Photon Correlation Spectroscopy
"Dynamic Light Scattering"

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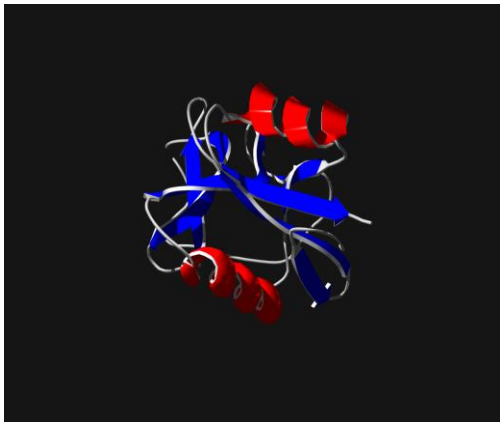
When?

Understanding the macromolecular scale in time for crystal growth phenomena



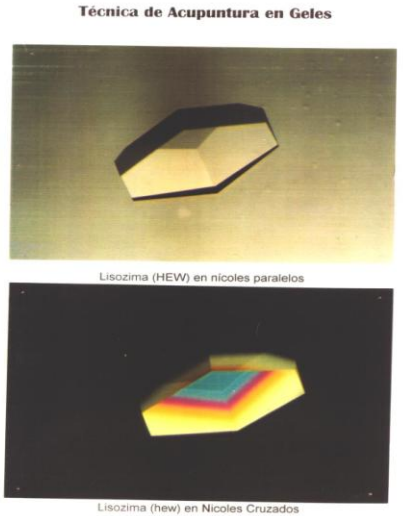
It is typical for crystal growth that many different length scales occur simultaneously: 0.01 ns for molecular conformations, ns for surface structure and defect displacements, μs for surface step displacement, ms for growth on one atomic layer, seconds for hydrodynamic transport, and minutes for homogenous nucleation.

Why are DLS & SLS needed?

For problems of homogeneity, nucleation, protein-protein interactions, precrystallization conditions, and recently to predict the induction time for growing protein crystals.

How?

Growth from solutions is usually predicted by hydrodynamic properties using dynamic and static light scattering methods.



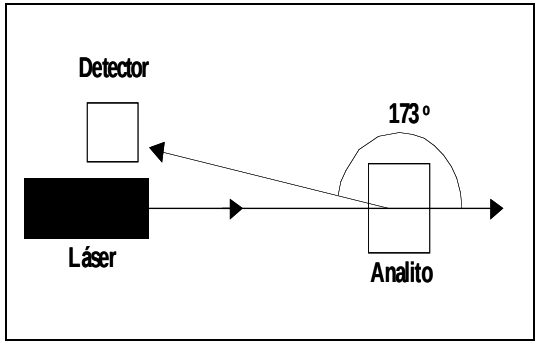
Dynamic Light Scattering

Photon correlation spectroscopy (PCS) also known as dynamic light scattering (DLS) is concerned with the investigation of correlation of photons.

The objective of PCS is to find any peculiar properties of the scattered signal which can be used to characterize and describe the seemingly random “noise” of the signal, and the correlation curve is used to achieve this objective.

What is needed from the experimental viewpoint?

Malvern Nano S



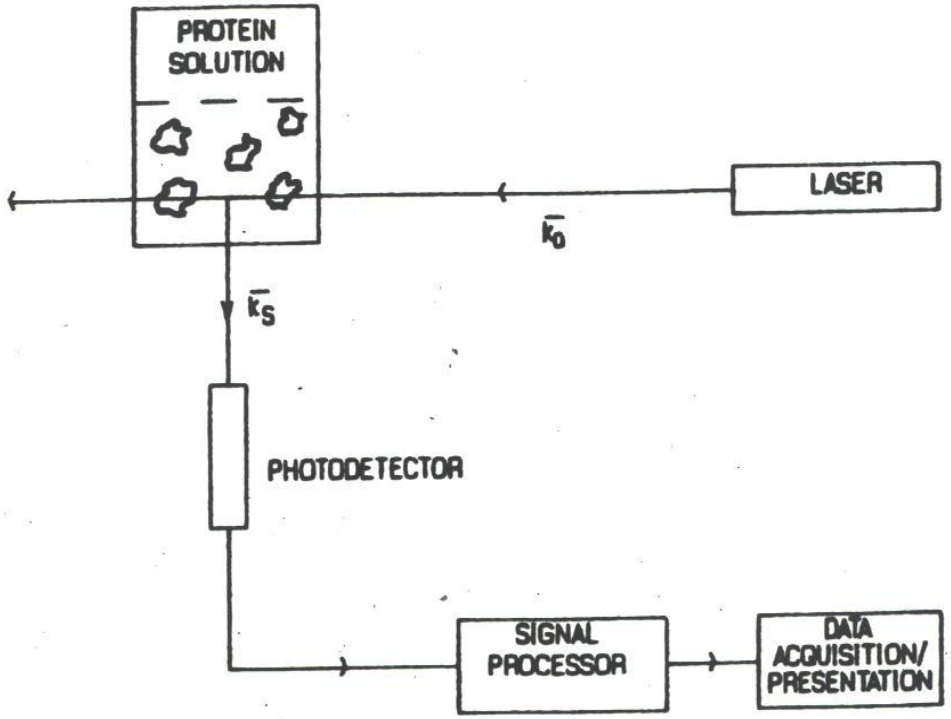


FIG. 1. Schematic of the DLS experiment showing the incident and scattered wave vectors designated as k_o and k_s, respectively. Photoelectric pulses from the detector are usually processed by a digital correlator.

How does DLS work?

In principle, dynamic light scattering (DLS) can be used to study any phenomenon that gives rise to an intensity fluctuation in the light that has been scattered from a scattering source:

a) Protein monomers

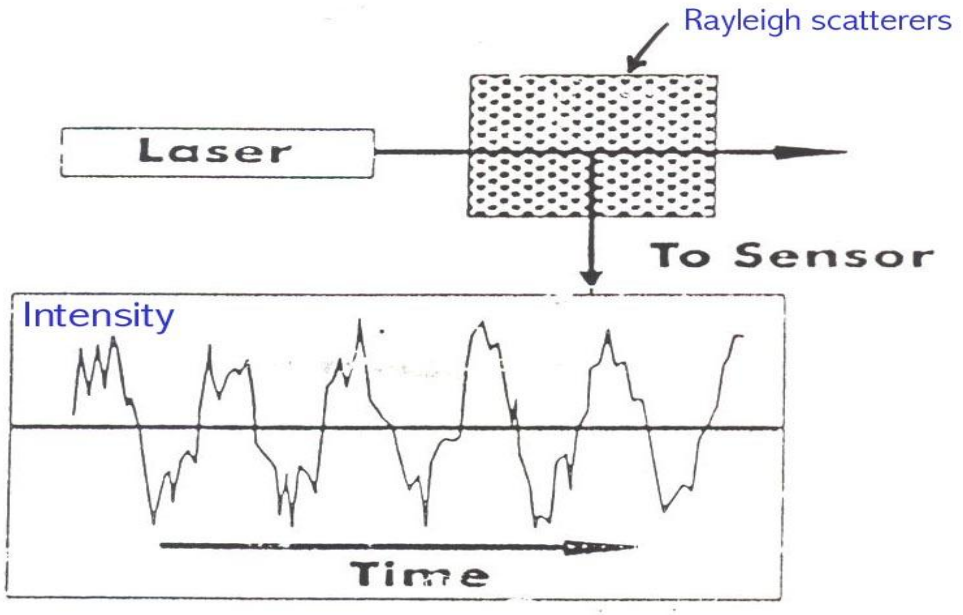
b) Protein aggregates

c) Micro-crystals

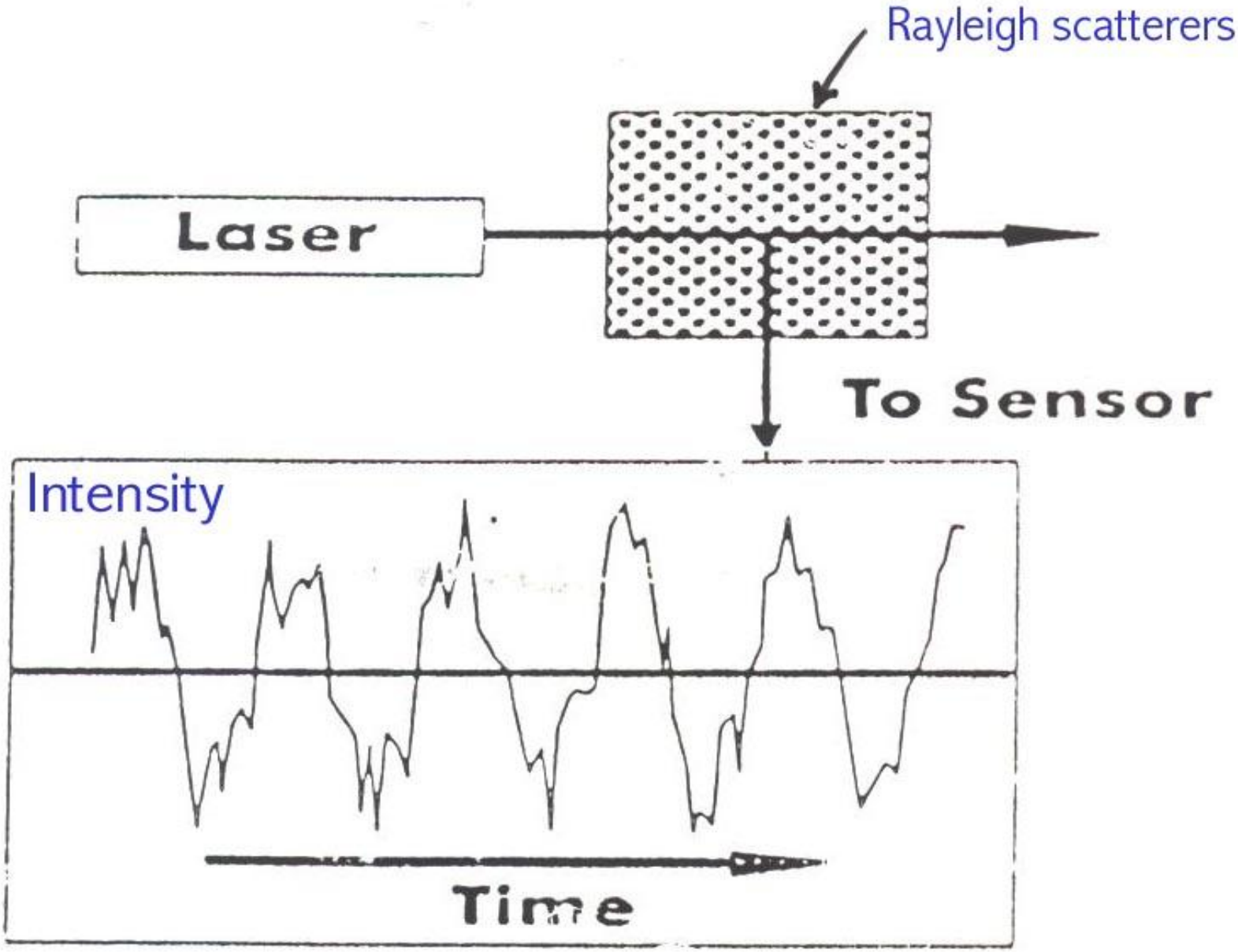
d) Precipitating agents

e) Additives

f) Even dust particles.



Each scattering source will scatter light with a characteristic intensity (**static property**), which we will give the static light scattering data and a characteristic scattered intensity fluctuation (**dynamic property**) giving us the dynamic light scattering data.


Rayleigh scatterers
Laser
To Sensor
Intensity
Time

Light scattering and crystal growth

Using dynamic light scattering to screen and improve protein crystal growth procedures, one does not need to employ overly sophisticated data reduction schemes. From the crystal growth point of view, one would like to be able to:

a) detect the formation of the small protein aggregates which allows nucleation and growth to be monitored,

b) estimate the mean value for the diffusion coefficient and hence the particle size of the protein molecules under various solutions conditions, and

c) characterize aggregates as protocrystals or protoprecipitates

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Crystallizing Proteins – a Rational Approach?

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Abstract

The advances in recombinant DNA technology in recent years have had a dramatic effect on the area of protein crystallization. Large amounts of pure protein produced in various expression systems have made it possible to conduct experiments that would have been impossible with material from natural sources. With many more laboratories becoming involved in crystallizing proteins a great deal of new information has been generated on techniques to eliminate the so called 'bottleneck of crystallization' in determining a three-dimensional protein structure. More and more new and interesting proteins are being submitted to this laboratory for crystallization. Certain criteria may be set before crystallization trials are started, such as solubility, purity and aggregation tendencies. The introduction of robots now facilitates the screening of crystallization conditions. In cases where no crystals have been obtained after initial screening it can now be decided which possible modifications can be made to the protein itself to improve the chances of obtaining crystals.

Table 1. *Light-scattering results*

Proteins measured	Size distribution	Crystals grown
Total 66	→	41 (100%)
	Narrow unimodal = 44	34 (83%)
	Broad unimodal = 10	6 (15%)
	Multimodal = 12	1 (2%)

Strategy for crystallization

Fig. 1 is a flow diagram illustrating the various steps towards crystallizing a protein and the options available to us when problems are encountered. After determining that a protein is soluble, SDS–PAGE (Laemmli, 1971) is used to determine the purity of the protein. We generally consider that a major band on a Coomassie blue stained gel is sufficient for initial screening. This does not exclude the possibility that subsequent purification may be necessary to improve crystal quality. The protein sample is then examined using laser light-scattering

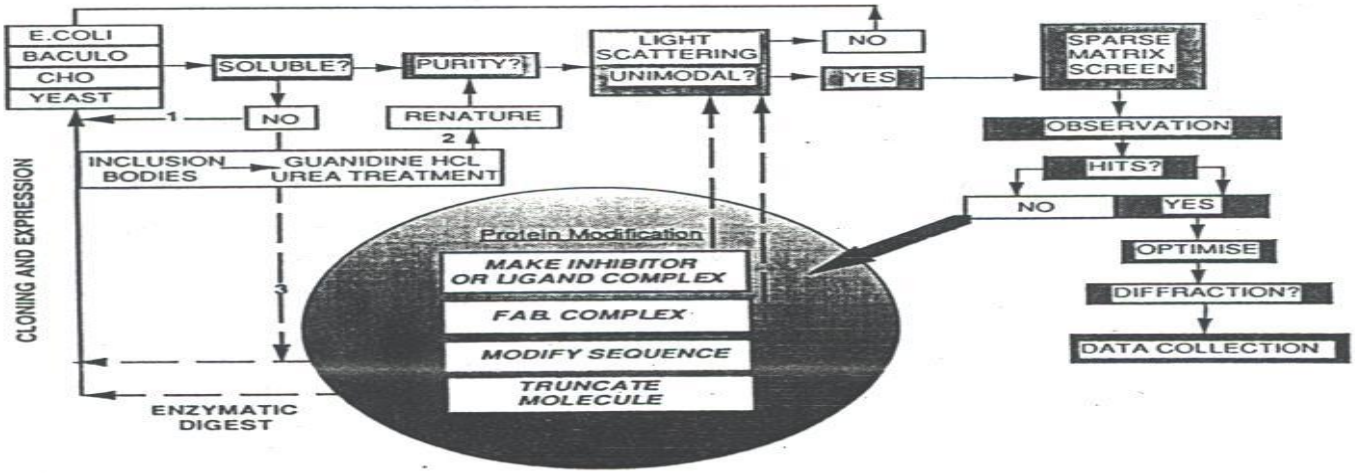


Fig. 1. Crystallization strategy.

Experiment name: BSA STD FILTER 0.20 micras
Date: Feb. 14, 1997
Time: 11:40 AM
Operator: Dr. Abel Moreno Carcamo
Comment 1: BSA 2 mg/ml Pierce No. 23209
Comment 2: Jueves 6 de Marzo de 1997.

#	Time min:sec	Amp.	Diffn Coeff	Radius (nm)	Polyd. (nm)	Est. MW	Temp ½C	Count Rate	Base Line	SOS error	PC ?
1	0:00	0.797	606	3.6	0.961	66k	21.3	105	1.007	1.132	
2	0:28	0.785	605	3.6	0.682	67k	21.6	100	1.001	0.424	
3	1:25	0.796	603	3.6	0.691	68k	21.7	95	1.001	0.423	
4	1:56	0.810	600	3.6	0.656	68k	21.4	92	1.001	0.393	
5	2:28	0.838	602	3.6	0.781	69k	21.7	90	1.000	0.612	
6	3:01	0.865	597	3.7	0.845	71k	21.9	87	1.000	0.718	
7	3:35	0.666	627	3.5	1.252	63k	21.9	92	1.000	2.195	
8	4:08	0.688	600	3.7	0.683	69k	21.8	91	1.000	0.433	

GEL FILTRATION CALIBRATION KIT INSTRUCTION MANUAL

• INTRODUCTION

The use of gel filtration chromatography for the determination of the molecular weight and size of proteins is well documented. The technique is based on the well-established ability of gel filtration media, such as Sephadex®, Sepharose®, and Sephacryl®, to separate molecules according to size. Molecular weight determinations by gel filtration are carried out by comparing some elution volume parameter, such as K_{av} of the substance of interest, with the values obtained for several known calibration standards.

In practice it is found that for a homologous series of compounds a sigmoidal relationship exists between their various elution volume parameters and the logarithm of their molecular weights. A calibration curve is prepared by measuring the elution volumes of several standard substances, calculating their corresponding K_{av} values (or similar parameter), and plotting their K_{av} values versus the logarithm of their molecular weight. The molecular weight of an unknown substance can then be determined from the calibration curve once its K_{av} value is calculated from its measured elution volume. For accurate determination of molecular weight, the calibration standards must have the same relationship between molecular weight and molecular size as the substance of interest. Pharmacia Calibration Kits provide highly purified, well-characterized, globular protein standards for protein molecular weight determination.

• CONTENTS OF CALIBRATION KITS

Low Molecular Weight Gel Filtration Calibration Kit

Protein	Molecular Weight	Stokes' Radius (Å)	Source
ribonuclease A	13,700	16.4	bovine pancreas
chymotrypsinogen A	25,000	20.9	bovine pancreas
ovalbumin	43,000	30.5	hen egg
albumin →	67,000	35.5	bovine serum
Blue Dextran 2000			

High Molecular Weight Gel Filtration Calibration Kit

Protein	Molecular Weight	Stokes' Radius (Å)	Source
aldolase *	158,000	48.1	rabbit muscle
catalase *	232,000	52.2	bovine liver
ferritin *	440,000	61.0	horse spleen
thyroglobulin	669,000	85.0	bovine thyroid
Blue Dextran 2000			

Each kit contains 50 mg of each protein and 50 mg of Blue Dextran 2000.

* These proteins are supplied mixed with sucrose or mannitol to maintain stability and aid in their solubilization. The percent of protein in each vial is indicated on

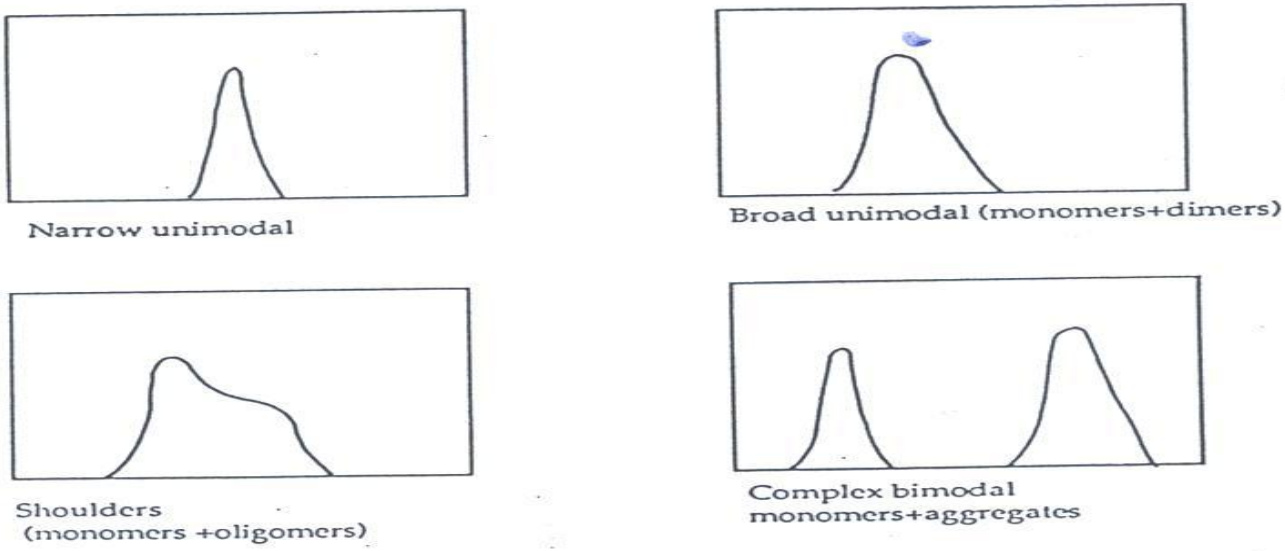


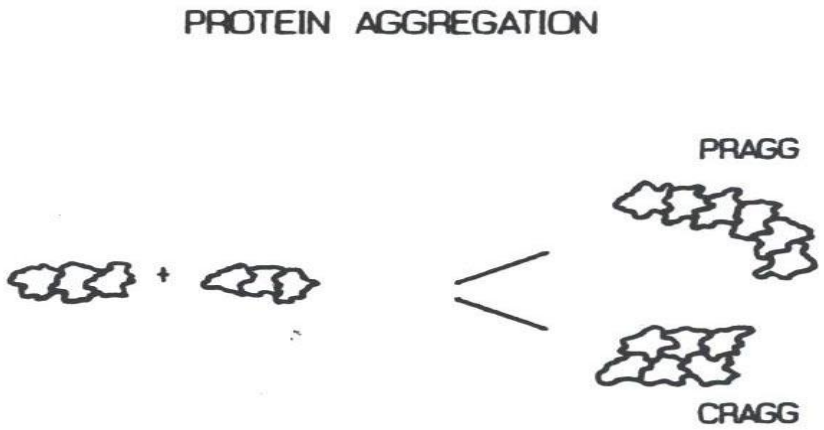
Fig. 1. Plot of $F(R)$ versus R.

Table 1
Results

Protein	MW (kdalton)	Predicted R_h (Å) mono/dimer	R_h (Å) Measured	Distribution	Comment
C. Freundii β-lactamase	29	22.4/23.8	22 ± 2	Narrow unimodal	Crystals
E. coli β-lactamase	29	22.4/28.3	24 ± 1	Narrow unimodal	Crystals
E. coli β-lactamase mutant	29	22.4/28.3	23 ± 2	Narrow unimodal	Crystals
Thrombin-ppack-hirudin	45	26.0/32.7	28 ± 1	Narrow unimodal	Crystals
iiuman pancreatic iipase	50	26.9/33.9	26 ± 2	Narrow unimodal	Crystals
Gamma interferon	18	19.1/24/1	22 ± 2	Narrow unimodal	Crystals
Platelet derived growth factor	28	22.2/27.9	24 ± 3	Narrow unimodal	Crystals
TNF-β	18.6	19.3/24.4	27 ± 4	Broad unimodal	Crystals
RV-endonuclease	28	22.2/27.9	26 ± 2	Narrow unimodal	Crystals
Dihydrofolate reductase *P. Carini*	24	21.0/26.5	20.0 ± 2	Narrow unimodal	Crystals
Catechol O methyl transferase	23	20.8/26.2	175 650	Bimodal	No crystals
Acetylcholinesterase	54	27.6/34.8	51 240	Bimodal	No crystals
HIV reverse transcriptase	66	29.5/37.2	38.0 ± 6	Broad unimodal	No crystals
Calcium binding protein	32	23.2/29.2	23 420	Bimodal	No crystals
Carboxyl ester lipase	100	33.9/42.7	57 ± 10 470 ± 100 860 ± 100	Trimodal	No crystals

What kind of solid phase is obtained?

Early in the evolution of a supersaturated solution of macromolecules, monomers assemble to form aggregates, which lead either to crystals or precipitates. In an obvious terminology it is convenient to refer to the former as CRAGGS (crystalline aggregates) and the latter as PRAGGS (precipitating aggregates).



DLS & SLS for PROTEINS

The reported uses of DLS & SLS for the specific purpose of studying crystallizing protein solutions have generally been for:

a) Characterize of the thermodynamic properties of the solutions where aggregates are forming

b) Monitoring the nucleation kinetics for control purpose.

Reference:

W.W. Wilson. Methods: A companion to methods in enzymology, 1, No.1 (1990) 110-117.

The electric field due to scattering of a single particle is

$$E_j(t) = A_j(t)e^{-iw_o t}e^{i\phi_j(t)}. \quad [1]$$

In close analogy with the equations describing X-ray diffraction, the amplitude of the electric field is given by $A_j(t)$, and $\Phi_j(t)$ is called the phase factor, which relates the optical path length difference for light scattered by particle (j) located at position r_j and another particle located at the origin. The phase factor and the position vector $r_j(t)$ denoting the instantaneous location of particle (j) are related by

$$\phi_j(t) = \mathbf{q} \cdot \mathbf{r}_j(t). \quad [2]$$

In Eq. [2], $\mathbf{q}$ is called the scattering vector and is defined as

$$\mathbf{q} = \mathbf{k}_o - \mathbf{k}_s. \quad [3]$$

The magnitude of the scattering vector is given by

$$\mathbf{q} = \frac{4\pi n}{\lambda_o} \sin\frac{\theta}{2}, \quad [4]$$

The total scattered electric field is given by the summation of eq (1) over all the the protein scatterers:

$$E_s(t) = \sum_j A_j(t) e^{-i w_o t} e^{i \mathbf{q} \cdot \mathbf{r}_j(t)}. \qquad [5]$$

Equation [5] clearly shows that the time dependence of $E_s(t)$ arises from the $\mathbf{q} \cdot \mathbf{r}_j(t)$ term, which reflects random motion of the protein molecules due to translational and rotational diffusion. One type of mathematical function that is often utilized to describe the time-dependent features of a random physical process is called a correlation function. The scattered electric field correlation function is defined as

$$\mathbf{G}^1(\tau) = \langle E_s(t) E_s^*(t + \tau) \rangle \qquad [6]$$

where * denotes complex conjugate, $\langle \quad \rangle$ indicates a time or ensemble average, and τ is called the delay time. The normalized form of the field correlation function is given by

$$g^1(\tau) = G^1(\tau)/G^1(0). \qquad [7]$$

The number of photoelectric pulses at the detector output is proportional to the intensity of the scattered light given by

$$I_s = |E_s|^2, \qquad [8]$$

so that the scattered intensity correlation function is written as

$$G^{(2)}(\tau) = \langle I_s(t) I_s(t+\tau) \rangle. \qquad [9]$$

For the type of problem considered here, the scattered electric field is a stationary Gaussian random process so that $G^2(\tau)$ is related to $g^1(\tau)$ by the Siegert relation

$$G^2(\tau) = \langle I \rangle [1 + |g^1(\tau)|^2]. \qquad [10]$$

The form of the experimentally measured intensity correlation function follows closely that represented by Eq. [10] and is most often written as

$$C(\tau) = B[1 + a|g^1(\tau)|^2], \qquad [11]$$

where B is the background and a is a constant depending on spatial coherence of the detected scattered light.

For the case in which the scatterers are a collection of identical globular protein molecules undergoing Brownian motion, $g^1(\tau)$ becomes

$$|g^1(\tau)| = e^{-\Gamma\tau}, \qquad [12]$$

where $\Gamma = Dq^2$ and D is the translational diffusion coefficient of the protein molecule. The experimentally measured intensity correlation function follows from Eq. [11] and takes the form of an exponential decay as a function of the delay time, τ. A large value for Γ and D corresponds to small particle size and causes the correlation function to decay rapidly, whereas a small value for Γ indicates larger particles and results in a slowly decaying function.

$$C(\tau) = B[1 + ae^{-2\Gamma\tau}] \qquad [13]$$

The usual way of obtaining D from $C(\tau)$ is by linear regression analysis on

$$\ln\left[\frac{C(\tau) - B}{B}\right] = \ln a - 2\Gamma\tau. \qquad [14]$$

The slope of the line represented by Eq. [14] is -2Γ and since q can be calculated from Eq. [4], D can be directly determined. Globular proteins are often assumed to be spherical in shape so that the Stokes–Einstein relation can be used to estimate the apparent hydrodynamic radius, $r_h(\text{app})$ of the protein by

$$r_h(\text{app}) = \frac{kT}{6\pi\eta D}, \qquad [15]$$

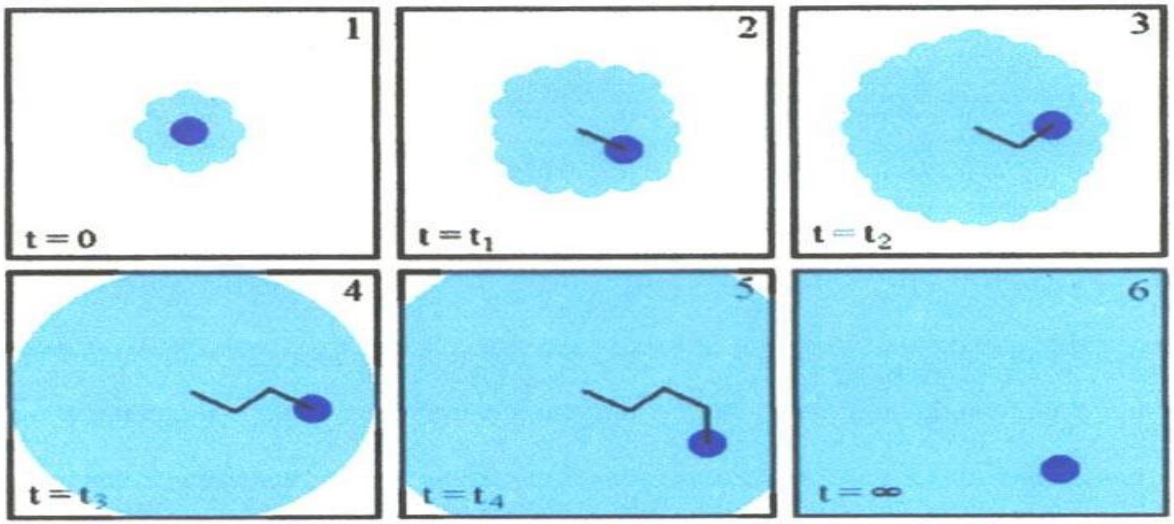
Polydispersity Effects

Since the intent is to use DLS as a diagnostic for precrystallization of proteins, the solutions studied will at some point during the growth process contain protein aggregates with correspondingly smaller translational diffusion coefficients along with protein monomers. For this case, the expression for $|g^1(\tau)|$ in Eq. [12] must be modified to account for the distribution of different species,

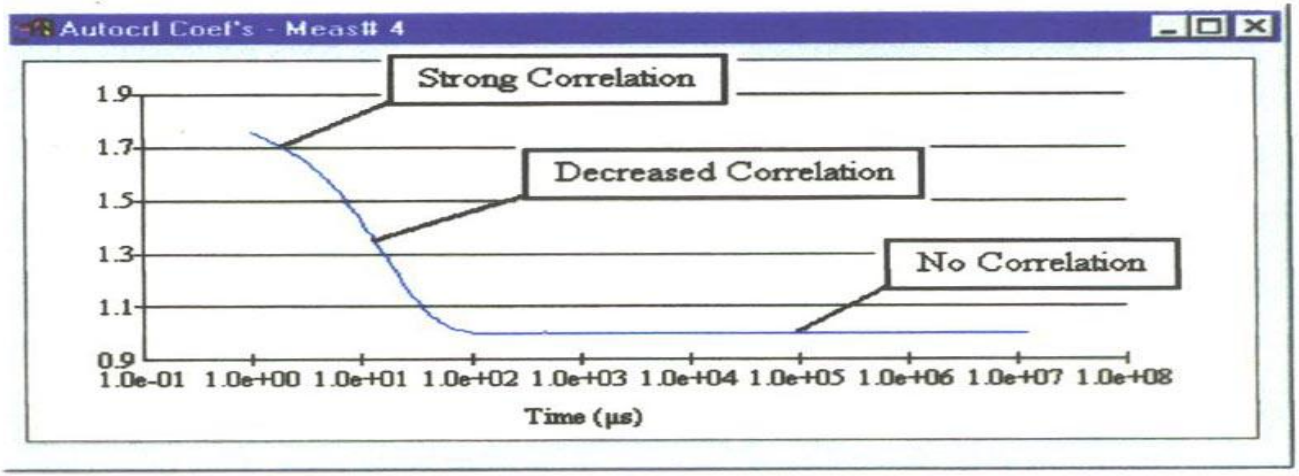
$$|g^1(\tau)| = \int_0^\infty f(\Gamma)e^{-\Gamma\tau}d\Gamma, \qquad [16]$$

where $f(\Gamma)d\Gamma$ is the fraction of the total light intensity scattered by protein aggregates having Γ in the range Γ to $\Gamma + d\Gamma$. The distribution of Γ's leads directly to the distribution of D's, so the problem then is to devise a method to extract the distribution $f(\Gamma)$ from the experimentally measured correlation function, $C(\tau)$. This particular problem in the field of light scattering has been vigorously attacked in several ways, including the

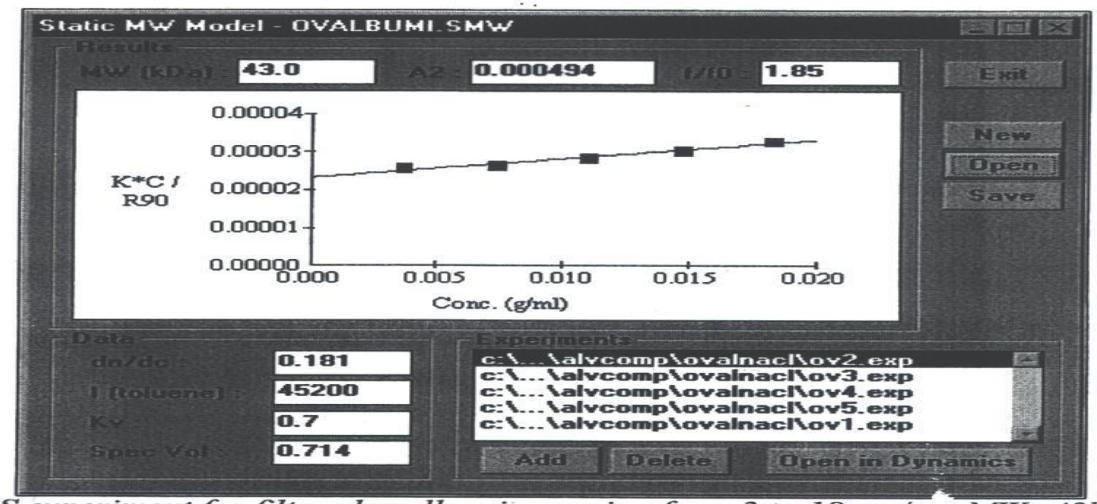
- Method of moments or cumulants
- Method of splines
- Laplace inversion
- Parametrised distributions
- Multiexponential and histogram methods.



The correlation in particle position at small shift times is contained within the measured intensity correlation function, an example of which is shown below. In the absence of any applied forces, the particle position is dictated by the degree of Brownian motion. As such, the measured intensity correlation curve is an indirect measure of the particle's diffusion coefficient.



Measuring Average MW with 90° Total Intensity Light Scattering (TILS)



TILS experiment for filtered ovalbumin ranging from 3 to 18 mg/m. MW=43kDa.

Parameters required for measurement and derivation of the molecular weight

$$K = K_v \left(\frac{dn}{dc}\right)^2$$

$$K_v = \frac{4\pi^2 n^2}{\lambda^2 N_A R_\theta}$$

where,

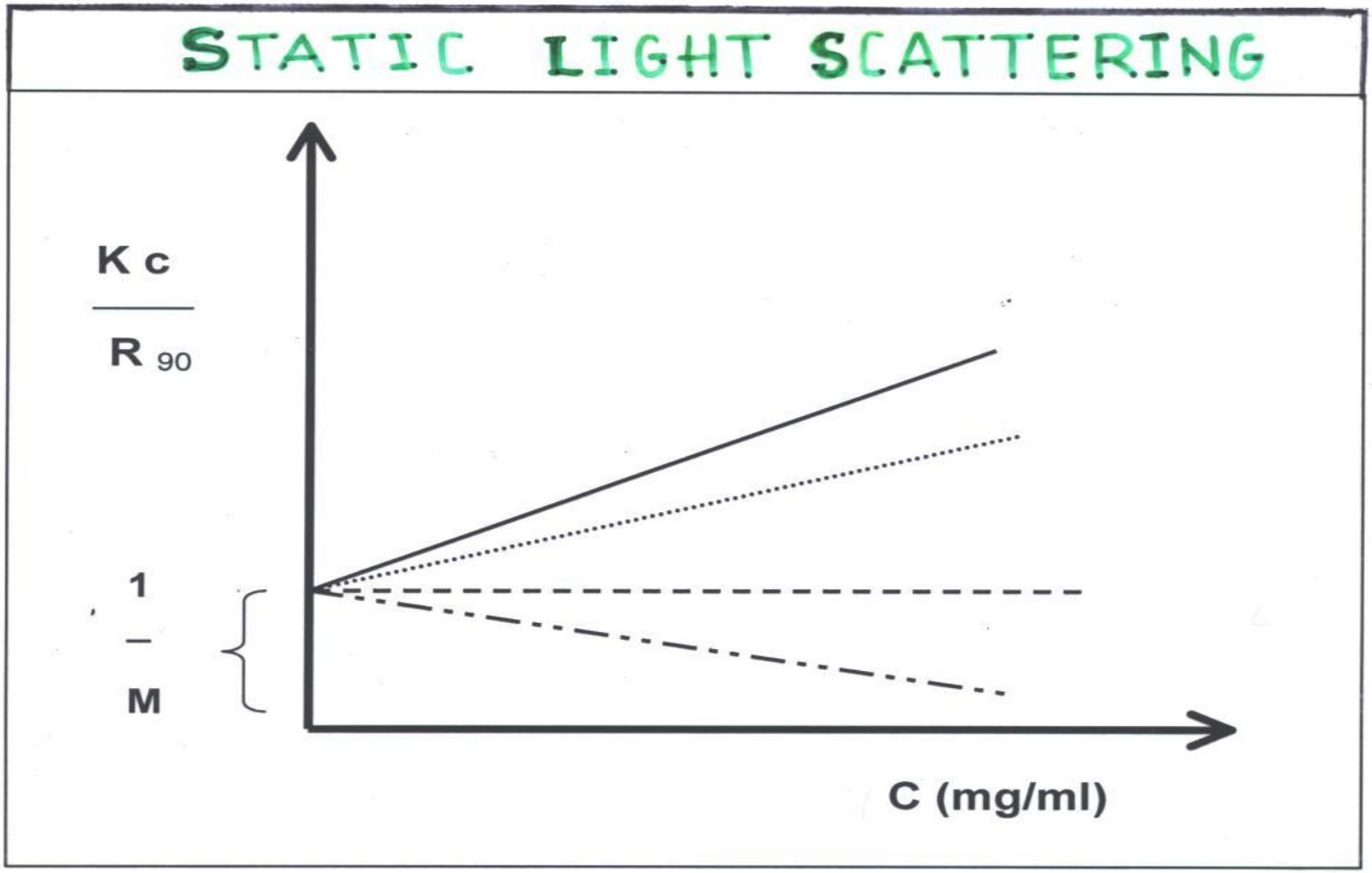
1.- K_v is an instrument constant.
2.- n is the index of refraction
3.- λ is λ of the laser
4.- N_A Avogadro's constant
5.- R_θ Rayleigh ratio for toluene.

Note:

$\frac{dn}{dc}$ is the refractive index increment for the particular sample/solvent system.

$$R_\theta = \frac{(\text{solution photon count rate} - \text{solvent photon count rate})}{\text{toluene count rate}}$$

STATIC LIGHT SCATTERING



$$\frac{Kc}{R_{90}} = \frac{1}{M} + 2B_2c$$

K is an instrument constant
C is the concentration of the solute.
R_θ is the Rayleigh ratio
B_2 is the second virial coefficient

Static Light Scattering

Kratochvil equation:

$$K^*c / R_\theta = 1 / MW + A_2 * c$$

1. K is an instrument constant
2. c is the concentration of the solute
3. R_θ is the Rayleigh ratio and describes the absolute intensity scattered at 90° in excess of the pure solvent.
4. MW is the molecular weight
5. A_2 is the second virial coefficient (a thermodynamic parameter).

Parameters required for measurement and derivation of the MW

$$K = K_V (dn/dc)^2$$

1. K_V is an instrument constant given by $(4 \Pi^2 * n^2) / \lambda^2 * N_A * R_\theta$

   with n = index of refraction, λ of the laser, N_A Avogadro's constant and R_θ Rayleigh ratio for toluene.

2. dn/dc is the refractive index increment for the particular sample/solvent system. This value is measured with a differential refractometer. For proteins and protein complexes that contain no carbohydrates, the dn/dc value is nearly constant (~ 0.186 ml/g).

c = concentration of protein, polymer, or other solute solution.

Note: the concentration of the solute can be determined from weight of dry solute in known volume of solvent, or also determined by UV-VIS spectrophotometer.

R_θ = (solution photon count rate - solvent photon count rate) / Toluene count rate.

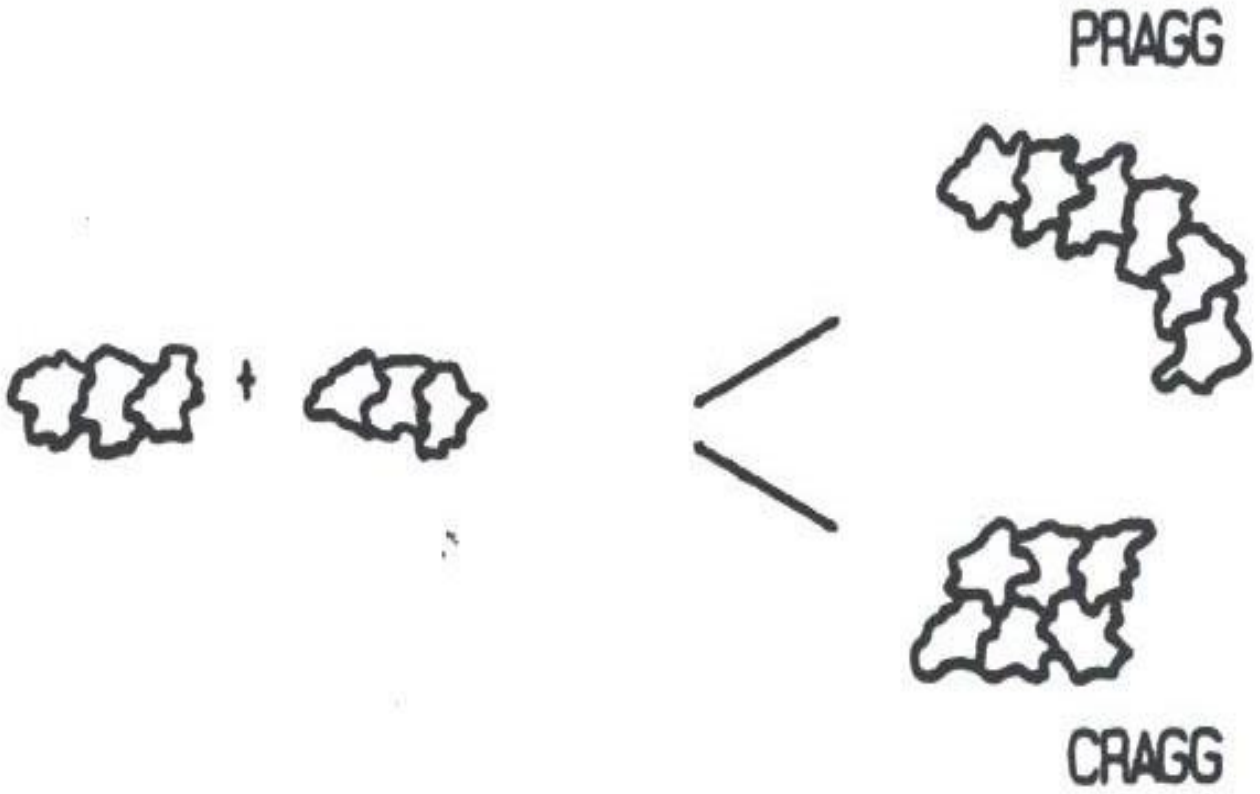
Note: the solvent count rate is the total scattering intensity of the pure solvent in which the protein, polymer, or other solute is contained. This term is also referred to as a 'base line' or the contribution of the solvent to the total intensity.

The expression used to describe the scattering of a dilute solution of particles is shown below, where K is an optical constant, "c" is the particle concentration, R_θ is the Rayleigh ratio of scattered to incident light intensity, M is the weight average molecular weight, A_2 is the second virial coefficient, R_g is the radius of gyration, λ is the vacuum wavelength of the incident radiation, and θ is the scattering angle.

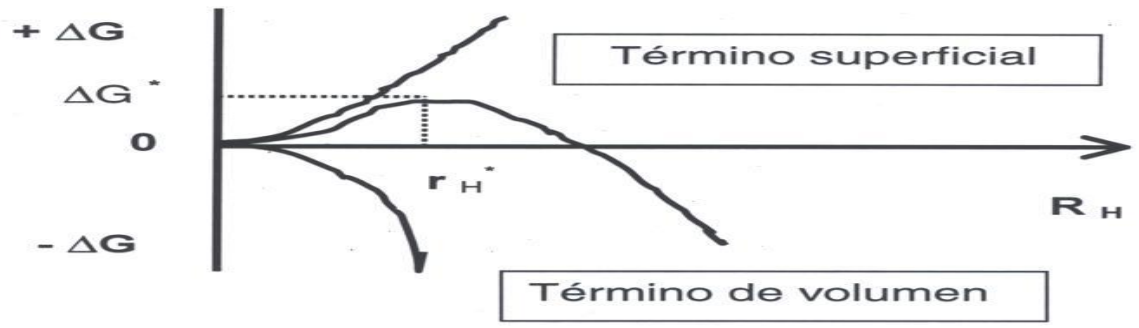
$$Kc/R_\theta = (1/M + 2A_2c) [1 + (16\pi^2R_g / 3\lambda^2) \sin^2 (\theta / 2)]$$

The angular dependence portion of the second term arises from the interference effects due to multiple scattering from a single particle. For particles much smaller than the wavelength of the incident radiation, this term goes to zero, and the angular dependence data is redundant. That is why the total intensity light scattering method is useful for static light scattering calculations using a single angle (90°). On the other hand, static light scattering measurements are based on Kratochvil's equation, which permits to obtain the molecular weight plotting Kc/R_θ versus concentration of protein in static mode [18].

PROTEIN AGGREGATION



Termodinámica



Sabemos que:

$$\Delta G = \Delta H - T\,\Delta S \qquad \ldots\ldots\ldots \quad (1)$$

Actualmente se ha establecido que la ecuación que permite determinar el calor de cristalización esta dado por la siguiente expresió:

$$\Delta H_{cryst} = - \frac{R\ ln\ (S_2 / S_1)}{(1/T_1) - (1/T_2)} \qquad \ldots\ldots\ldots\ldots \quad (2)$$

Los valores que se han encontrado para la cristalización de proteínas están en el intervalo comprendido entre:

-7800 cal/mol a 18000 cal/mol

También es posible calcular la entropía basado en la dependencia con la temperatura y teniendo la curva de solubilidad:

$$\Delta S = - \frac{\Delta\mu_A\ (pH, C, T_2) - \Delta\mu_A\ (pH, C, T_1)}{T_2 - T_1} \qquad \ldots\ (3)$$

También: $\Delta\mu = KT\ ln\ \beta$, donde: β = sobresaturación = $C / C_{equilibrium}$

Reference: Sandra B. Howard et al ***J. Cryst. Growth 90 (1988) 94-104***

El calor ganado o perdido sobre la transferencia de una molécula de A, a un pH constante y una concentració dada es:

$$\Delta H_A = -\{(T_1 + T_2)/2\}^2 \left[\frac{\Delta\mu_A(pH,C,T_2)}{T_2} - \frac{\Delta\mu_A(pH,C,T_1)}{T_1}\right]\left[1/(T_2 - T_1)\right]$$

Algunos datos acerca de los ΔH_{cryst} para lisozima encontrados experimentalmente tenemos: (las medidas fueron hechas a pH 4.2, 0.5 M NaCl a 25 °C y 35 °C).

$\Delta H_{cryst} = -105 \pm 21$ kJ / mol FASE TETRAGONAL

$\Delta H_{cryst} = 54 \pm 13$ kJ / mol FASE ORTORROMBICA

Empleando la ecuación anterior y empleando las diferencias de temperatura de 15 a 20 °C y entonces de 25 a 35 °C Howard et al., calcularon:

$\Delta H_{cryst} = -79$ kJ / mol FASE TETRAGONAL

$\Delta H_{cryst} = 22$ kJ / mol FASE ORTORROMBICA

Esta diferencia sustancial probablemente está relacionada al tipo de contribución de cada molécula de lisozima al formar el empaquetamiento del cristal. Existe actualmente una controversia acerca de la formación de cristales, no se ha explicado en detalle si la unión de macromoléculas procede a través de monómeros o dímeros.