G^2}{\lambda^2}\cdot\sin^2\frac{\theta}{2}\right)$$

Visible light, λ ~ 400 – 700 nm

For molecules with a few nm, scattering of visible light have a very week angular dependence.

↓

Difficult to determine the size of the molecules by scattering of visible light.

Biomolecules absorbs UV light.

↓

x-ray scattering

X-ray scattering

The major scatterers of x-ray in a molecule are electrons.

$$\frac{I(\theta)}{I_0} = \frac{8\pi^4\alpha^2(1+\cos^2\theta)}{\lambda^4 r^2}$$

Light scattering by a small molecule

↓

$$\frac{I(\theta)}{I_0} = \left(\frac{e^2}{mc^2}\right)^2 \cdot \frac{(1+\cos^2\theta)}{2r^2}$$

X-ray scattering by an electron

X-ray scattering

In a solution of molecules that each has Z electrons, and a molecular weight of M, and concentration of C

$$\frac{I(\theta)}{I_0} = \frac{2\pi^2 n_0^2}{A\lambda^4 r^2}\left(\frac{dn}{dC}\right)^2 CM\ (1+\cos^2\theta)$$

Light scattering

↓

$$\frac{I(\theta)}{I_0} = \frac{1}{2r^2}\left(\frac{e^2}{mc^2}\right)^2\left(\frac{Z-Z_0}{M}\right)^2 AMC(1+\cos^2\theta)$$

X-ray scattering

Z_0: number of solvent electrons in the volume of a solute molecule

$$\frac{I(\theta=0)}{I_0} = \frac{1}{r^2}\left(\frac{e^2}{mc^2}\right)^2\left(\frac{Z-Z_0}{M}\right)^2 AMC$$

X-ray scattering can be used to determine the molecular weight.

Small angle X-ray scattering (SAXS)

The size of the molecules are always larger than $\lambda \sim 0.1$ nm.

$$P(\theta) = \frac{\langle I(\vec{S}) \rangle}{\langle I(0) \rangle} = \frac{1}{N^2} \sum_{j=1}^{N} \sum_{i=1}^{N} \frac{\sin 2\pi S r_{ij}}{2\pi S r_{ij}} \qquad S = \frac{2}{\lambda} \sin \frac{\theta}{2}$$

$Sr_{ij} << 1$ only at very low angle

At very low angle $SR_G << 1$

$$P(\theta) = \frac{\langle I(\theta) \rangle}{\langle I(0) \rangle} = \exp\left(- \frac{4\pi^2 S^2 R_G^2}{3} \right)$$

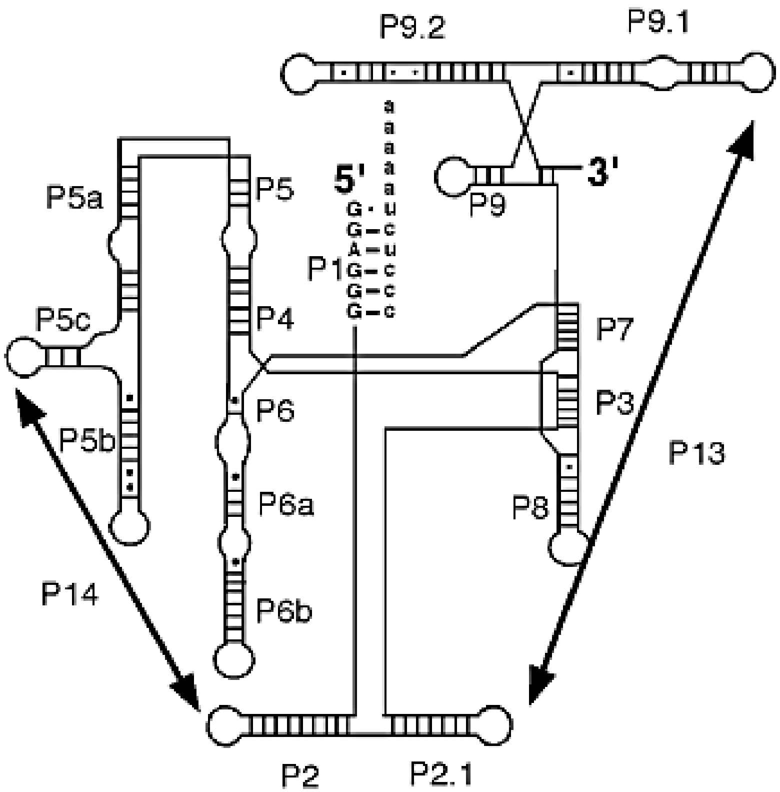
Guinier formula

Molecular weight and size measured by Light scattering or SAXS

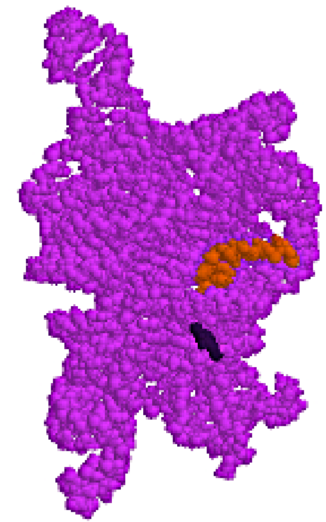
Material	M_w	R_G (nm)
Ribonuclease	*12,700*	*1.48*
α-Lactalbumin	*13,500*	*1.45*
Lysozyme	*13,600*	*1.43*
β-Lactoglobulin	36,000	
	36,700	*2.17*
Serum albumin	70,000	*2.98*
Myosin	493,000	46.8
Turnip yellow mosaic virus		*10.4*
Tobacco mosaic virus	39×10^6	92.4

Values in *italic* are from low angle X-ray scattering.

RNA folding



+ Mg^{2+}

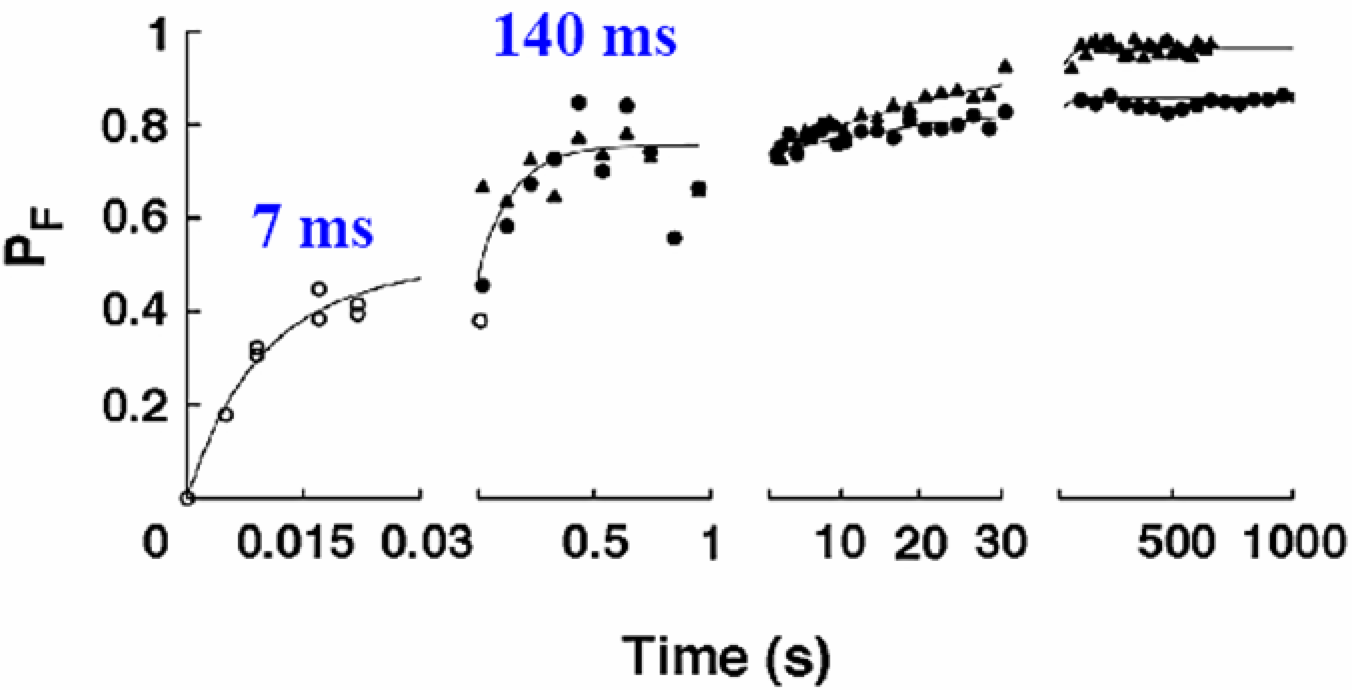


Unfolded state
Secondary structure

Folded state with tertiary structure

SAXS has a potential to answer how fast RNA molecule compacts.

RNA folding studied by SAXS



- Major compaction happen very fast in two phase: 7 ms and 140 ms