Biotech and Nanotech

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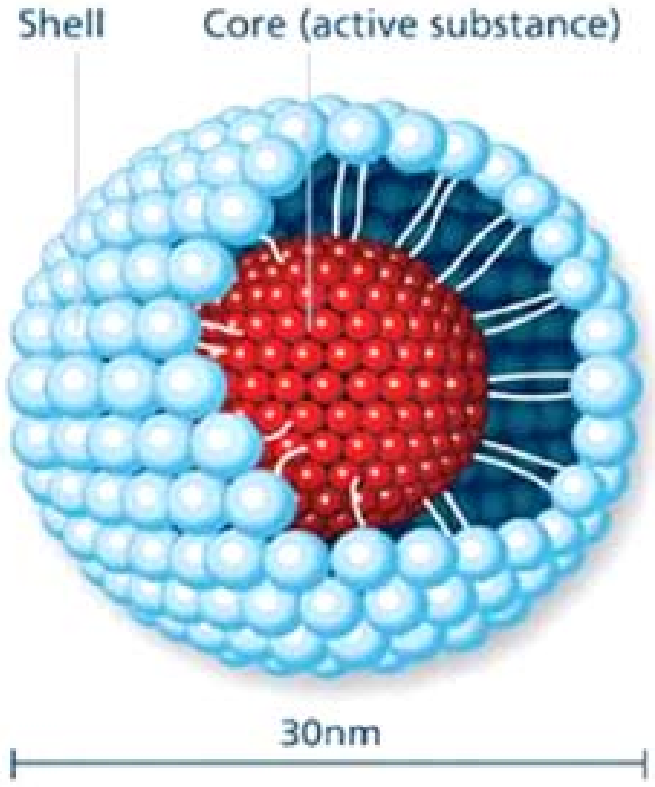
Nanotechnology and Biotech

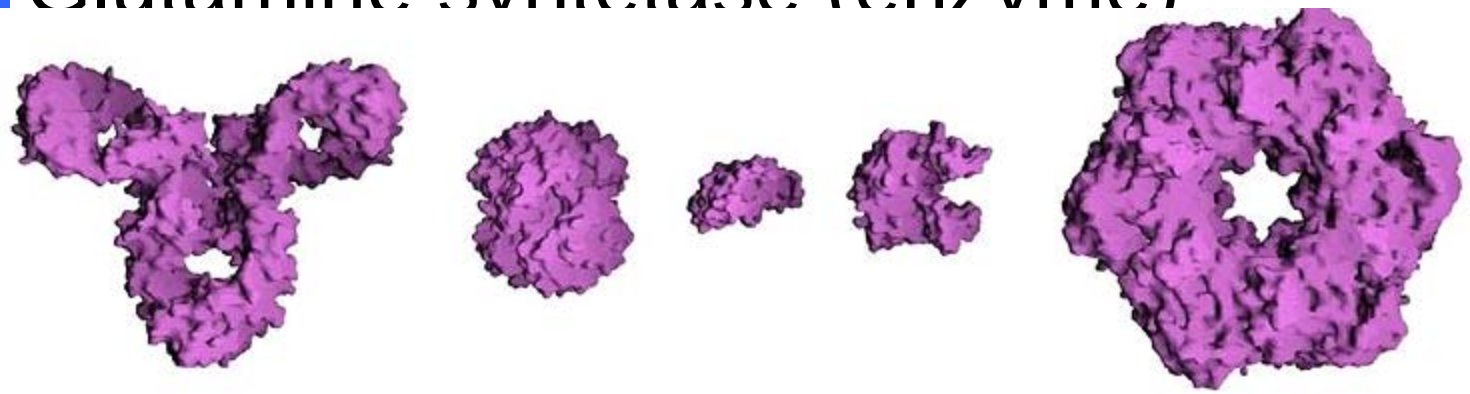
- Micelles
- Liposome
- Proteins
- Gold Nano particles
- DLS
- Zeta Potential



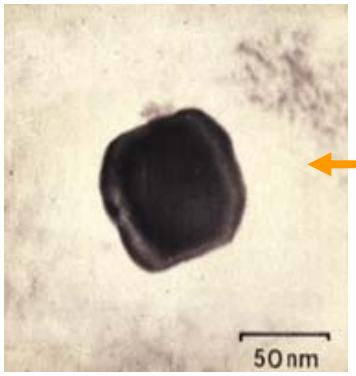
HORIBA

Proteins and Size

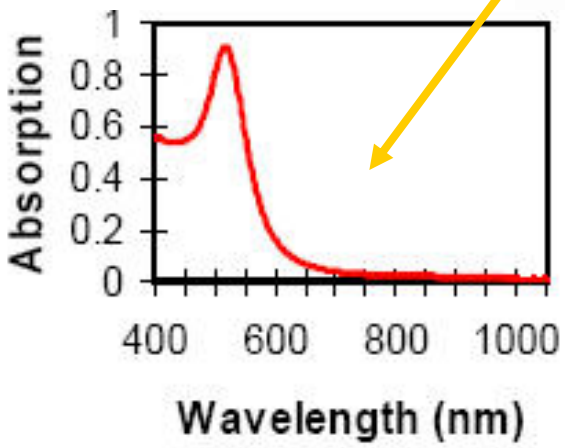
- Immunglobin G (antibody)
- Hemoglobin
- Insulin
- Andeylate kinase (enzyme)
- Glutamine syntetase (enzyme)



Gold Nanoparticles



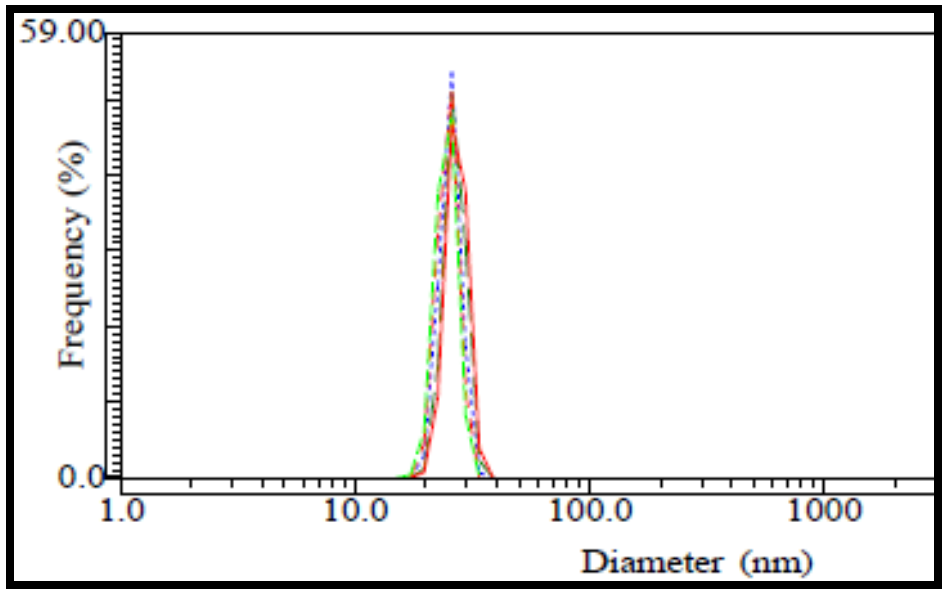
Surface Plasmon Resonance



Gustav Mie, Ann. Physik25, 377 (1908)

Gold Nano-Particles

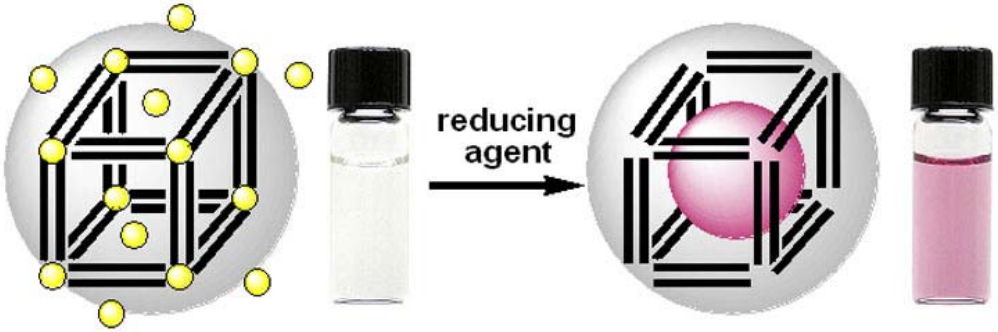
- Useful Biomarker
- Color Changes with Size
- Polymers and Proteins easily bind to gold
- Gold is chemically inert

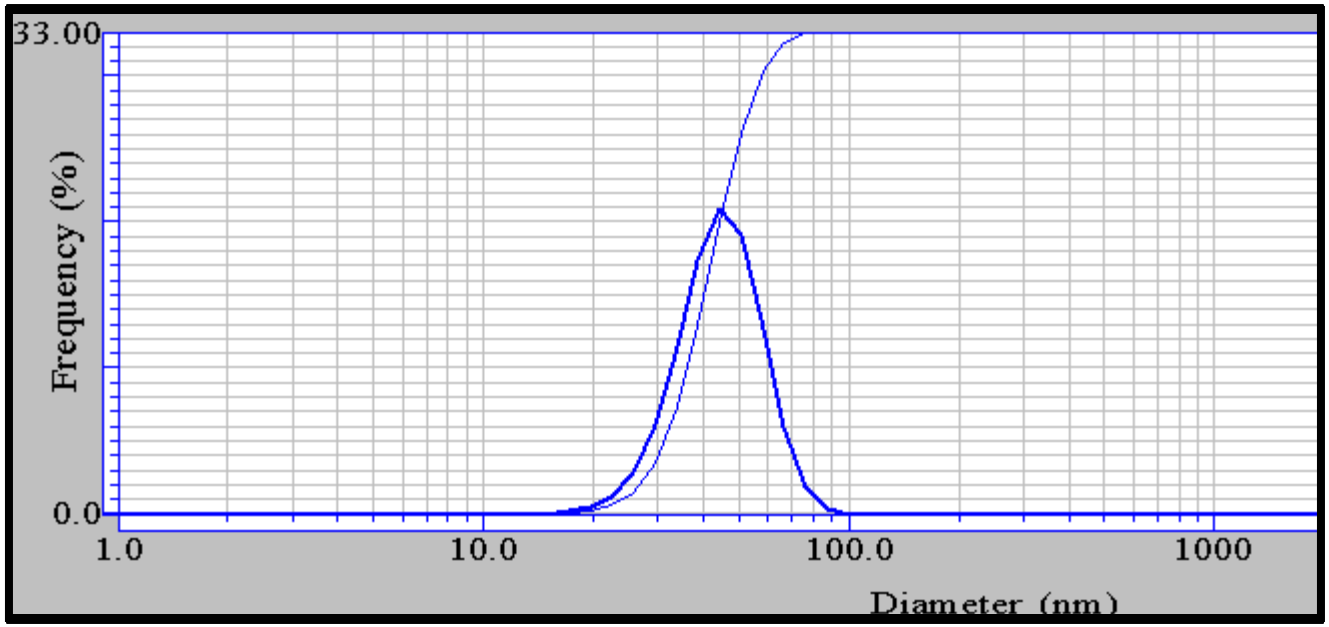


Memory No.	Graph Type	Filename	Mean
Memory 1	-------	23nm-meas visc-vol-01	23.8(nm)
Memory 2	— —	23nm-meas visc-vol-02	24.8(nm)
Memory 3	– – –	23nm-meas visc-vol-03	23.2(nm)
Memory 4	—–—–	23nm-meas visc-vol-04	22.8(nm)
Memory 5	———	23nm-meas visc-vol-05	25.3(nm)

Gold Colloid

- Dilute Gold Colloid
- Varian Sample
- Used in protein screening
- Mie studied gold colloids

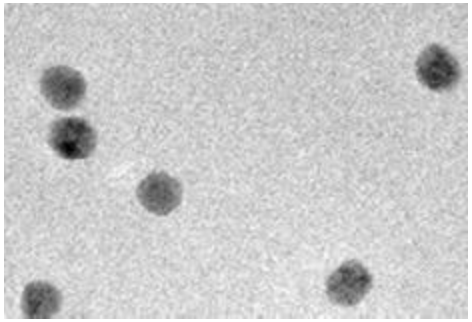


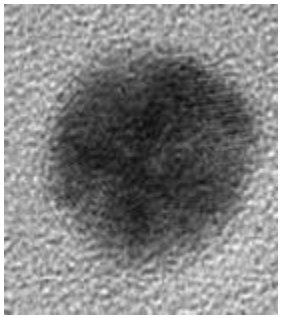


Gold Colloids

8011	Z ave	PDI
Sample 1	9.63 nm	0.079
Sample 2	10.51 nm	0.19

Technique	Size nm
Atomic Force Microscopy	8.5 ± 0.3
Scanning Electron Microscopy	9.9 ± 0.1
Transmission Electron Microscopy	8.9 ± 0.1
Differential Mobility Analysis	11.3 ± 0.1
Dynamic Light Scattering liquid	13.5 ± 0.1
Small-Angle X-ray Scattering	9.1 ± 1.8





SEM (above) and TEM (below) images for RM 8011

Nano-technology and Horiba

Research of materials with dimensions from 1 to 100 nanometers(nm) = Nanoscience

LA-950: 30nm – 3 mm

LB-550 3 nm- 1 µm

DT-1201 3 nm – 300 µm

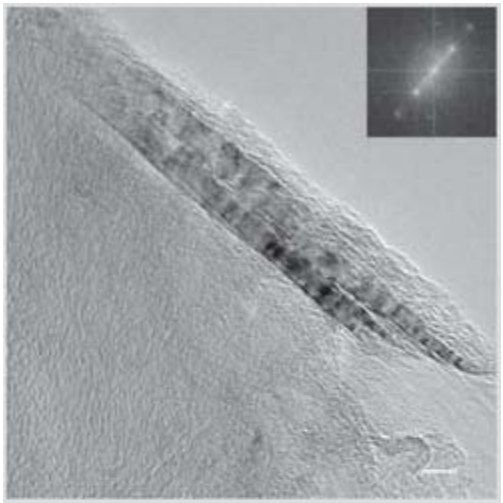
Damascus sabre

- Multiwalled tubes
- Scale bars: 5 nm (**a**) and 10 nm (**b**)
- In **b**, the tubes are bent like a rope.

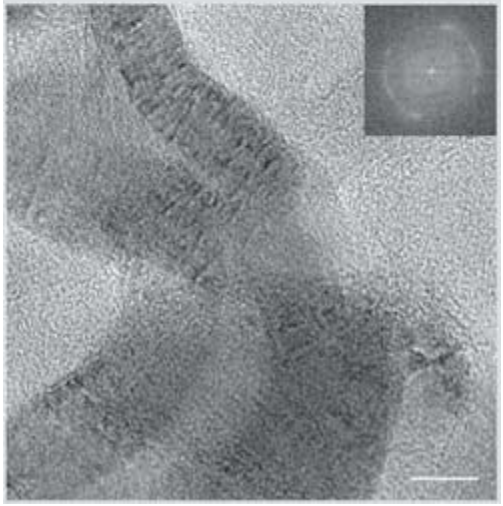
Materials: Carbon nanotubes in an ancient Damascus sabre

Nature 444, 286(16 November 2006)

a



b



Dynamic Light Scattering

- QELS – Quasi Elastic Light Scattering
- PCS – Photon Correlation Spectroscopy
- Light Scattering
  - Incident momochromatic light
  - Light Scattered from moving particles
  - Wavelength shifted scattered light measured at a stationary detector
  - Particle Size is calculated from the information contained in the fluctuating scattered light signal

Benefits of DLS

- Rapid
- Sensitive to aggregates – R^6 scattering dependence
- Non-invasive
- Quantitative

Proper Measurement

- **Large Particles or Dust**
  - The presence of a few large particles or Dust can cause the scattering intensity to fluctuate significantly
  - These fluctuations can make measurements unusable
- **In order to overcome these problems**
  - Introduce sample into the bottom of the cuvett to avoid washing dust off the cuvett walls
  - Do not vortex the partially filled cuvett.
  - Do not wash disposable cuvetts
  - Filtration. The easiest way to remove large impurities from solution is by filtration.
  - Centrifugation is another effective way to remove large impurities from the

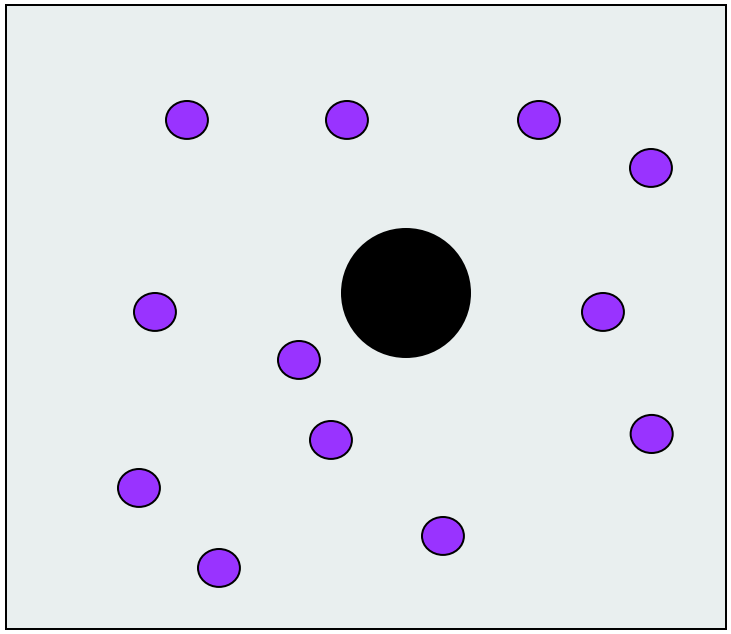
Methods to Measure Size

- There are many methods that can be used to measure size or aggregation state, including
  - Sedimentation equilibrium
  - Size exclusion chromatography
  - Native gel electrophoresis
  - Light scattering
- Light scattering
  - Easiest to implement, the
  - Quickest to perform, and the
  - Least destructive to the sample

Dynamic Light Scattering:

Brownian motion 1 nm – 1 µm

Particles in suspension undergo Brownian motion due to solvent molecule bombardment in random thermal motion.



- Brownian Motion
  - Random
  - Related to Size
  - Related to viscosity
  - Related to temperature

Brownian Motion

HORIBA

Having found motion in the particles of the pollen of all the living plants which I had examined, I was led next to inquire whether this property continued after the death of the plant, and for what length of time it was retained.

In plants, either dried or immersed in spirit for a few days only, the particles of pollen of both kinds were found in motion equally evident with that observed in the living plant; specimens of several plants, some of which had been dried and preserved in an herbarium for upwards of twenty years, and others not less than a century, still exhibited the molecules or smaller spherical particles in considerable numbers, and in evident motion, along with a few of the larger particles, whose motions were much less manifest, and in some cases not observable.[1]

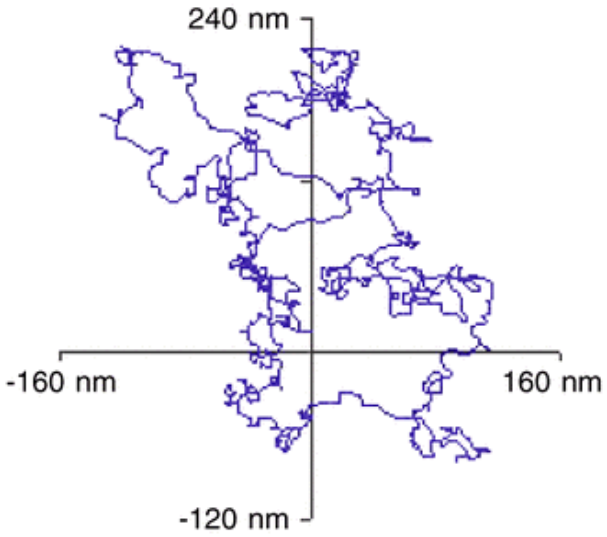


FIGURE 1. The trajectory of a molten lead particle in solid aluminum at 438°C was determined from 1056 video frames.

LB550

- Why should one consider Dynamic Light Scattering?

- Non-invasive measurement
- Can Measure Low quantities of material
- Can Measure Concentrated Samples
- Good for detecting trace amounts of aggregate
- Good technique for macro-molecular sizing

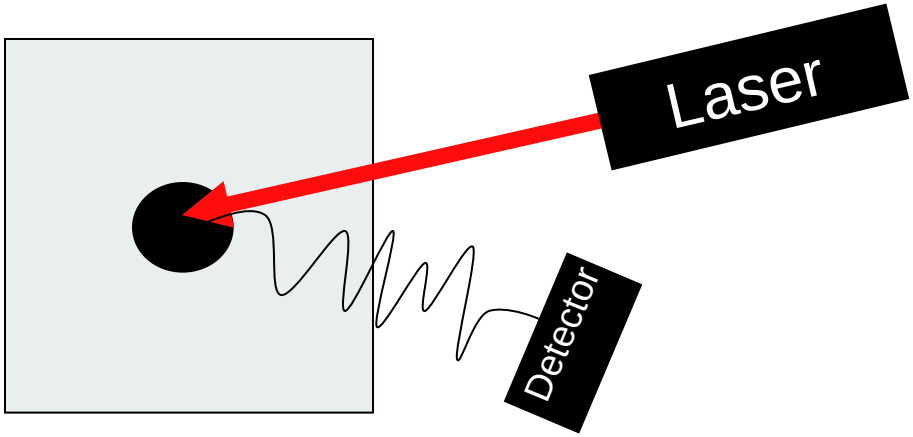
Cost of Materials

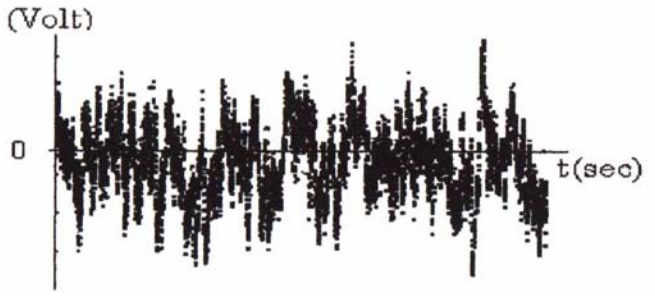
Aredia®	cost/30mg	cost/mg	cost/kg
Powder and solvent for solution for infusion	222.11	7.40	$ 7,403,667
Composition and pharmaceutical forms			
Active ingredient: disodium 3-amino-1-hydroxypropylidene-1, 1-bisphosphonate pentahydrate	Bulk Cost of Materials used to make 1kg		
(pamidronate disodium).	Aredia	$50	
One vial contains 30 mg or 90 mg of sterile, lyophilised pamidronate disodium. Vials are supplied with			
solvent ampoules. One solvent ampoule contains 10 mL of sterile water for injection.			
Cocaine US value per/kg	$135,000		
Polished 1 karat diamonds value/kg	$80,000,000		
Kopi Luwaki Coffee (most expensive coffee in the world) value/kg	$1,000		

- Must characterize using small quantities
- DLS useful here
- Final product cost drives analysis tool

Diffusion

- Particle is randomly diffusing
  - Larger particles will diffuse more slowly
  - Larger particles have more Inertia
- Scatter light off this diffusing particle
- Measure the Frequency Shift of the signal



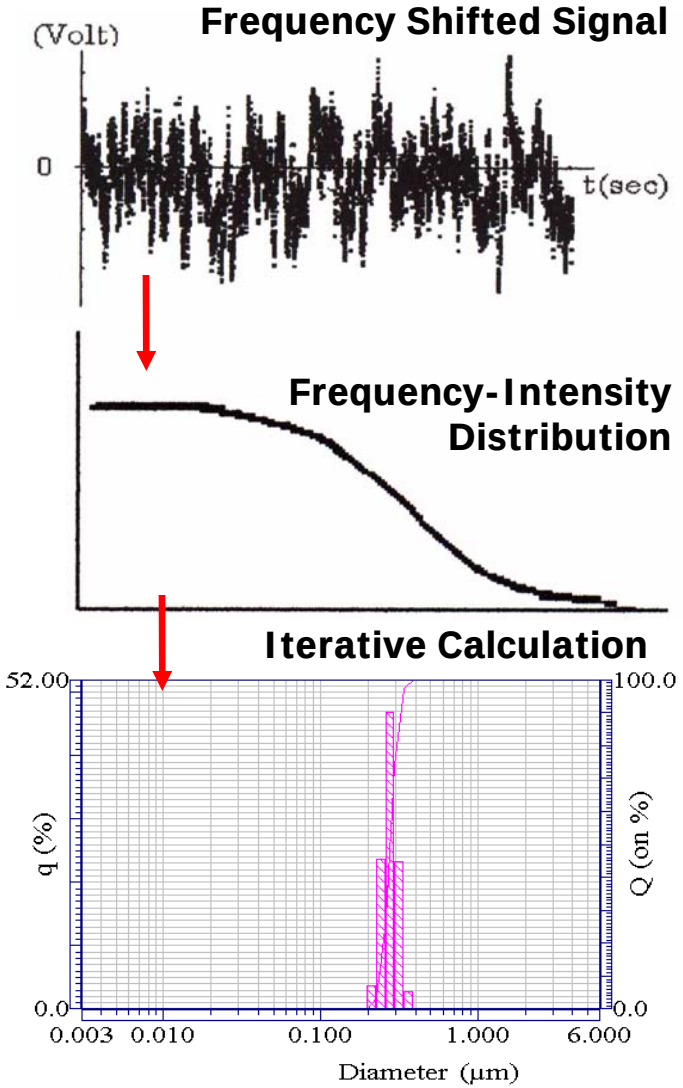


Frequency Shifted Signal

Dynamic Light Scattering

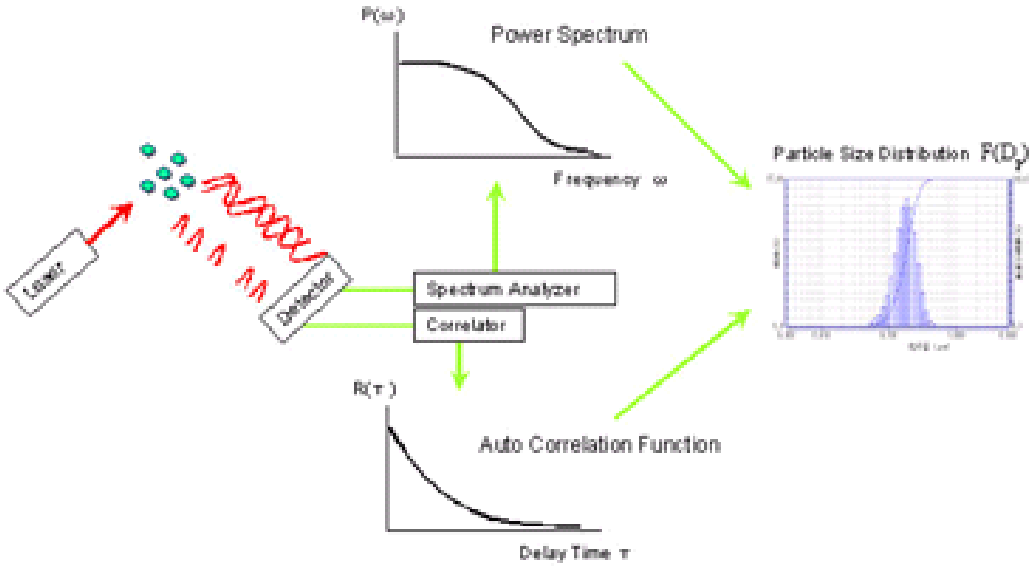
- Measured frequency-intensity distribution (**power spectrum**)
- Power spectrum takes form of Lorentz distribution, whose half-value width can be expressed as **$2Dq^2$**
- All parameters in the half-width are known or measured
- The Diffusion Coefficient D is related to the Particle Size

Stokes-Einstein $$R_H = \frac{kT}{6\pi\eta D}$$



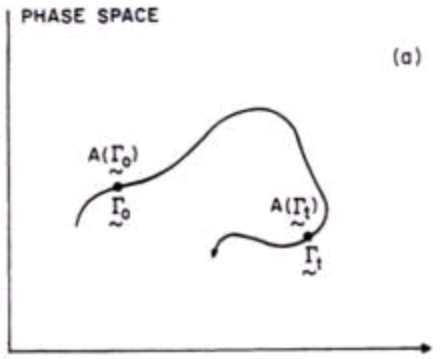
DLS Spectrum Analyzer

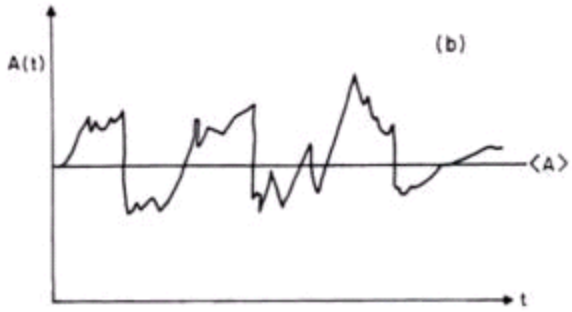
- Spectrum Analyzer
  - Operates in the frequency domain of scattered light
- Auto Correlation Function
  - Operates in the time domain of scattered light

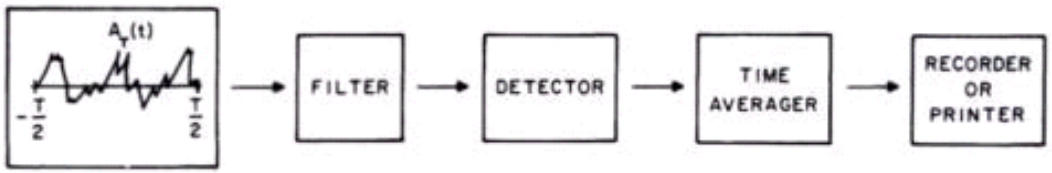


Spectrum Analyzer

- Trajectory of a particle in phase space
- Variation of the particles position with time
- Spectrum analysis of fluctuating variable

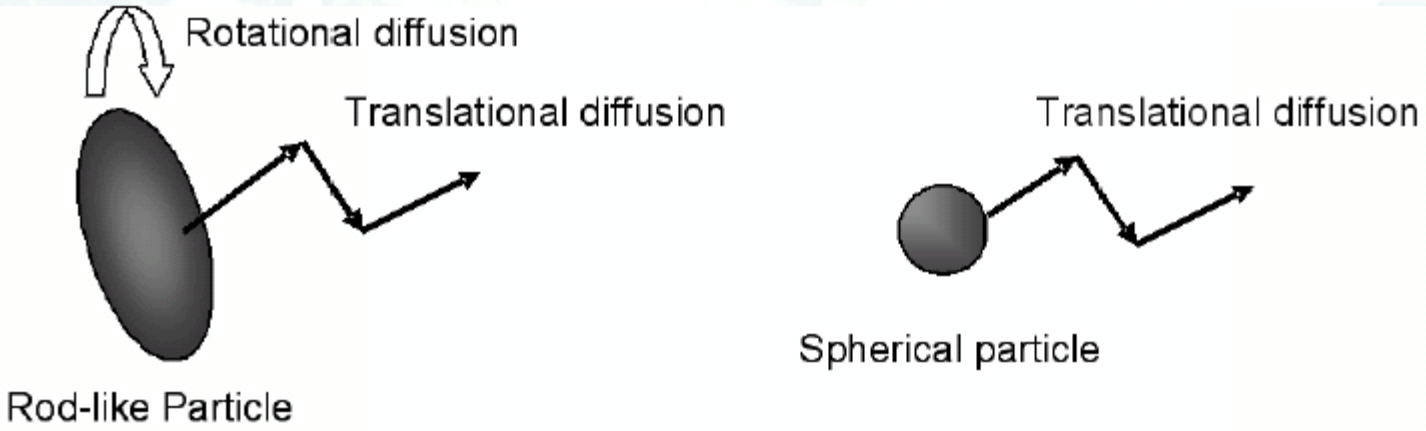






Hydrodynamic Radius

- Shape Information
- Particles with shape
  - Diffuse More slowly
  - Over estimation of size



LB550

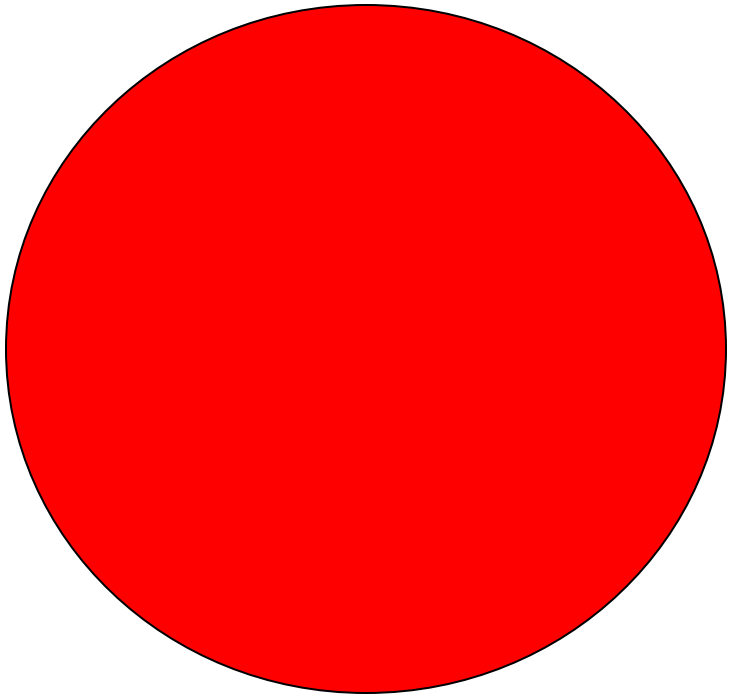
- The range of instrument 1nm to 6µm
- Temperature setting up to 70°C
- Concentration range up to 40wt%
- Low volume cuvettes – 30µL
- Viscometer attachment

Range of Sizes

- Two particles 1nm and 1μm

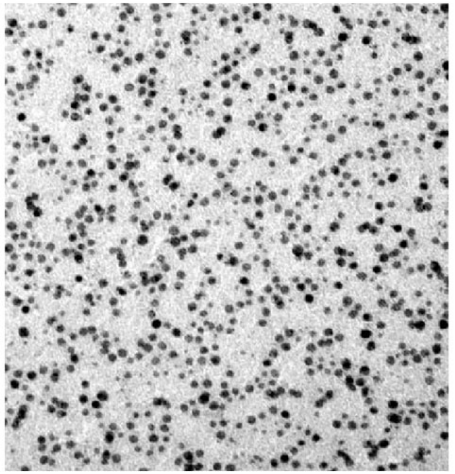
- Volume of the 1nm particle is $1nm^3$

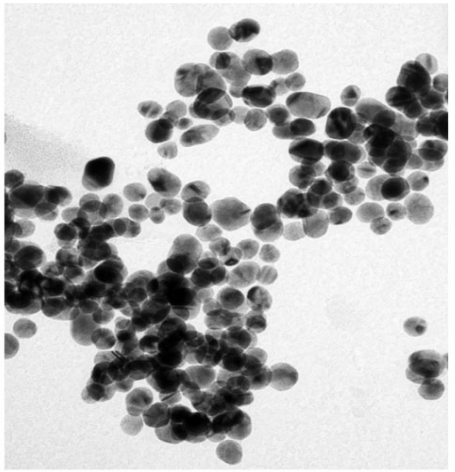
- Volume of the 1μm particle is $1{,}000{,}000{,}000nm^3$



Mixed Samples

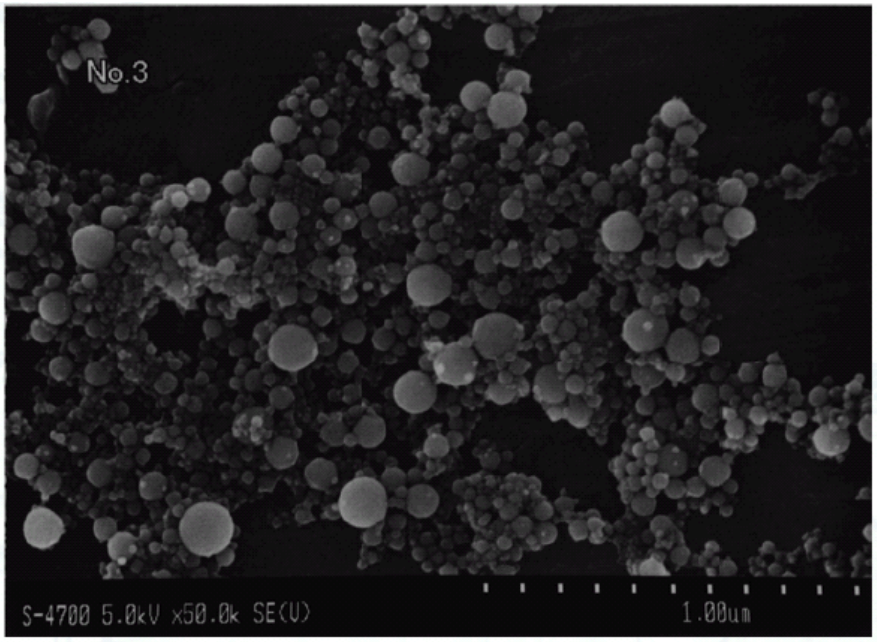
- You need 1 Billion 1nm particles to equal the scattering from One 1µm particle!
- DLS is useful for detecting these aggregates
- Electron Microscopy would miss these aggregates: AFM, TEM, SEM, etc…

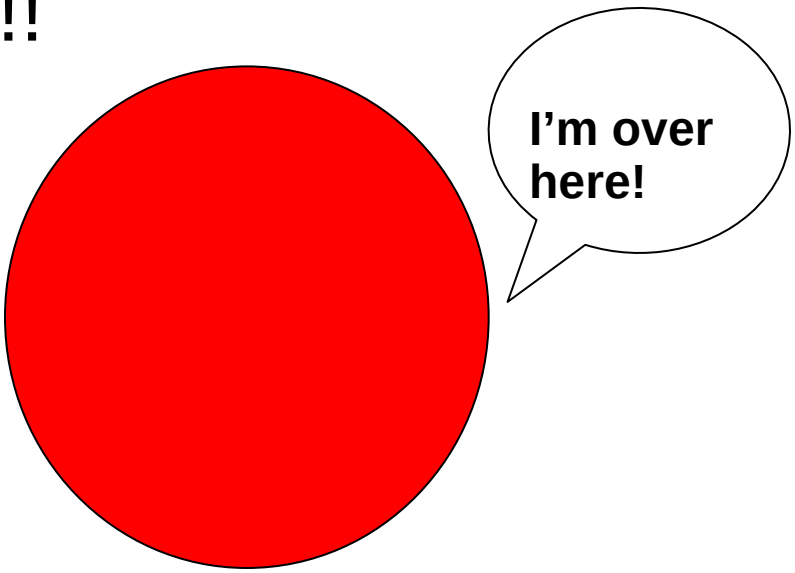




You get the idea

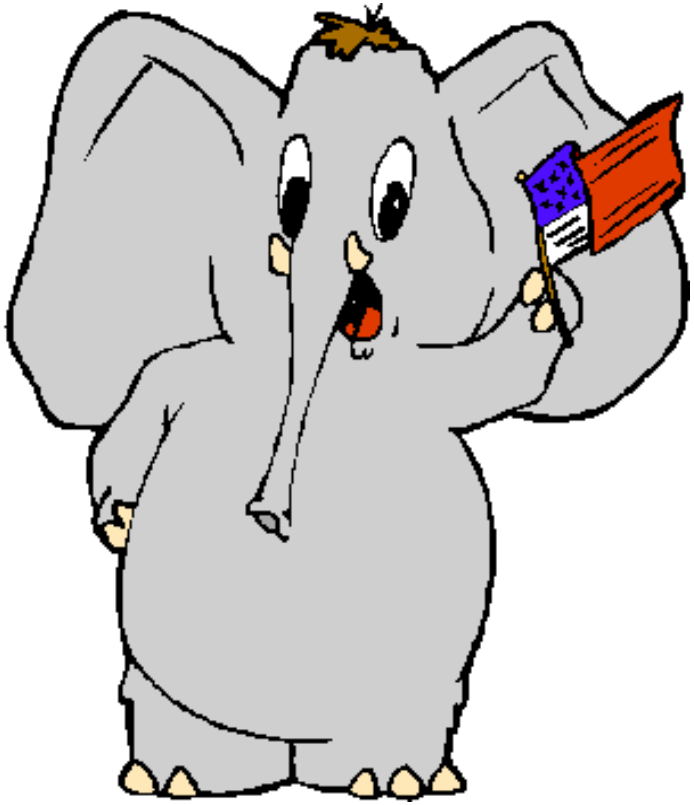
So Light Scattering is an excellent technique for uncovering that single large outlier in a distribution!!!





The difference between

1nm and 1μm in scale is the same as the difference between a mosquito and an elephant



Don’t Believe Me?

- African elephants weigh on average 3000kg
- An unfed Mosquito weighs 0.0016g
- A Well fed Mosquito can weigh 0.003g

There is a 1 billion times difference in size

The same difference between 1μm and 1nm

What happens?

- Say we don't care about the aggregates
- We want to know the size of our smallest particles
- That is like saying we want to know the size our mosquitoes in a herd of elephants
- Even if we only care about the smallest particles, can we use DLS?

Filter to monitor aggregation

- A filter will remove our aggregates
- Filters available in sizes 20nm to 2μm
- We can also centrifuge the sample and extract the supernatant

Settling and DLS

Particle Diameter (μm)	Movement due to Brownian Motion		Movement due to Gravitational Settling
0.01	2.36	>>	0.005
0.25	1.49	>	0.0346
0.50	1.052	>	0.1384
1.0	0.745	~	0.554
2.5	0.334	<	13.84
10.0	0.236	<<	55.4

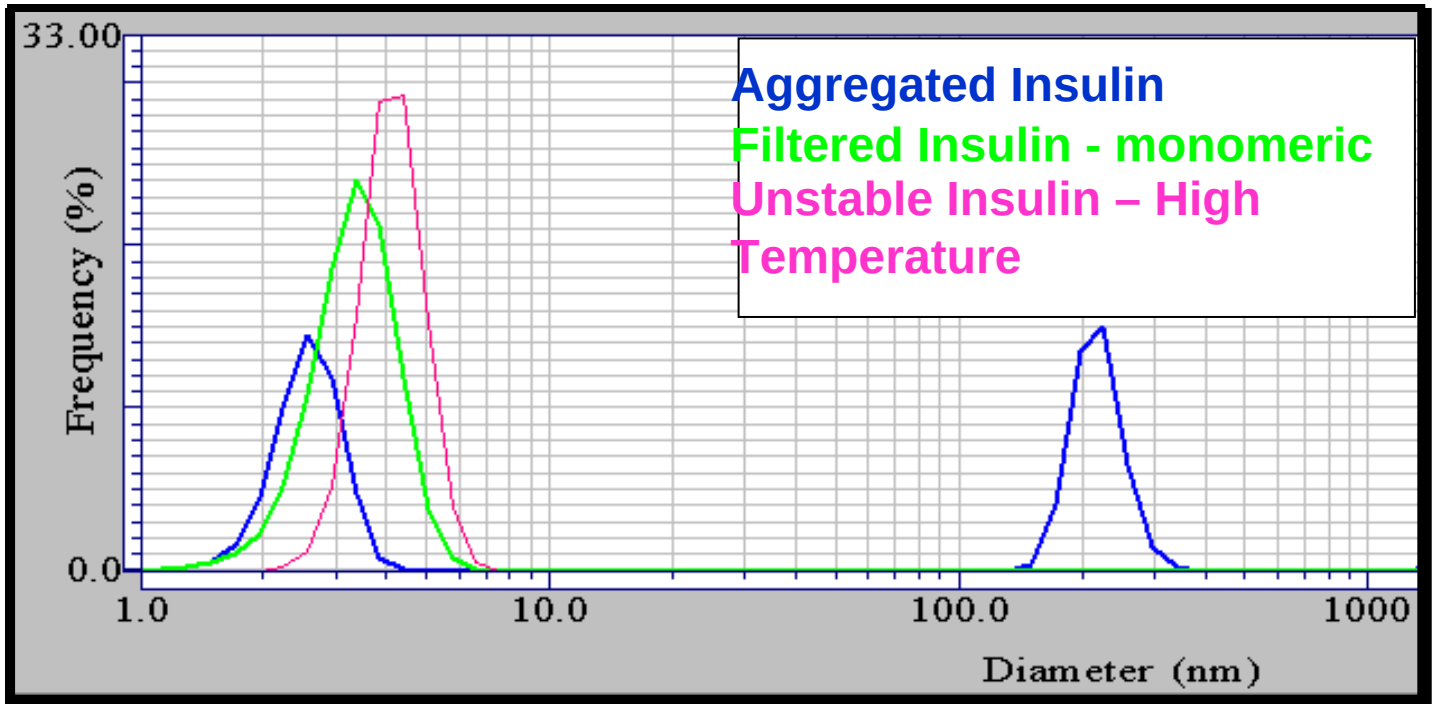
The Natural limit for Dynamic Light Scattering: Gravitational Settling

Gravitational Settling occurs at about 1μm

Filtration in Action

- Filtered Aggregated insulin with 20nm filter
- Temperature ramp
- up to 60ºC

Sample Name	Rh	Diameter	T (K)	η
	nm	nm		
Insulin - monomeric	1.57	3.1	295.6	0.0037
Unfiltered Insulin	77.65	155.3	294.9	0.0037
Insulin at 60C	1.92	3.8	335.3	0.0037



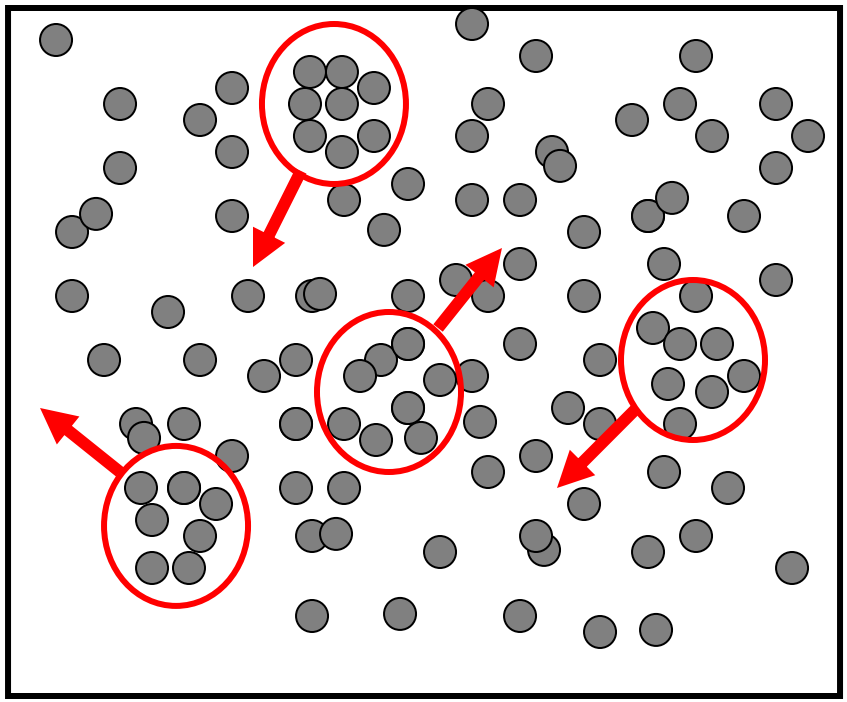
How does Concentration Affect Analysis

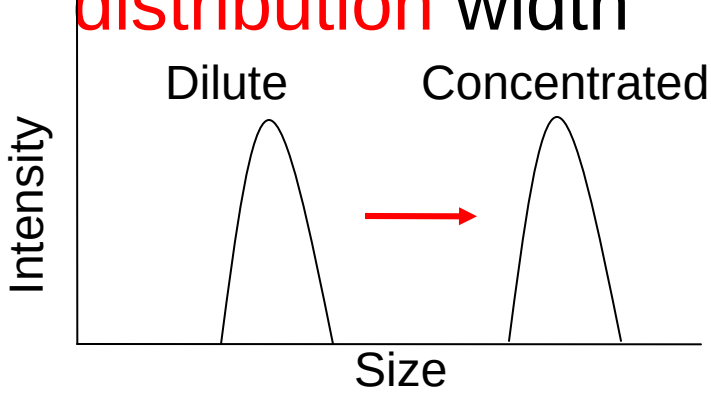
- Some ways
  - Diffusion Drag
    - Measured Alcholoic Emulsion LB550
  - Multiple Scattering
    - Concentration limit of technique
  - Aggregation Equilibrium
    - Concentration limit of material
    - Filtration has no affects

Diffusion Drag

- Bulk Viscosity Change
- Particles appear to diffuse together
- Apparent Increase in particle size
- No Change in distribution width

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



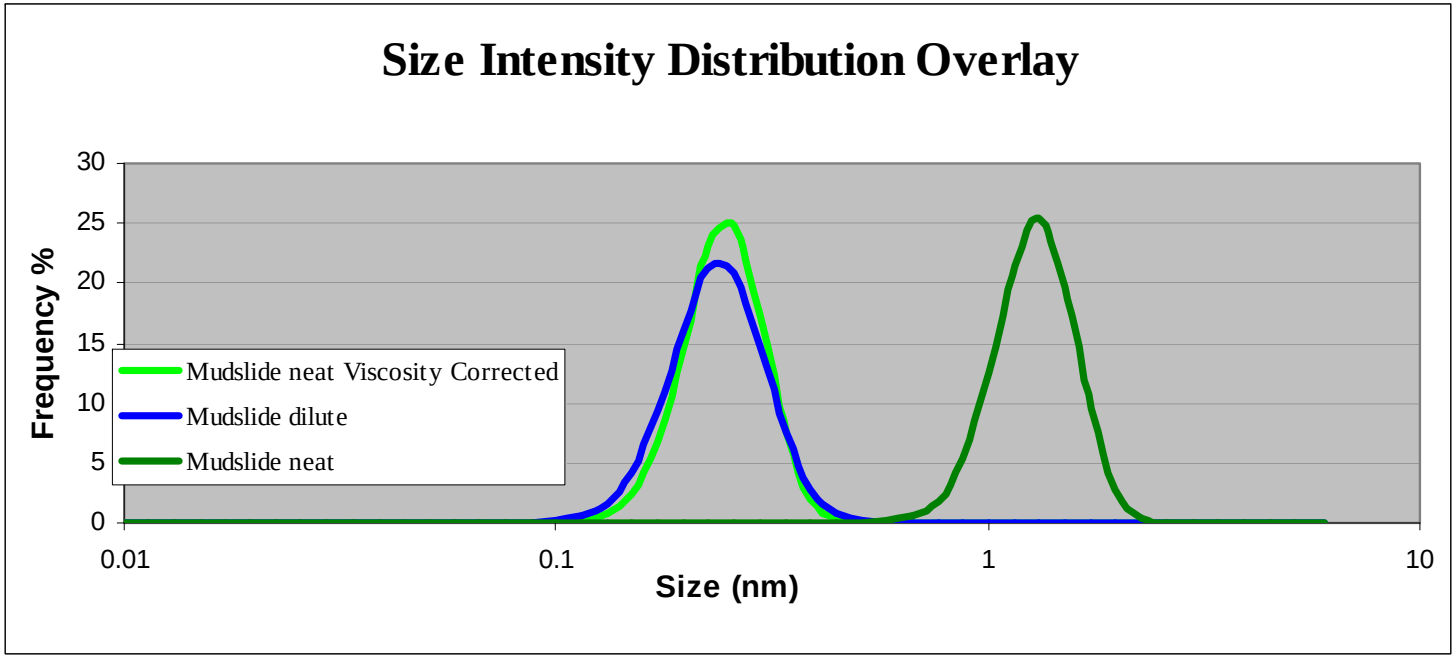


Data

- Mexican Mudslide
  - Milk Emulsion
  - Alcoholic Beverage off the Shelf at the Grocery Store
  - Well understood sample
  - 200nm size with a high zeta potential at pH 7
  - Extremely stable sample

LB550 Data

- Diffusion Drag
  - Use bulk viscosity for Concentrated sample
  - Apparent size shift upwards with concentration
  - Polydespersity- distribution width is constant



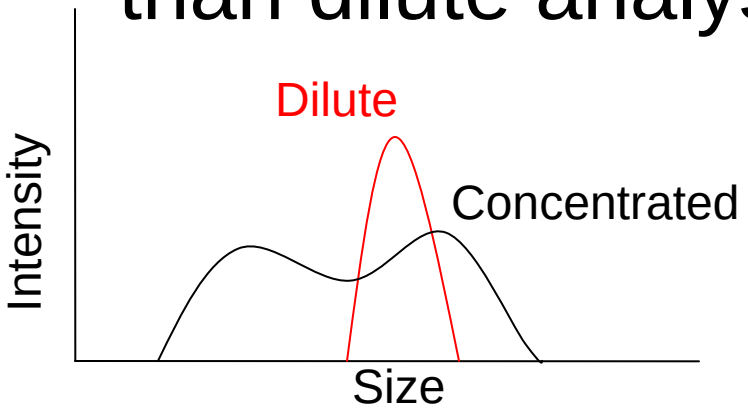
Tabular Data

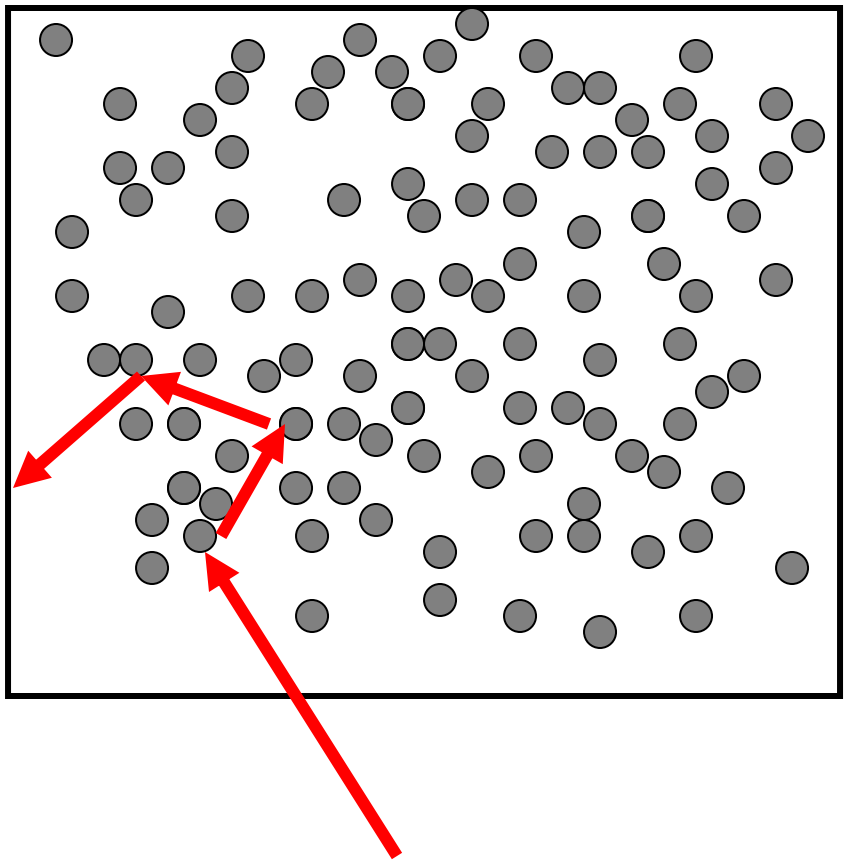
Filename	200807171548077New Visc	200807171601079	200807171548077
Sample Name	**Mudslide neat Corrected Viscosity**	**Mudslide dilute**	**Mudslide neat**
Viscosity (mPa s)	5	0.952	0.952
Median (nm)	228.1	220.9	1201.5
Mean (nm)	**231.5**	**226.7**	**1218.9**
CV	21.604	25.083	21.517
Polydespersity Index	**0.093**	**0.126**	**0.093**
Diffusion Coefficient (m2/s)	2.8174E-15 (m2/s)	1.4831E-14 (m2/s)	1.4798E-14 (m2/s)

- Adjust viscosity parameter
- No change in distribution width
- Apparent change in size is viscosity dependent

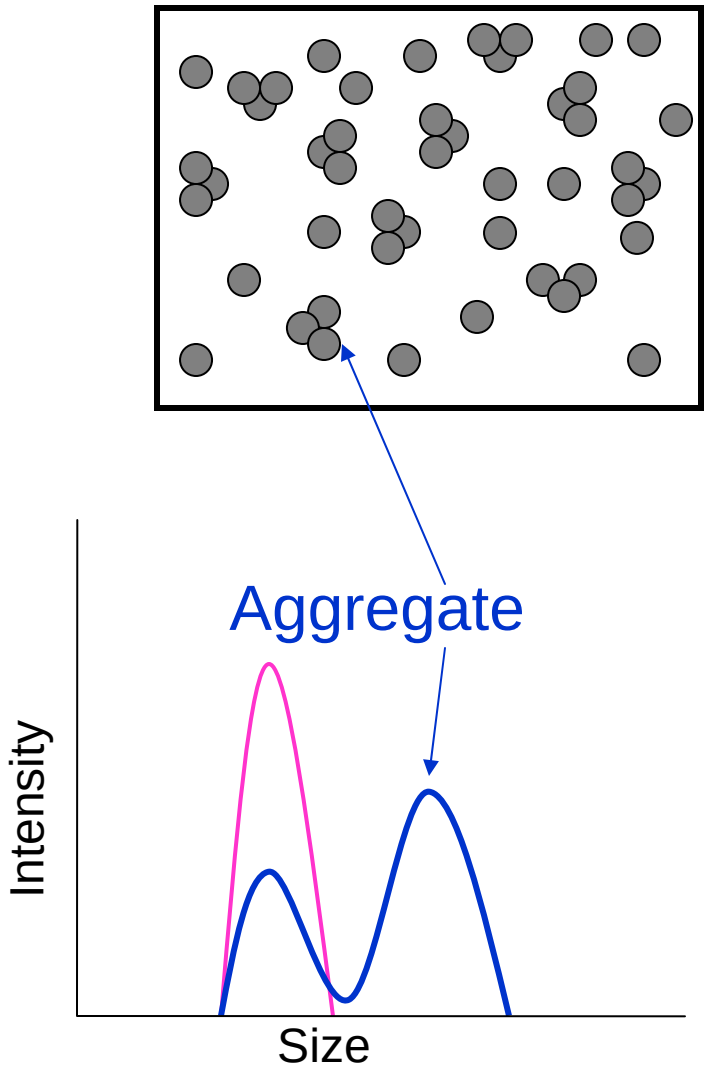
Multiple Scattering

- Incident Light Scatters off of more than one particle
- Particles appear smaller in size
- Distribution is wider than dilute analysis





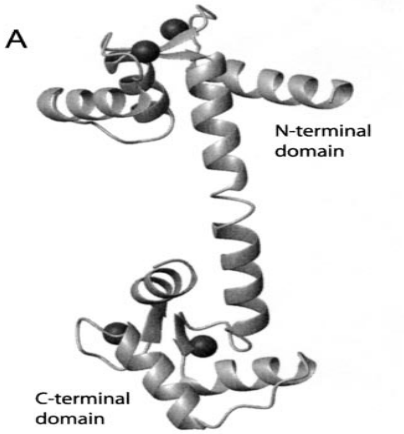
Aggregation Equilibrium

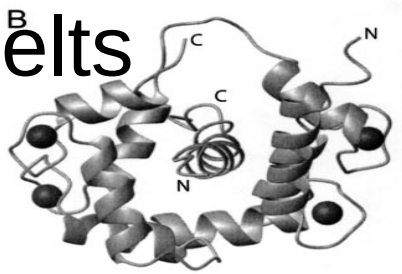


- You cannot filter out aggregates in concentrated state – Equilibrium has been reached
- Filtration will cause aggregates to reform
- Point where equilibrium occurs is important in understanding formulation stability

Small Molecule Applications

- Protein Crystallization
- Protein Denaturation
- Protein Formulation
- Protein Folding
- Enzyme-Substrate reactions
- Macro-Molecular temperature melts
- Estimated Molecular Weight
- Lipid Micelle formation- CMC
- Macro-Molecular Characterization





Starburst Polymers: Dendrimers

Dendrimer	Mean	Intensity	PDI
	7.4	9.53	0.062
	7.1	9.72	0.101
	7	9.56	0.075
	7.7	9.56	0.062
	7.1	9.61	0.073
	7.6	11.99	0.033
	7.5	10.24	0.035
	7	7.94	0.059
	7.6	8.24	0.059
AVG	**7.3**	**9.60**	**0.062**

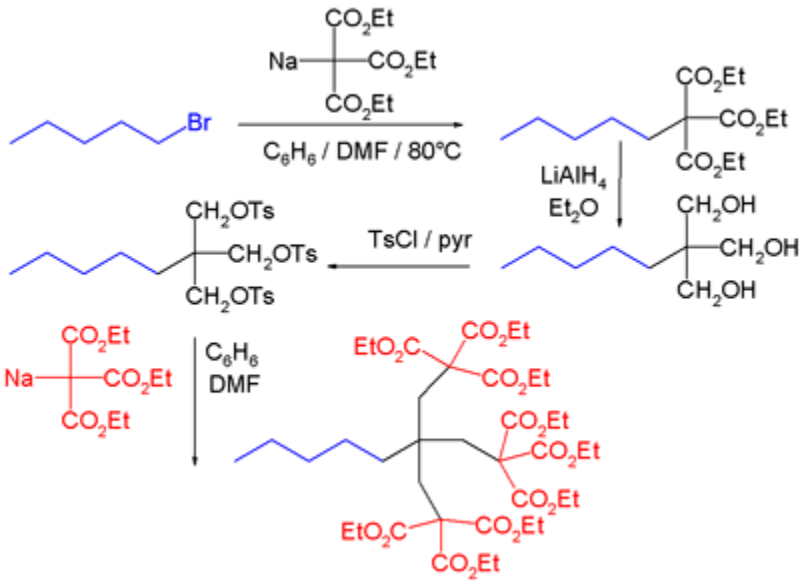
Expected Dendrimer Values

256 surface groups

MW 58 kDa

Size 7.2nm

- Dendrimers are repeatedly branched chain polymeric molecules



Size Data for Dendrimer

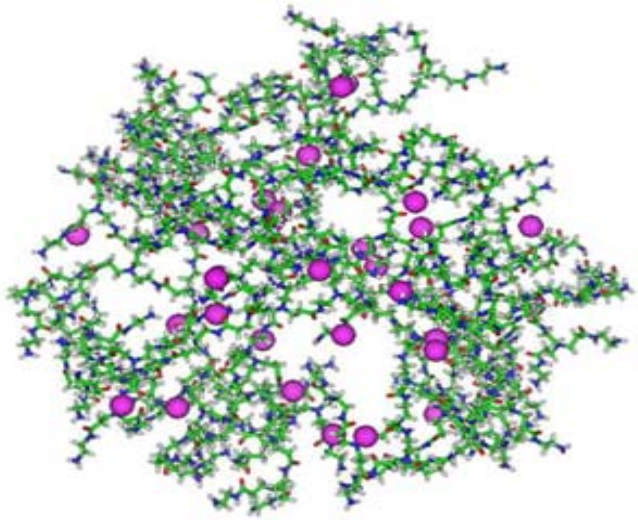
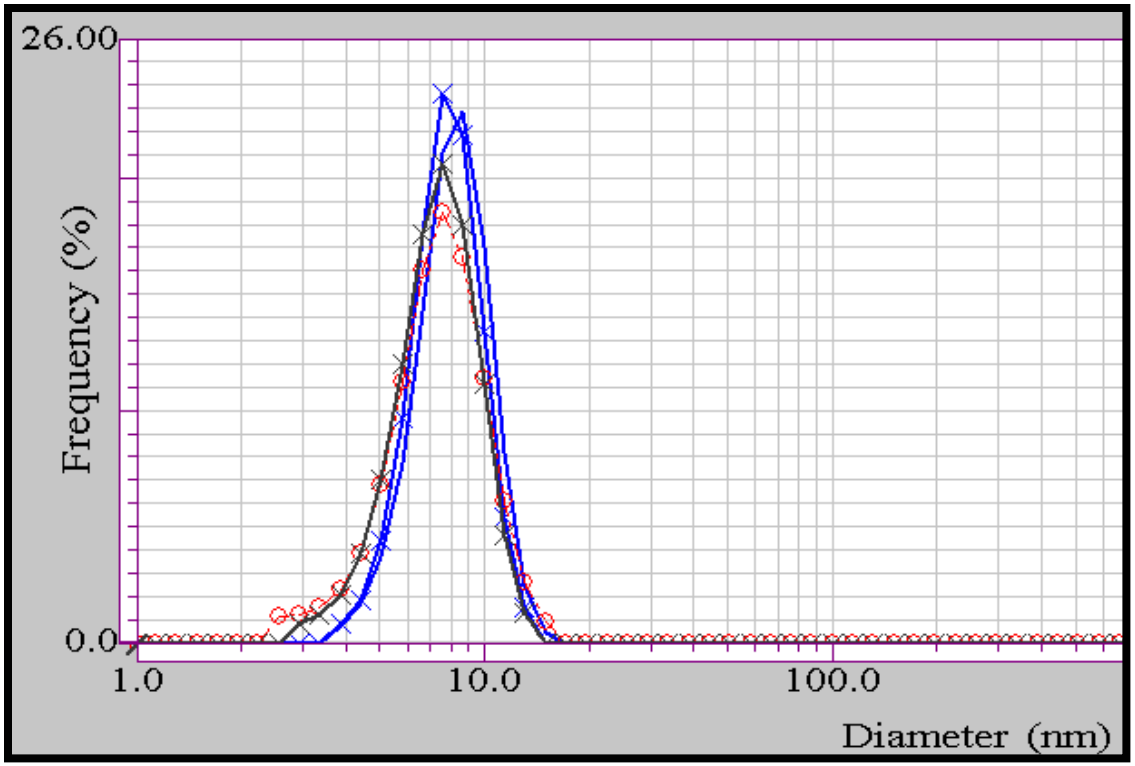
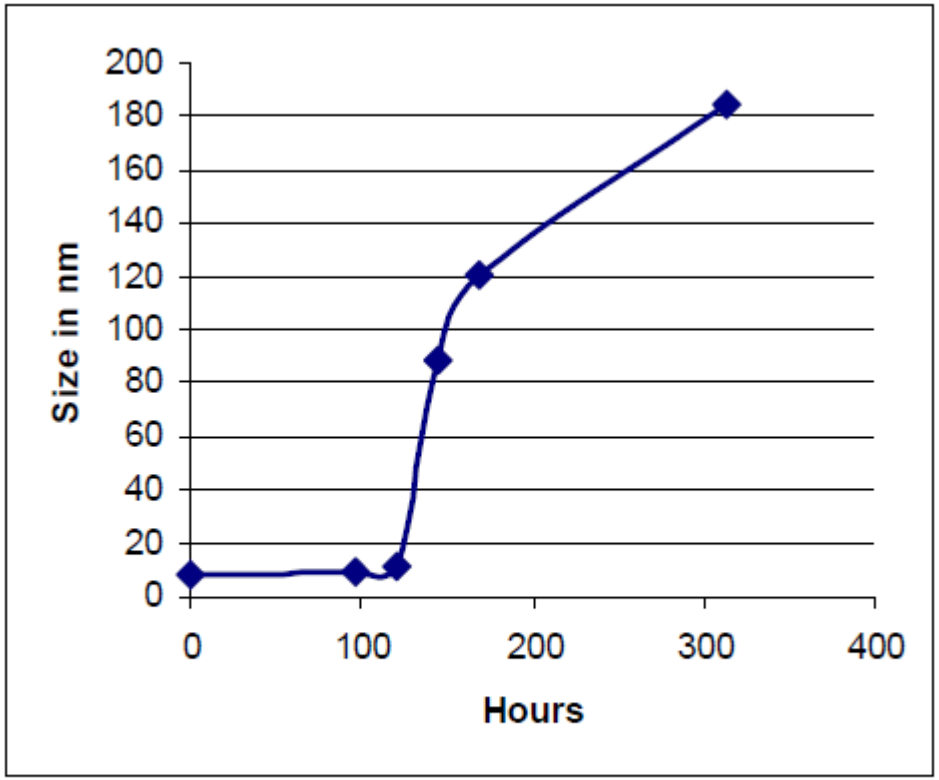


Figure 1. Computer simulation of the gold atom distribution in a dendrimer nanocomposite. (Courtesy of Inhan Lee, University of Michigan in Ann Arbor.)



Protein Aggregation Time Study

- Unstabilized 10mg/ml lysozyme at pH 2
- Lisa Cole and Ben Burnett at the Florida Institute of Technology



Protein size in nm vs. time in hours

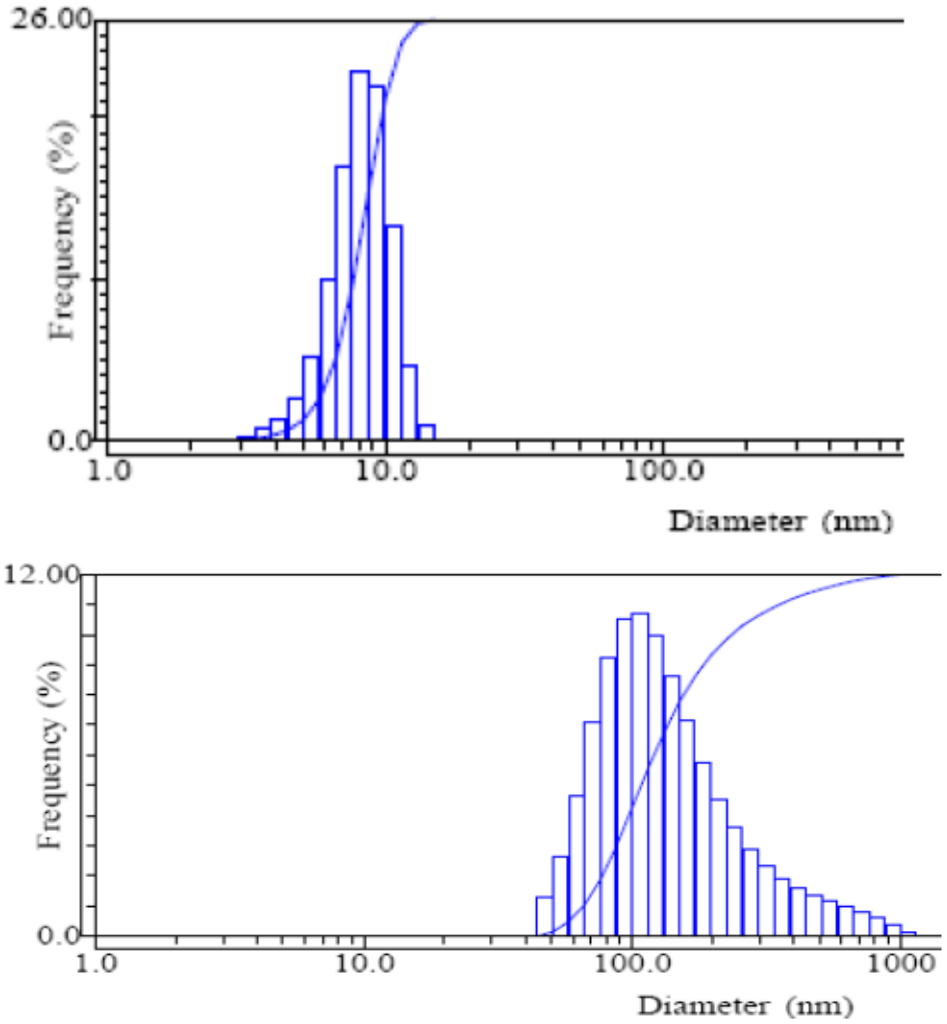
Time Evolution

Stability influenced by:

- Temperature
- Protein concentration
- pH
- Ionic strength

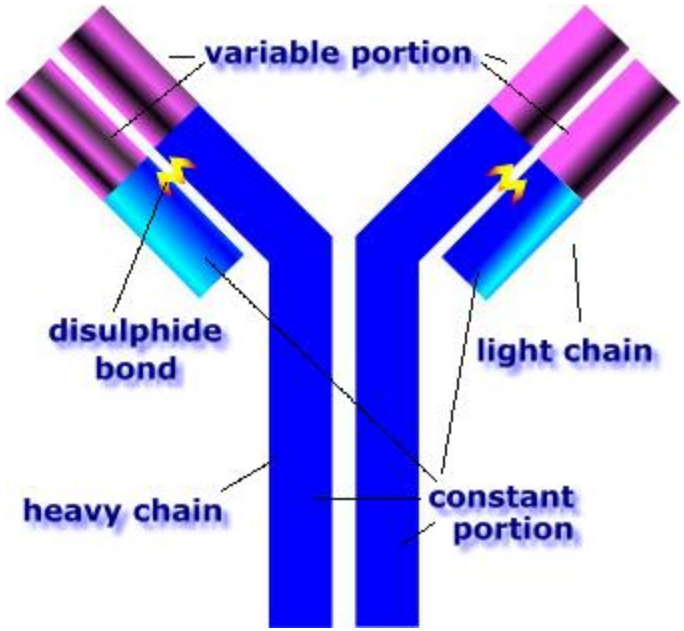
Aggregation influenced by:

- Freezing
- Exposure to air
- Interactions with metal surfaces



Small Molecules

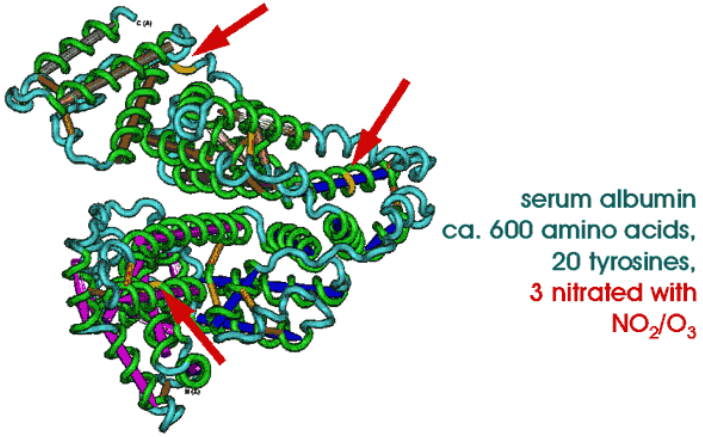
- Antibody characterization
- Dynamic light scattering for molecular weight determination
- Protein formulation stability
- Quaternary Structure of Protein

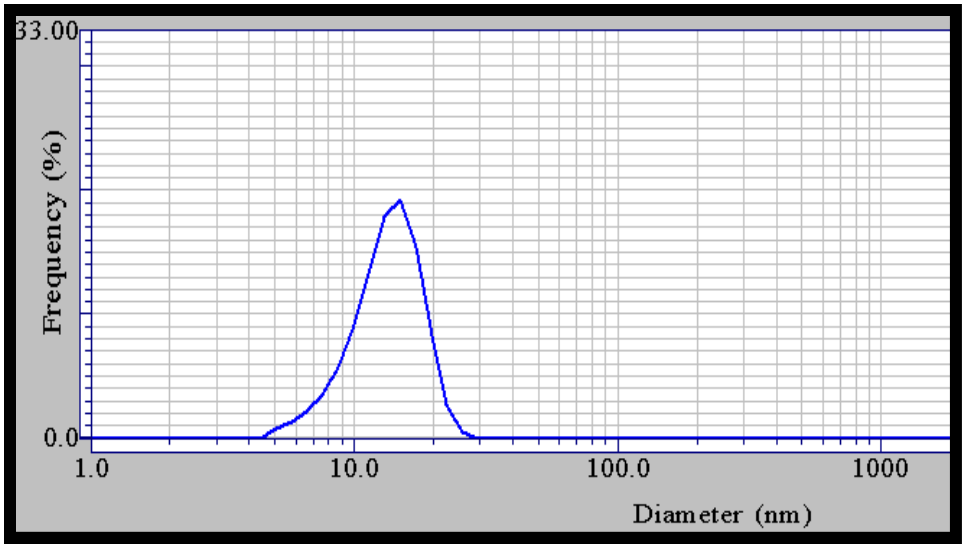


2mg/mL filtered BSA

- BSA- well characterized protein
- DLS – Can be used to determine

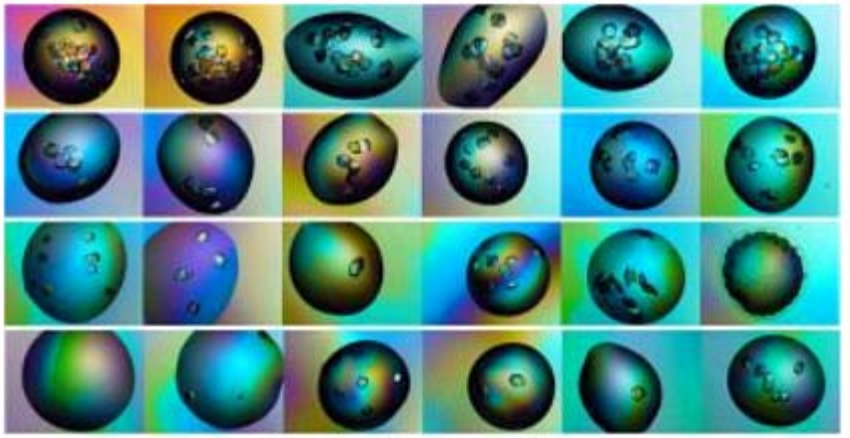
  the aggregation state of the protein





DLS a Crystalliztion Monitor

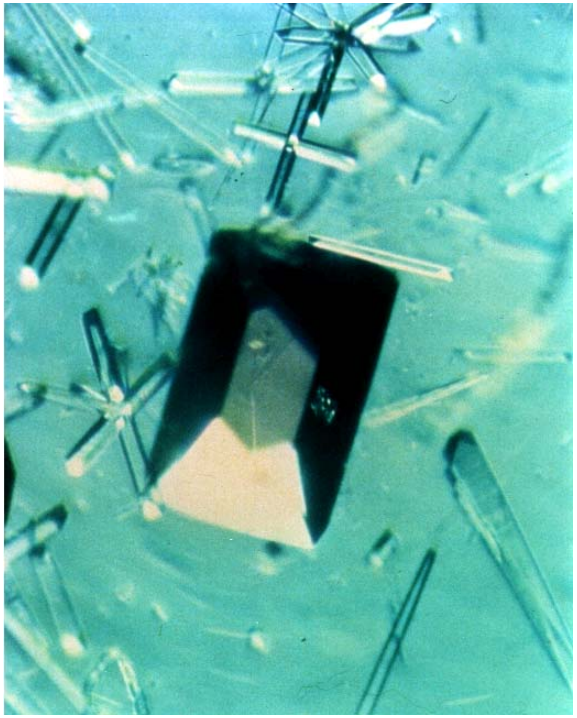
- Size variations as a function solution properties
  - protein concentration
  - pH
  - precipitant concentration
  - temperature
- Monomers assemble
  - Crystals
  - Precipitates
- DLS quantifies the aggregates state
- Early predictions about the crystallization outcome



Growing Protein Crystals

Protein Crystallization Screening

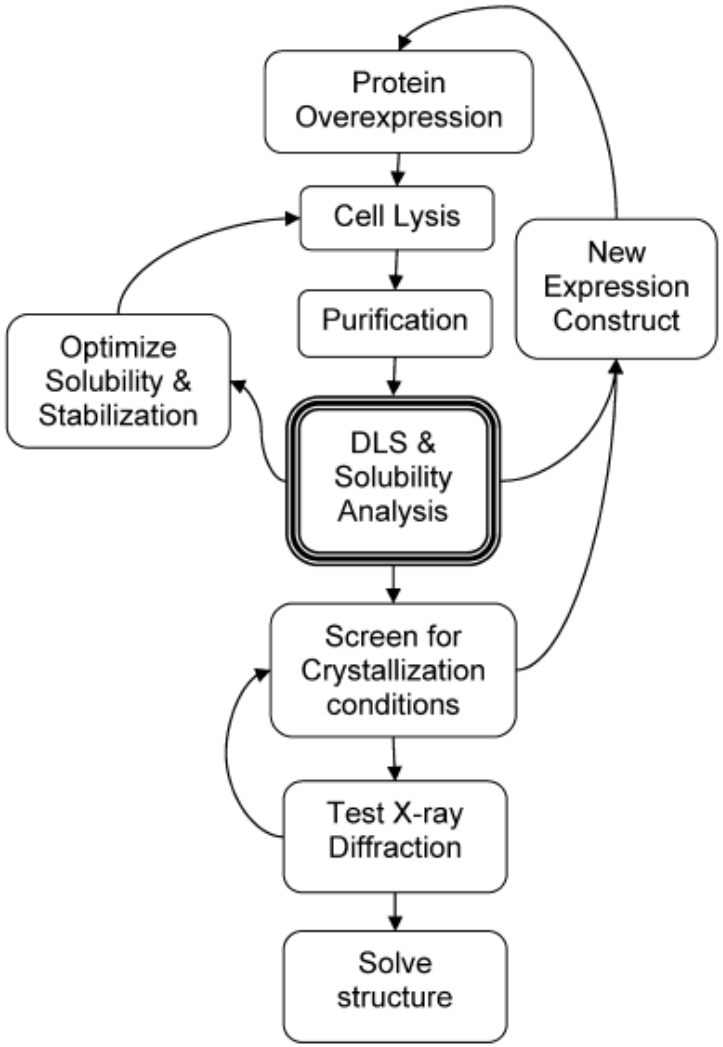
- Empirical correlation between DLS distribution breadth and successful crystallization
- When PDI – polydispersity index > 0.500, only 8% chance of crystal growth
- When PDI is less than 0.200, then 70% chance of crystals



DLS and Crystallization

- With DLS at the center of the protein screening process the chances of growing crystals is optimized

- **Gloria E.O. Borgstahl** "How to Use of Dynamic Light Scattering to Improve the Likelihood of Growing Macromolecular Crystals"



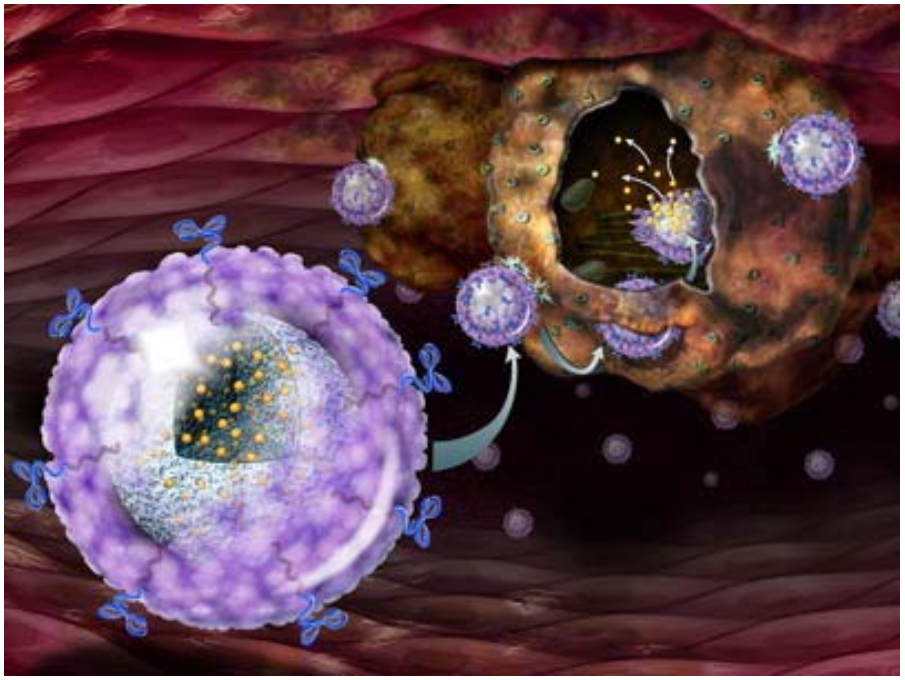
Estimating Molecular Weight

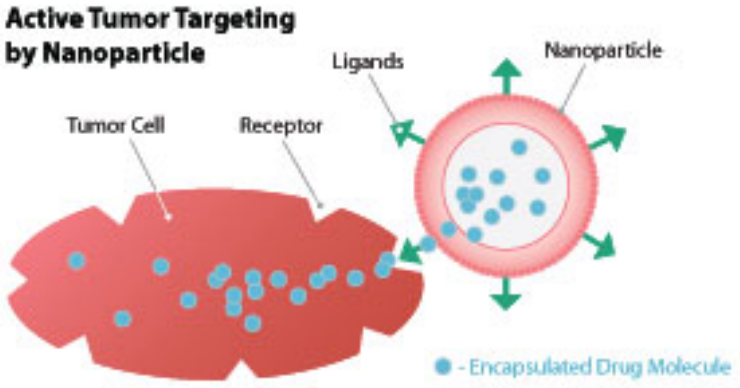
- Empirical Models
- Some models take into account shape factors
- Deviations from Expected values – indicate aggregation and dimerization

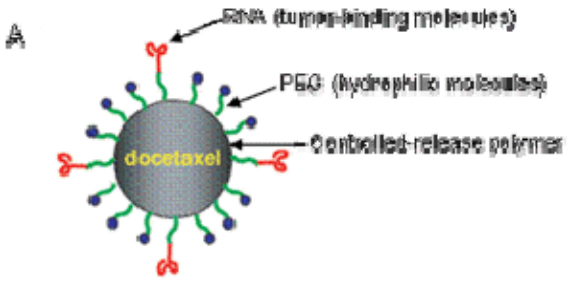
$$MW = (d * \alpha)^{\beta}$$

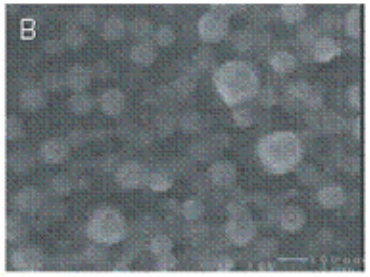
d = sphere diameter in nm
a = correction factor 1 = 1.68
b = correction factor 2 = 2.3398
MW = mol. weight in kDa

Drug Delivery Applications

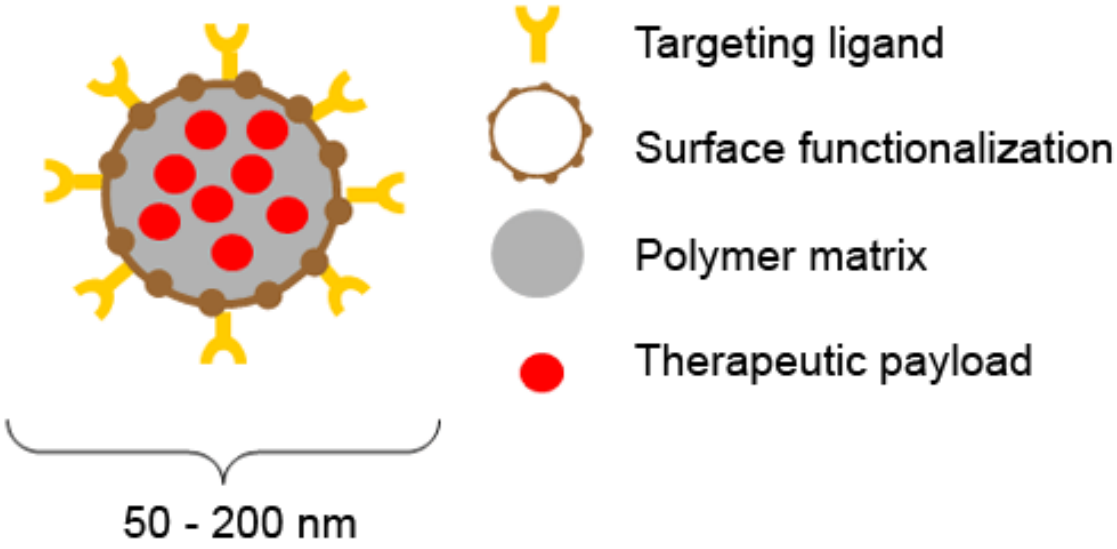




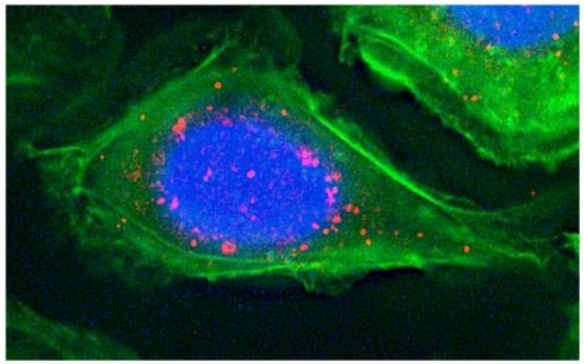




Bind Biosciences*



Targeting ligand provides recognition, enabling targeted nanoparticles to identify and bind to their intended target site.
Surface functionalization shields targeted nanoparticles from the immune system.
Polymer matrix encapsulates payload molecules in a matrix of biodegradable polymers .
Therapeutic payloads include small molecules, peptides, proteins, etc.

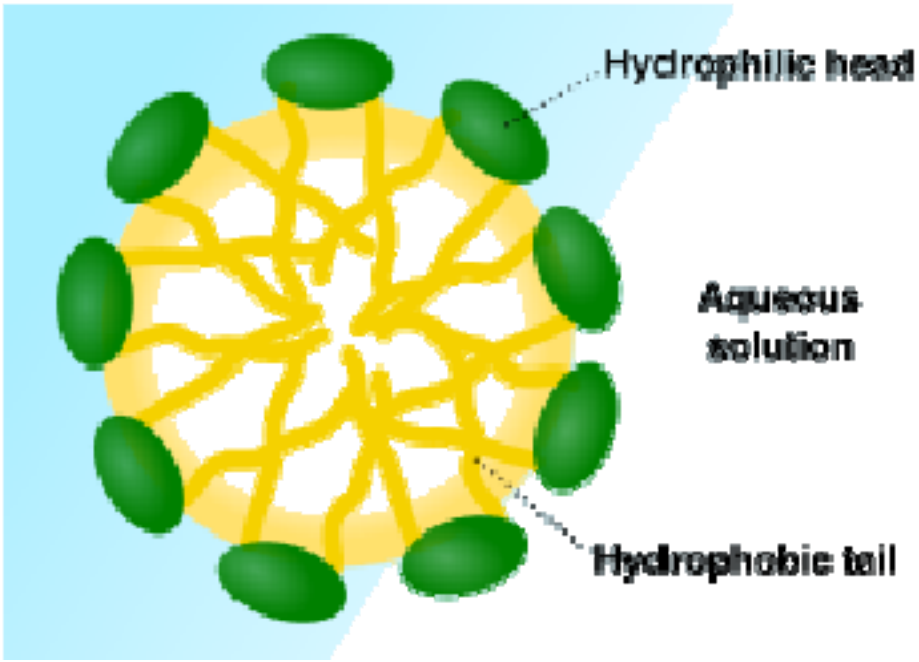


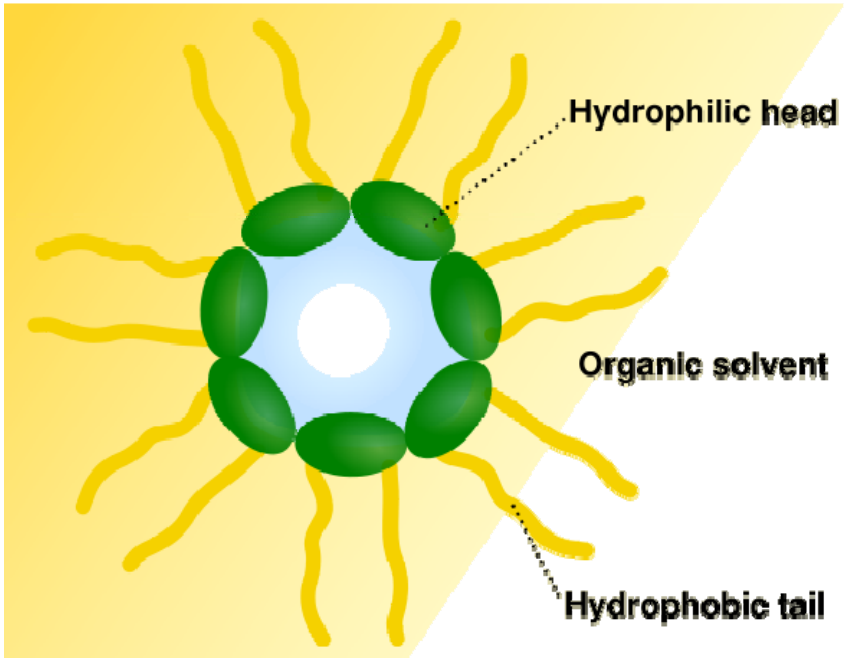
* Cambridge, MA, recent LA-950 customer

Self Assembly: Micelles

hates water Hydrophobic tail Hydrophilic head loves water

-c-h-c-h-c-h- R non polar +/- polar

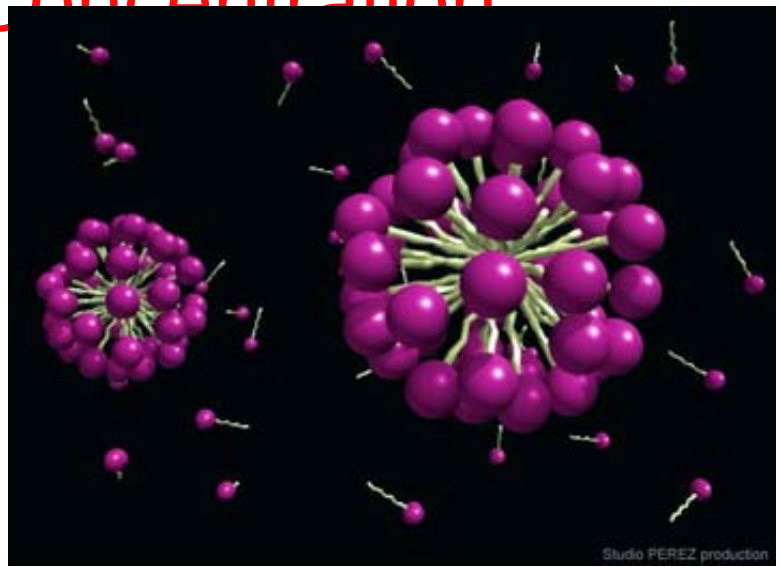




Biotech Applications

- Micelles- self-assembly
  - colloidal aggregated surfactant molecules
  - DLS – can characterize CMC

- CMC- Critical Micelle Concentration
  - Point at which surfactants emulsify to form micelles



CMC Values

TABLE 1 PHYSICAL PROPERTIES OF COMMONLY USED DETERGENTS

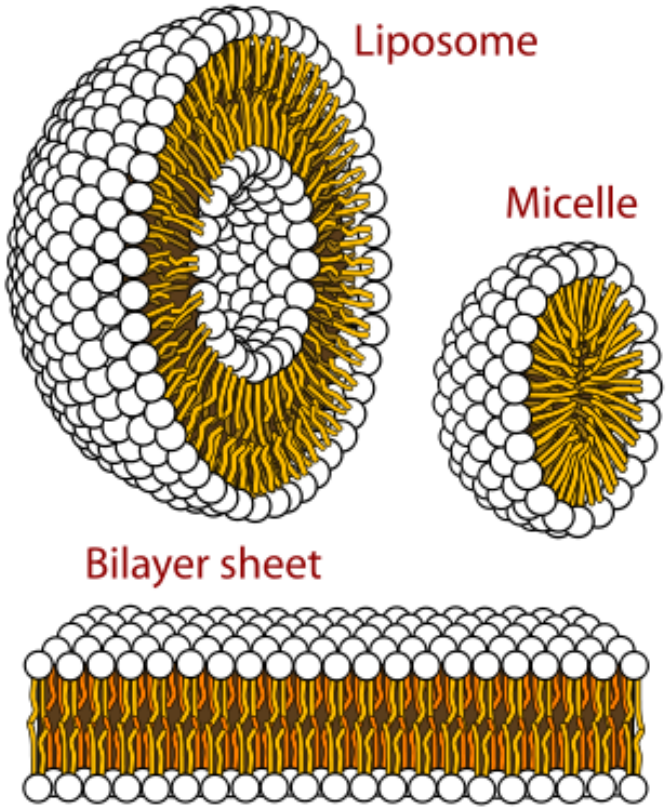
Detergent	Monomer, Da mw	Micelle, Da mw	CMC % (w/v)	CMC Molarity
Anionic				
SDS	288	18,000	0.23	8.0×10^{-3}
Cholate	430	4,300	0.60	1.4×10^{-2}
Deoxycholate	432	4,200	0.21	5.0×10^{-3}
Cationic				
C_{16}TAB	365	62,000	0.04	1×10^{-3}
Amphoteric				
LysoPC	495	92,000	0.0004	7×10^{-6}
CHAPS	615	6,150	0.49	1.4×10^{-3}
Zwittergent 3-14	364	30,000	0.011	3.0×10^{-4}
Nonionic				
Octylglucoside	292	8,000	0.73	2.3×10^{-2}
Digitonin	1,229	70,000	-----	----
$C_{12}E_8$	542	65,000	0.005	8.7×10^{-5}
Lubrol	582	64,000	0.006	1.0×10^{-4}
Triton X-100	650	90,000	0.021	3.0×10^{-4}
Nonidet P-40	650	90,000	0.017	3.0×10^{-4}
Tween 80	1,310	76,000	0.002	1.2×10^{15}

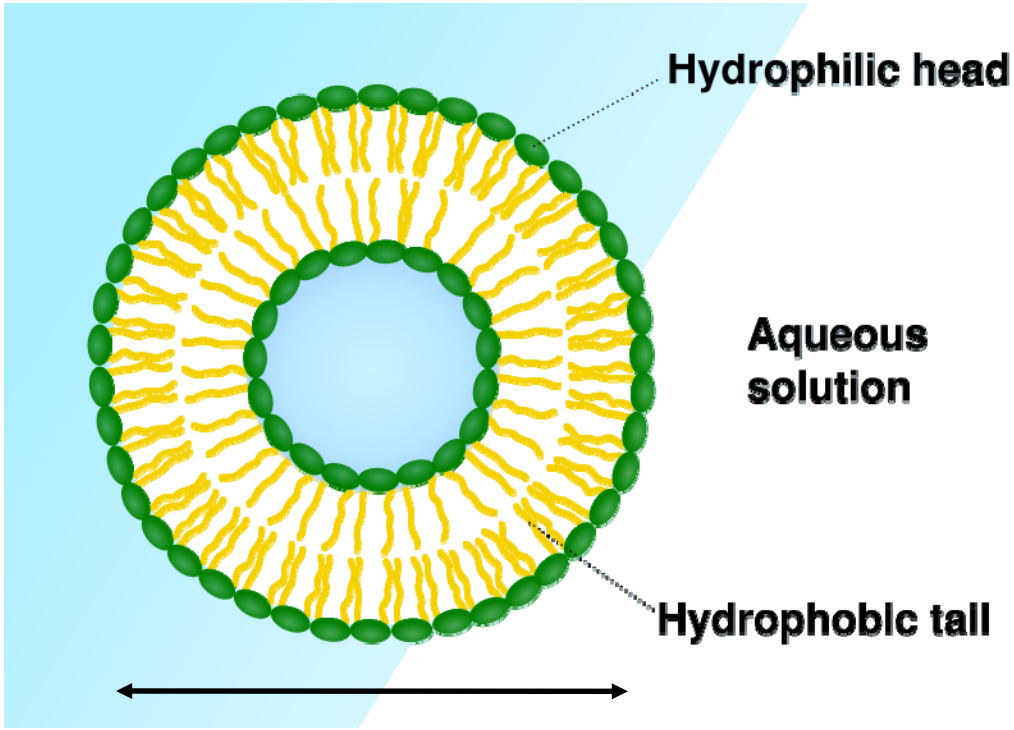
Triton-x100 measured CMC

- CMC value for Triton-x100
- Measured using the LB550
- The expected value is 0.021%

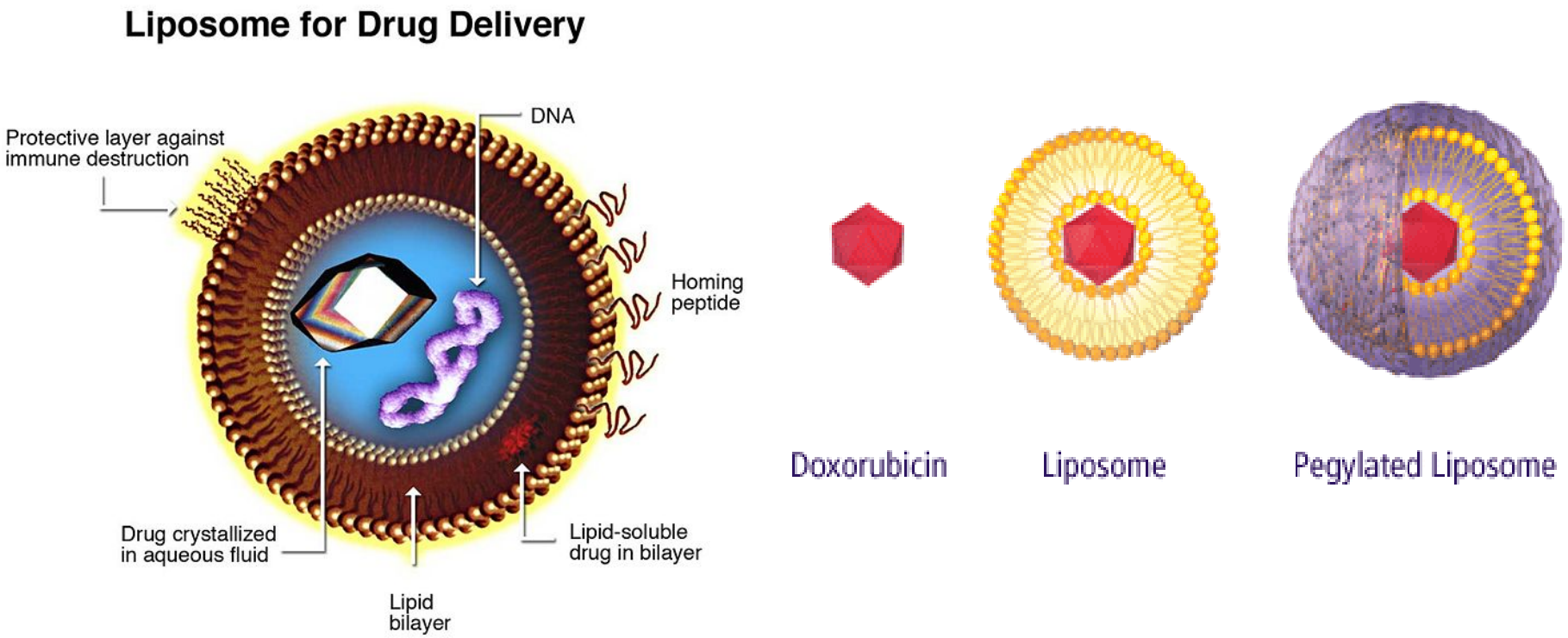
CMC	Concentration	Intensity	Size
Triton x-100	**wt%**		**(nm)**
10mMol NaCl	0.00	0.94	-
1 drop	0.0017	1.78	-
5 drops	0.0086	2.35	-
10 drops	0.0172	3.18	-
15 drops	**0.0255**	**4.78**	**9**

Liposomes



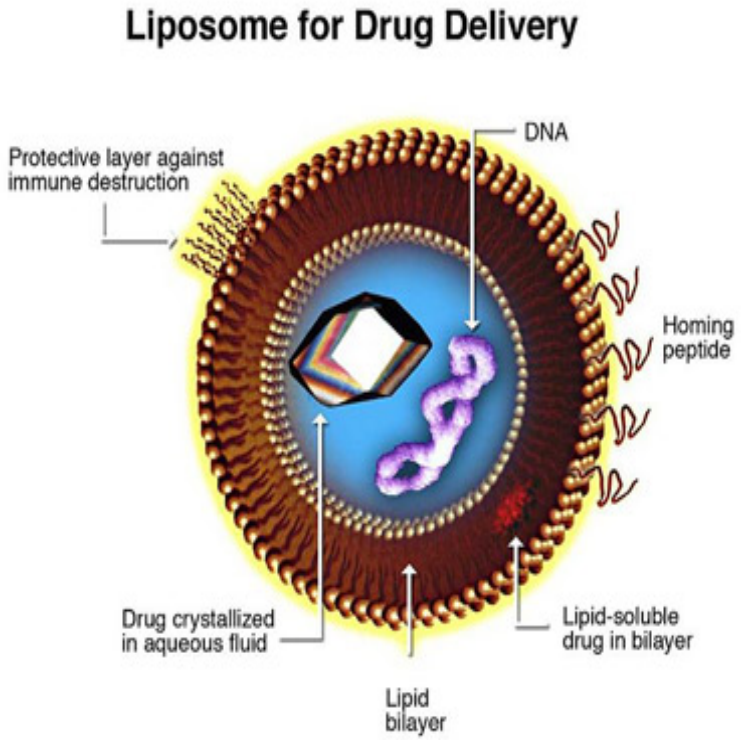


Liposomes - Doxil



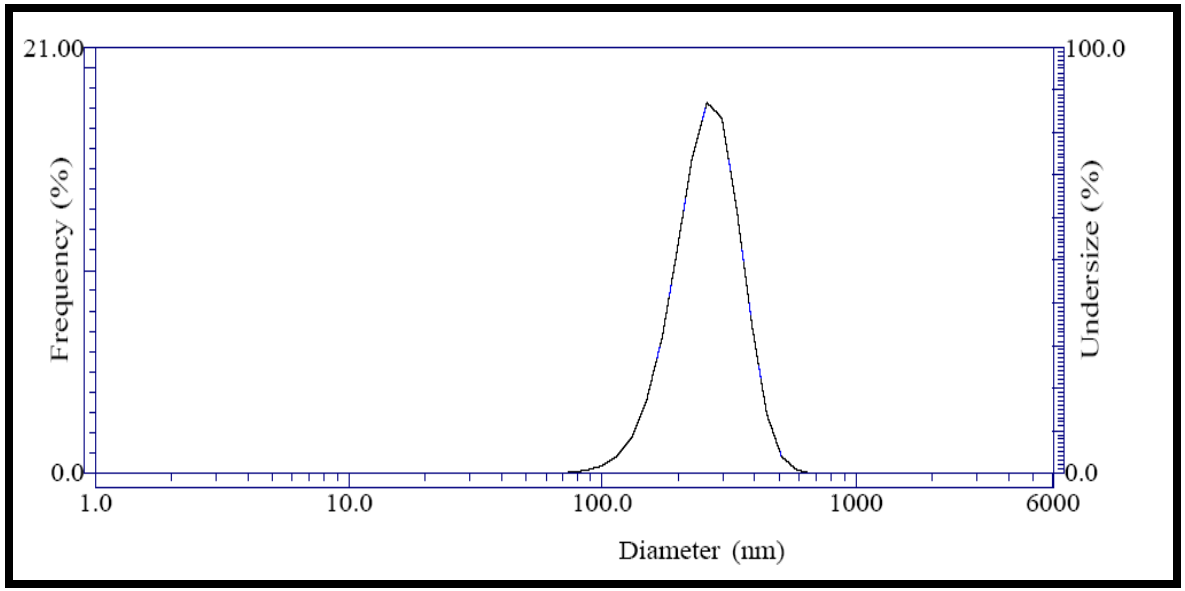
Applications

- Liposomes
  - Lipid bilayer vesicles
  - Sub-micron
  - Encapsulates API (active pharmaceutical ingredients)
  - Used in creams, emulsions
  - and drug delivery



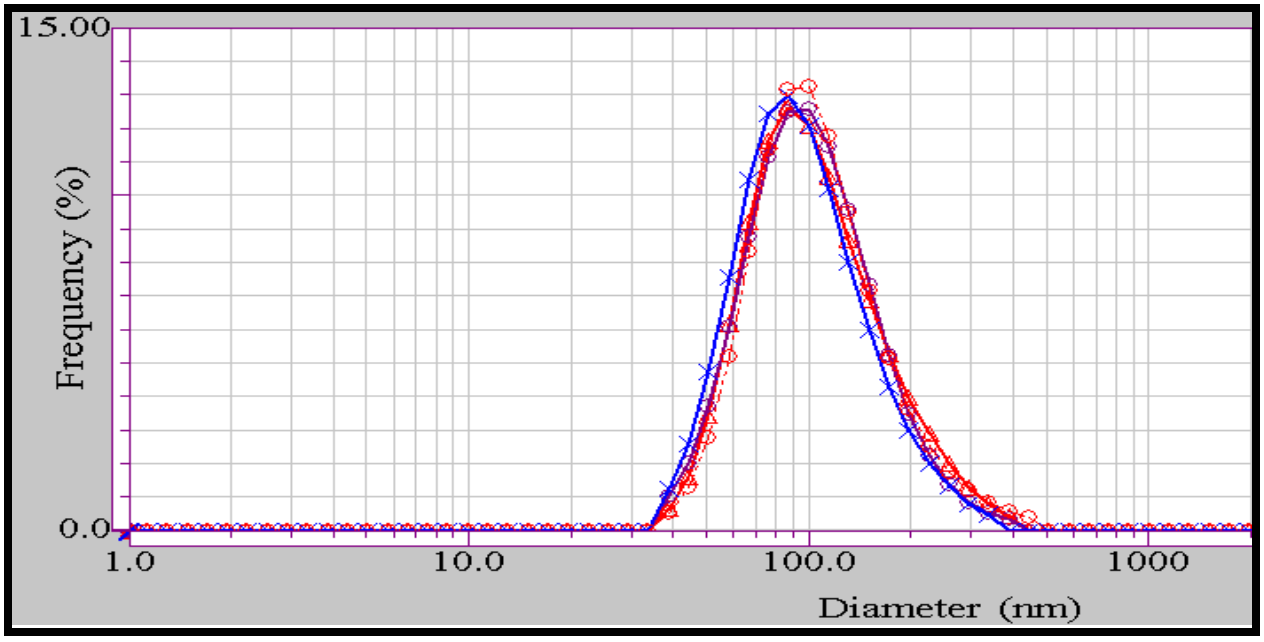
Novartis Liposome Data

- Liposomes to target tumor growth
- Size is critical to how the liposome
  - Encapsulates protein
  - Functions within body
  - Remains stable over time
  - Delivers the protein



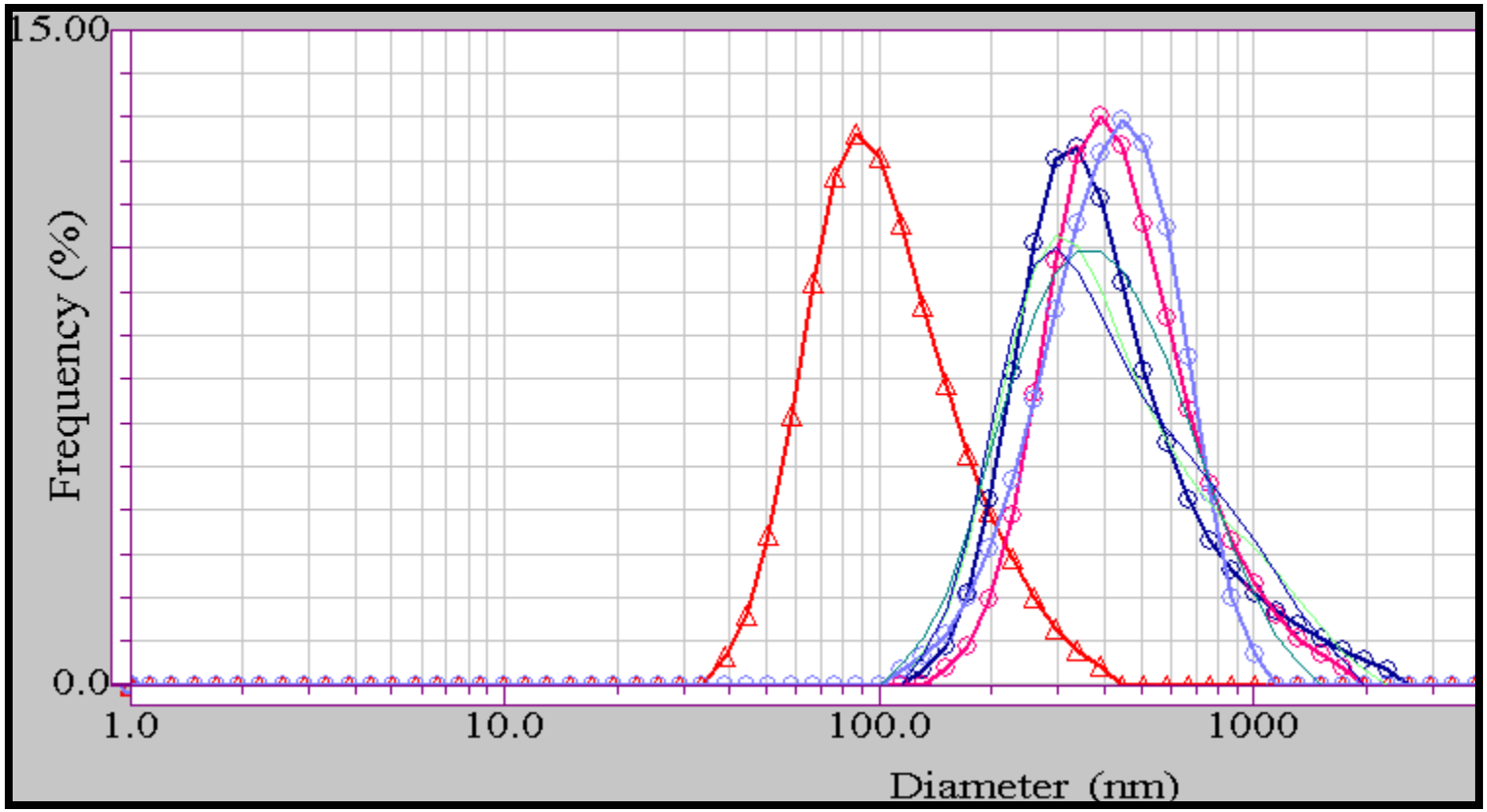
Liposome and Microfluidizer

- After One Pass through a Microfluidics fluidizer
- http://www.microfluidicscorp.com



Liposome Fluidization

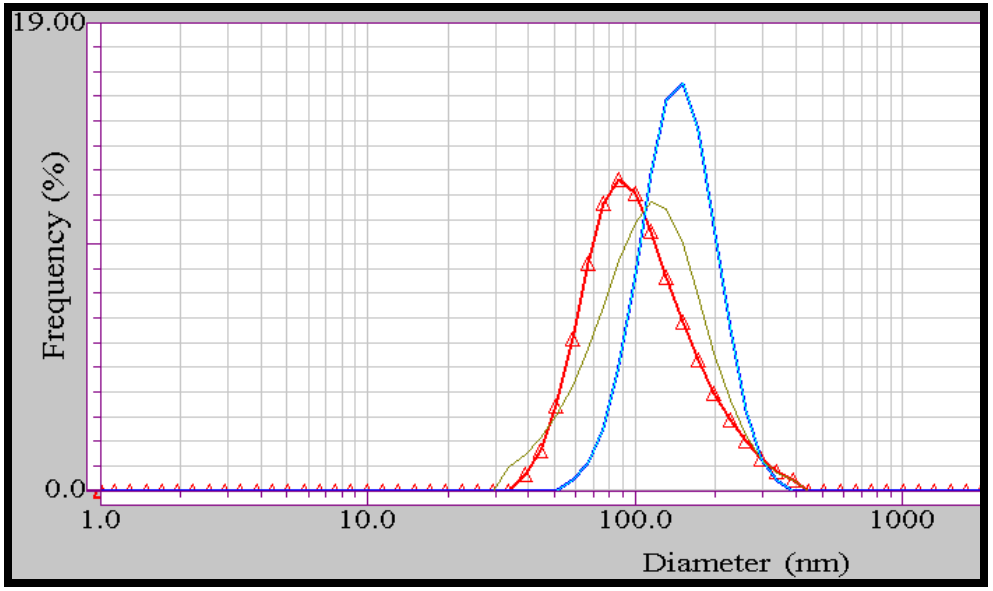
- Before fluidization
- After fluidization – decrease in size



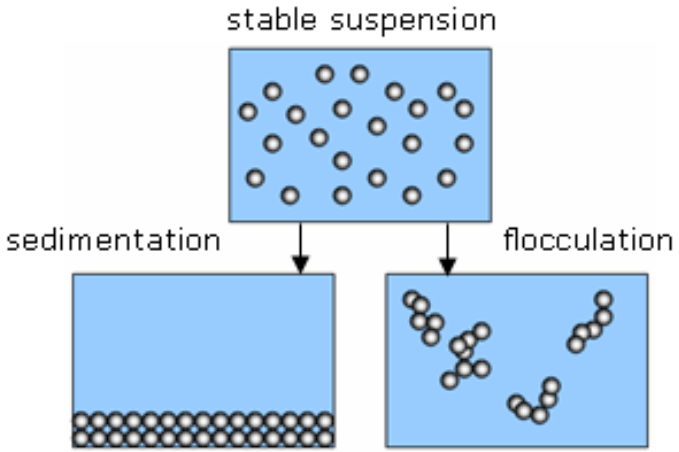
Long Term Liposome Stabilization

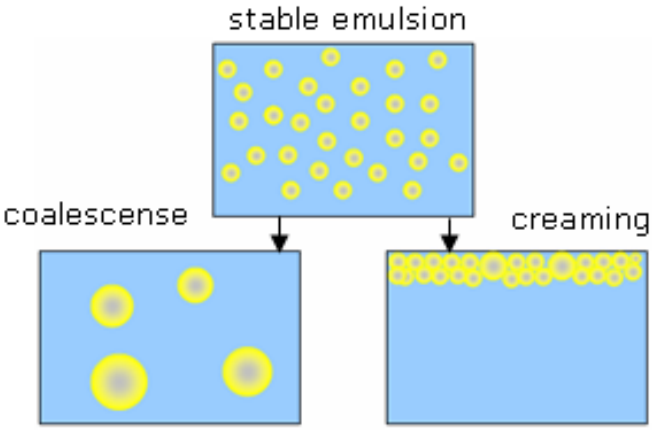
- Change in Stability as a function of pH
- pH adjusted to induce agglomeration
- Liposome sensitive to ionic and salt environment

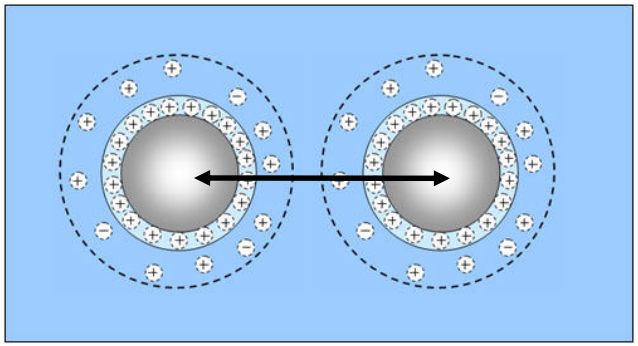
Liposome	Mean (nm)	PDI
1 Pass	107	0.520
1 Pass	104	0.461
1 Pass	98	0.465
1 Pass	109	0.537
Before	438	0.932
Before	456	0.518
Before	451	0.894
Before	444	0.810
1 Pass pH 12	141	0.340
1 Pass pH 2	116	0.462

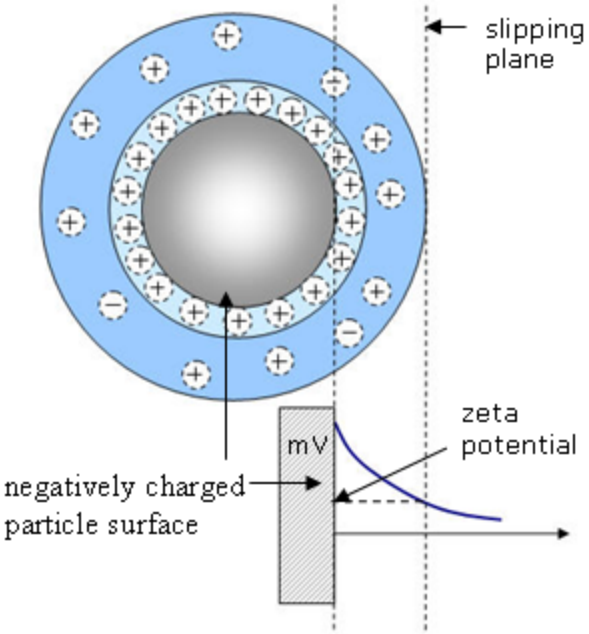


Particle Stability



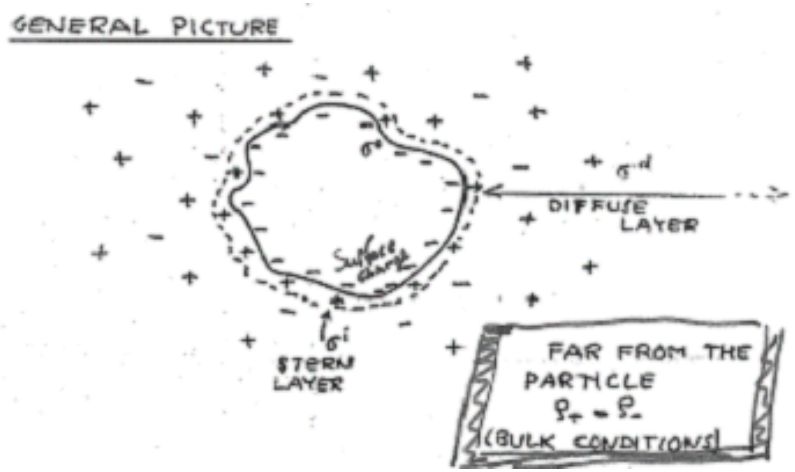






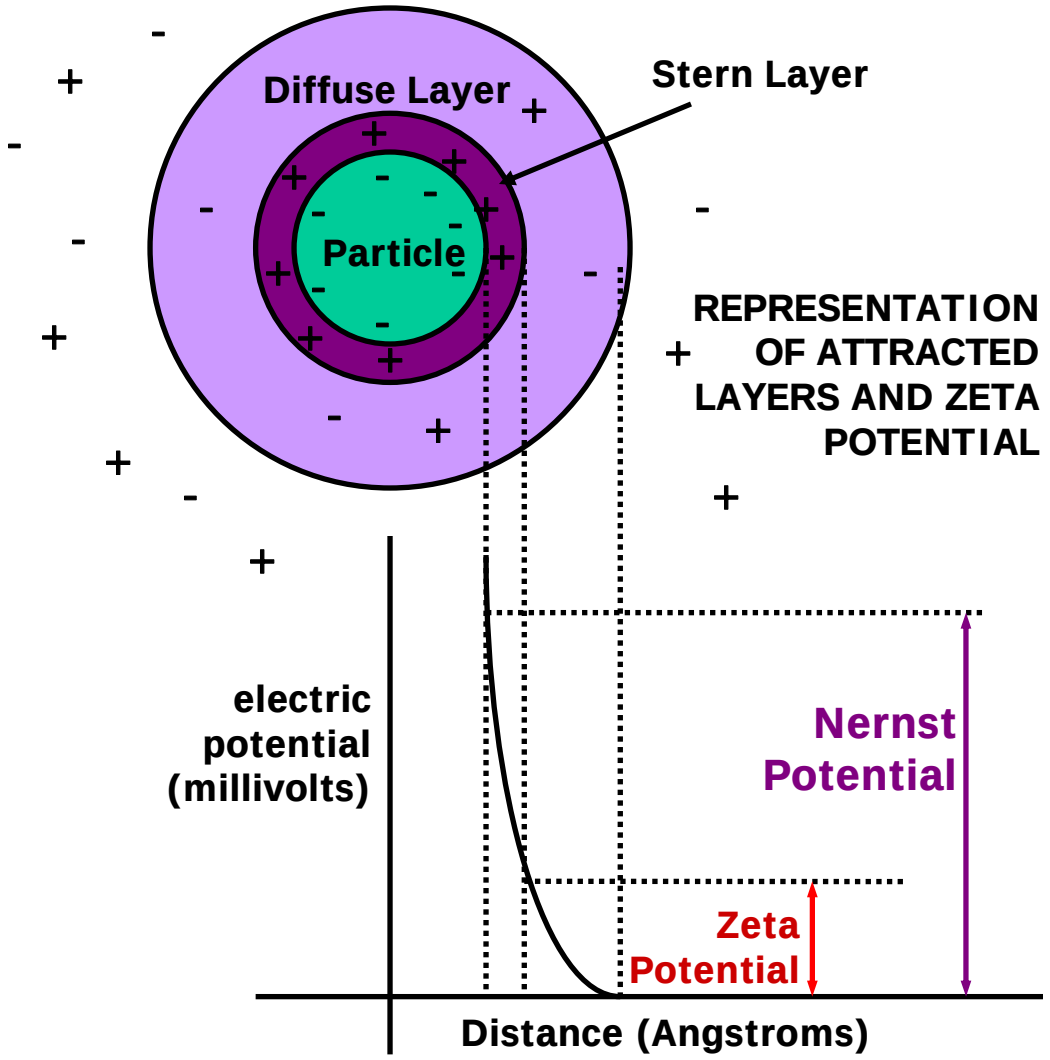
Zeta Potential and Stability

- An Electric Double Layer forms spontaneously around charged particles in an ionic matrix
- The more Diffusely the counter charge is distributed around the particle the stronger the chemical potential



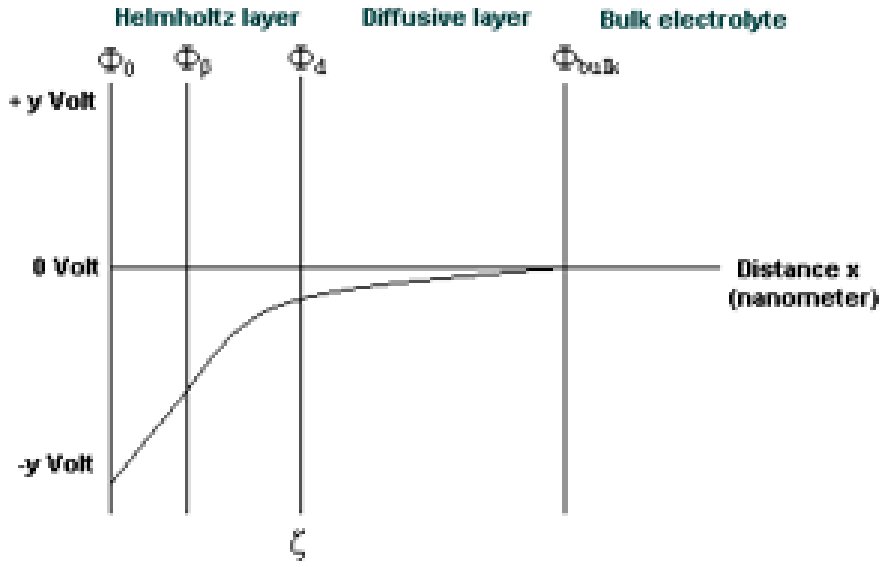
ZETA POTENTIAL

- If a particle is negatively charged, a thin layer of positive charge forms around the particle (the **Stern Layer**).
- Beyond the Stern Layer, is the **DIFFUSE Layer** where there is a wider layer of mostly opposite charge.
- The potential at the surface of the particle is designated the **NERNST Potential**, and the potential at the outside of the Stern layer is designated the ZETA Potential.
- **ZETA Potential** is a useful because it quantifies the surface activity of colloidal particles.



Zeta Potential and Stability

- The Diffuse Layer contains only a small fraction of counter charge 10%
- But, it extends far into the solution
- Therefore, it is of prime relevance for interactions



Van der Waals or London Forces

- Short range
- Attractive forces
- Strong force inversely proportional to size
- Drives Aggregation

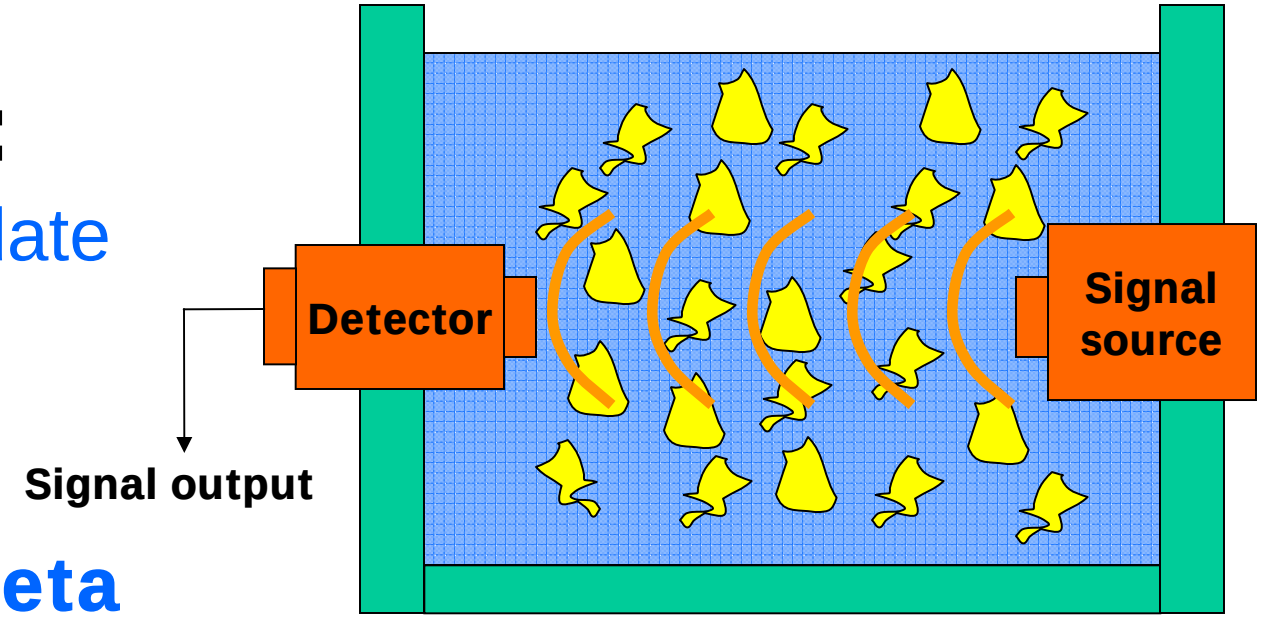
The Strength of Electrostatic Interactions

- If all the electrons were removed from one-tenth of a cubic millimeter from the nose cone of the space-shuttle and placed on the pad
- The attraction would be so great between the positive charge on the cone and negative charge on the pad
- The shuttle would remain locked in place despite full thrust of the rockets

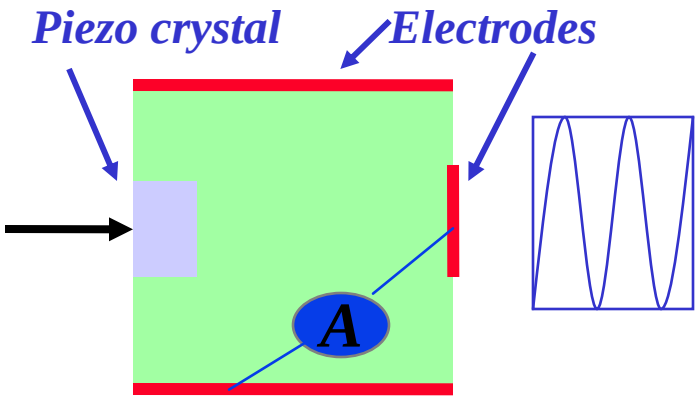
Acoustic Spectroscopy

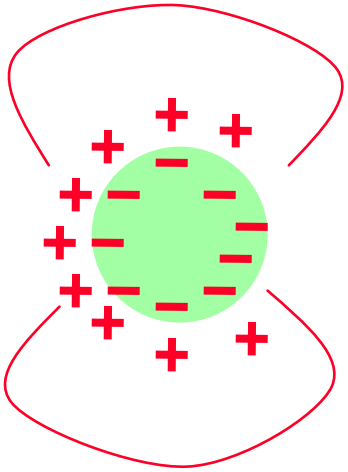
Advantages:

- Can accommodate high sample concentrations
- Can measure **Zeta Potential – in native concentration**

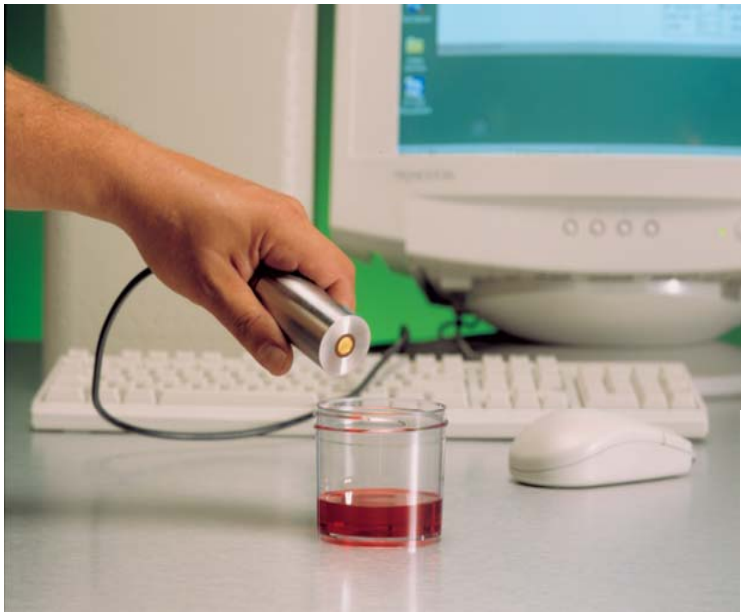


Electroacoustics – Zeta Potential





Zeta Potential Probe



Colloid Vibration Current

$$CVI = C\frac{\rho_p - \rho_m}{\rho_m}\varphi\,\mu_d\nabla P$$

DynamicMobility

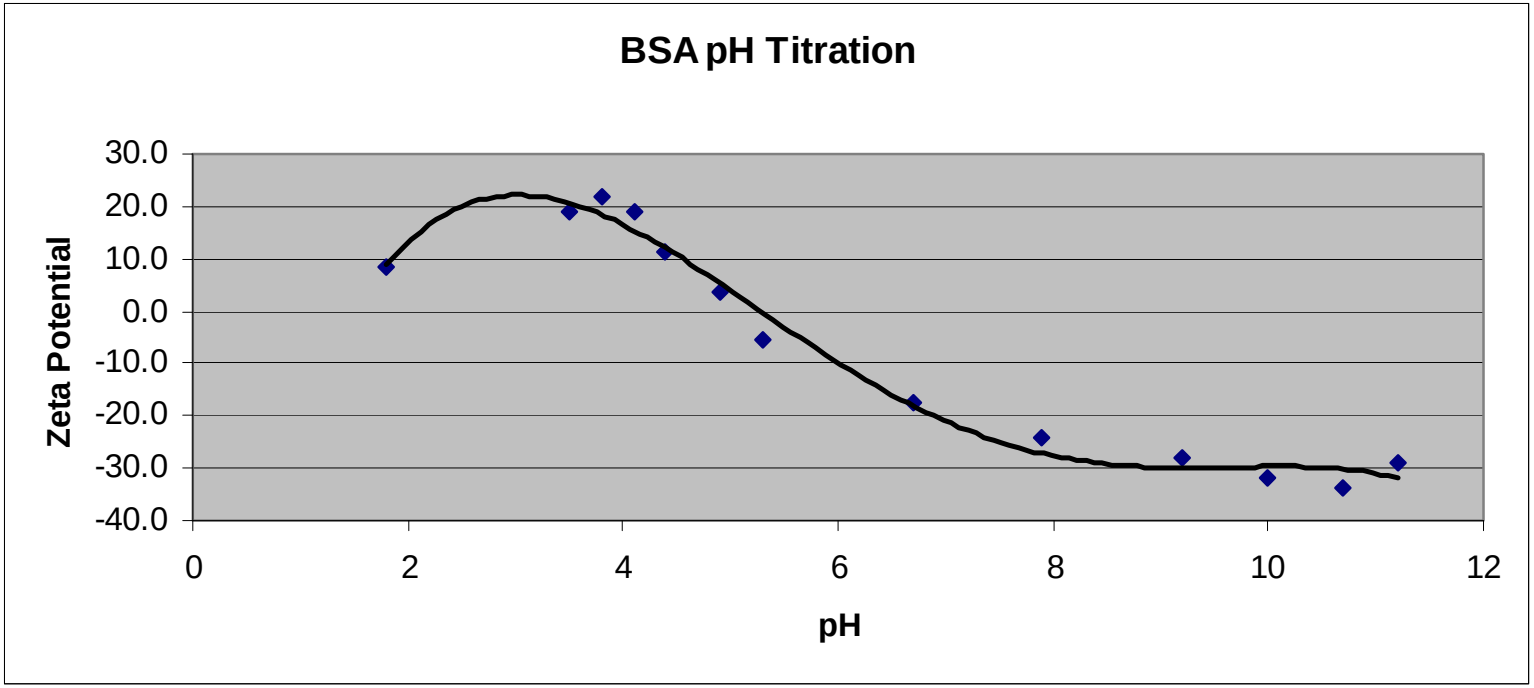
$$\mu_d = \frac{\varepsilon_m\varepsilon_o\varsigma}{\eta}\frac{(\rho_p - \rho_s)\rho_m K_s}{(\rho_p - \rho_m)\rho_s K_m}$$

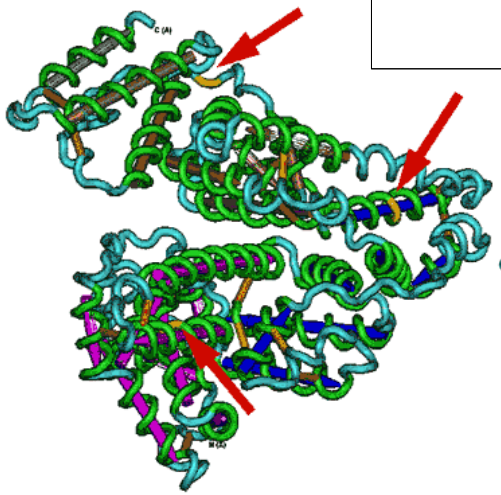
HORIBA

Acoustic Spectroscopy Benefits

- Dilution will disrupt the Zeta Potential
- Acoustic Spectroscopy the sample is not diluted
- Electro-acoustics measures Zeta Potential without dilution
- Non-invasive – no voltage is applied to the sample- no deleterious current

Titration Curve BSA



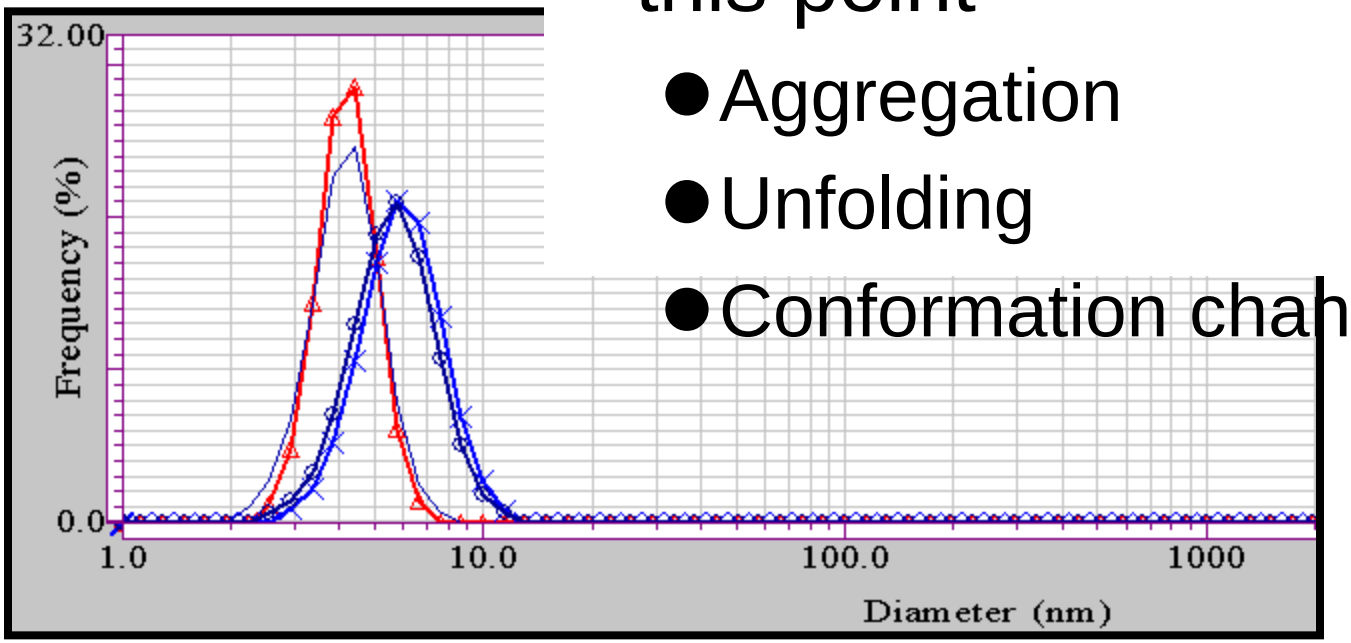


serum albumin
ca. 600 amino acids,
20 tyrosines,
3 nitrated with
NO_2/O_3

BSA at pH 5.6 and 4.2

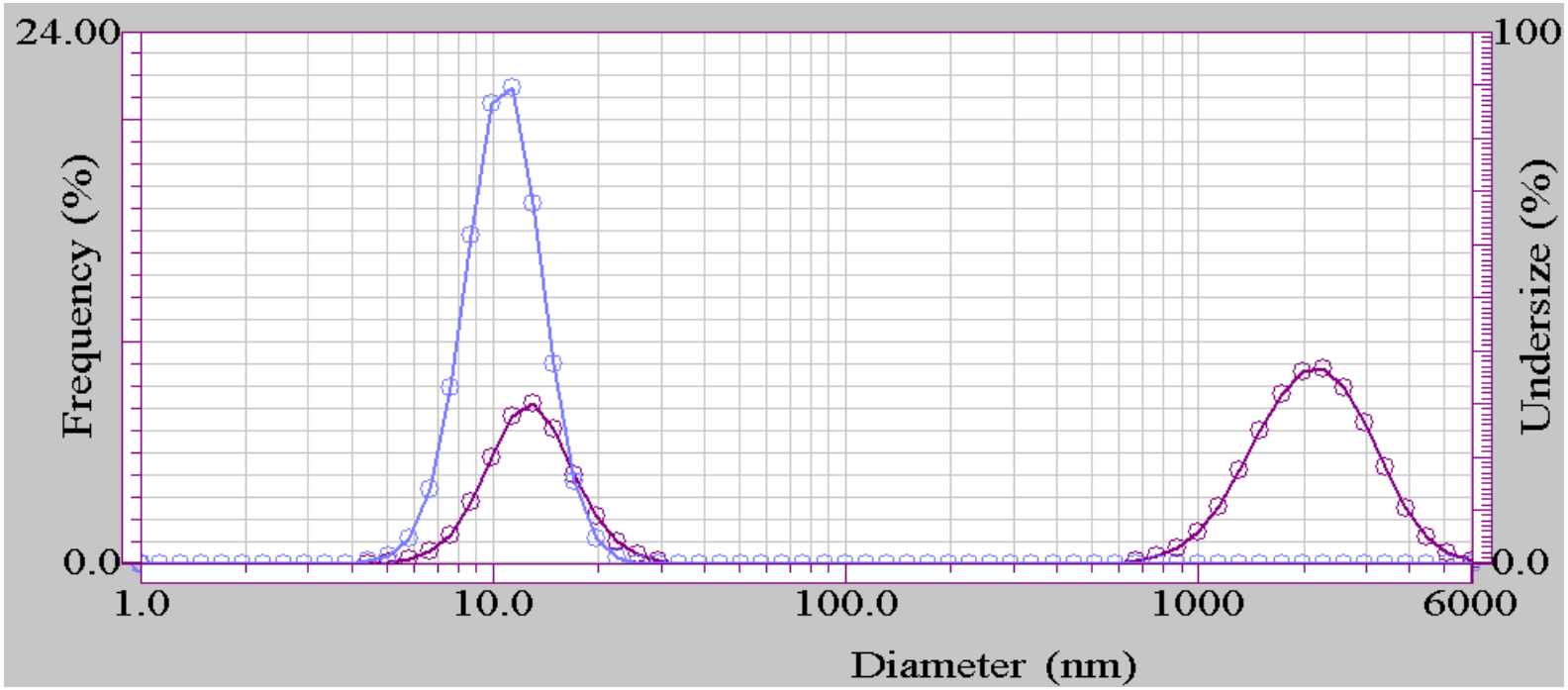
BSA	Mean	pH
	5.4	5.6
	5.7	5.6
	4.3	4.2
	4.4	4.2

- Conformational changes in BSA
- BSA iso electric point is 5.5
- There is a change in size near this point
  - Aggregation
  - Unfolding
  - Conformation changes

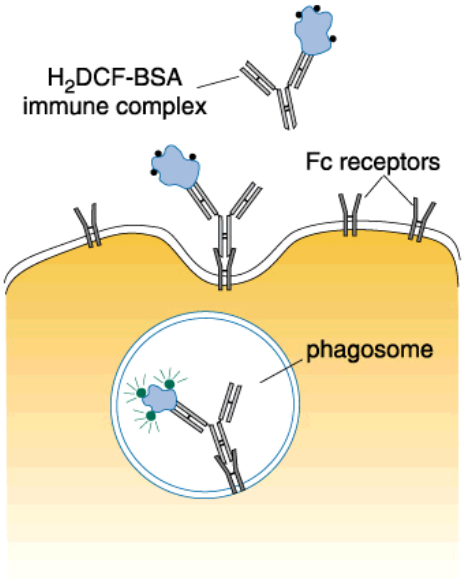


Overlay dimer and aggregate

- Change in Size as pH is Adjusted
  - Near the IEP (dimerized)
  - BSA at pH extremes (aggregated)



Tabulated Size Data

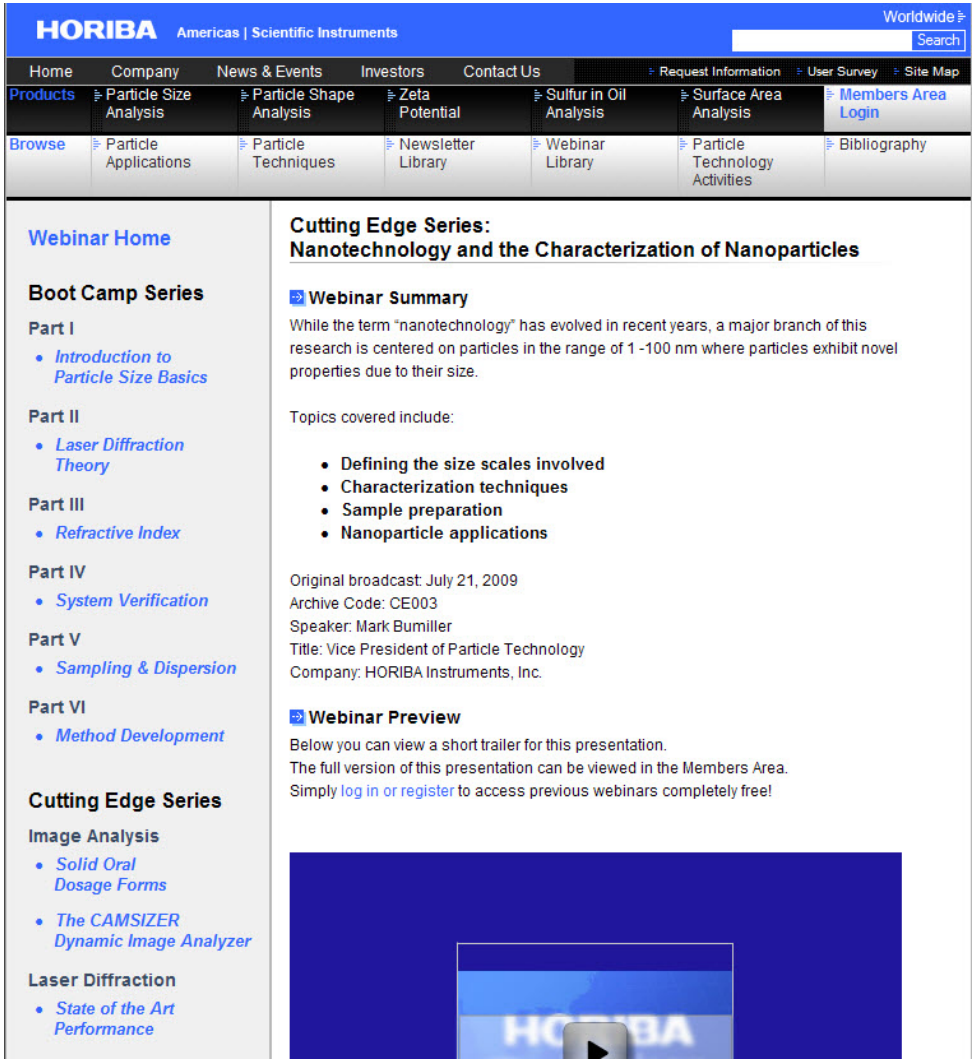


	Mean (nm)	CV	PDI
BSA pH 5.5	10.3	25.032	0.125
BSA pH 5.5	10.7	25.3542	0.129
BSA pH 5.5	10.6	37.9427	0.288
BSA pH 5.5	10.2	28.1969	0.159
BSA pH 5.5	10.3	26.578	0.141
BSA pH 5.5	10.1	28.5343	0.163
BSA pH 5.5	10.3	25.0322	0.125
BSA pH 1.7	1552	84.2164	1.418
BSA pH 1.7	1333	89.1802	1.591
BSA pH 1.7	1914	75.3278	1.135

Review

- DLS
  - Quick
  - Robust
  - Quantitative
  - Non-invasive
- Zeta Potential
  - Formulation Stability Information
  - Information on charge distribution
  - Completely Non-invasive

Webinar Library



Questions?

The End

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