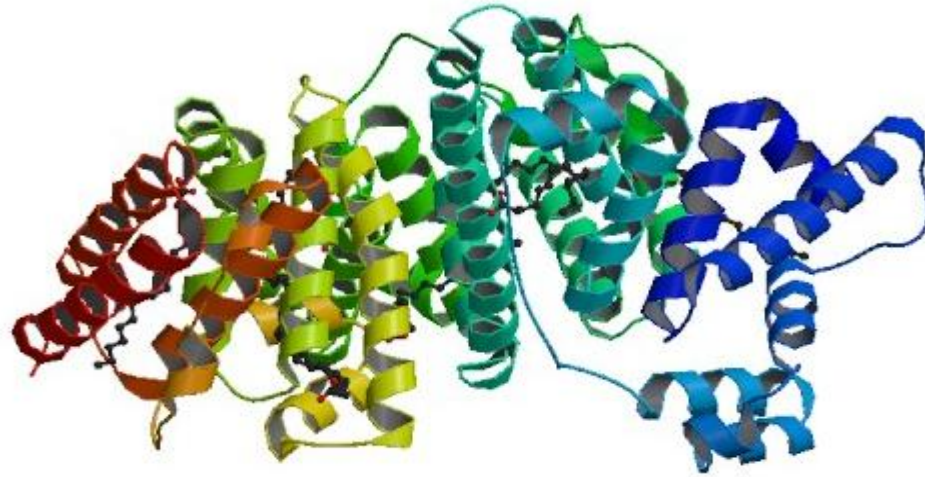


Analytical Techniques for Probing Protein Aggregation



(Source: <http://www.pdb.org>)

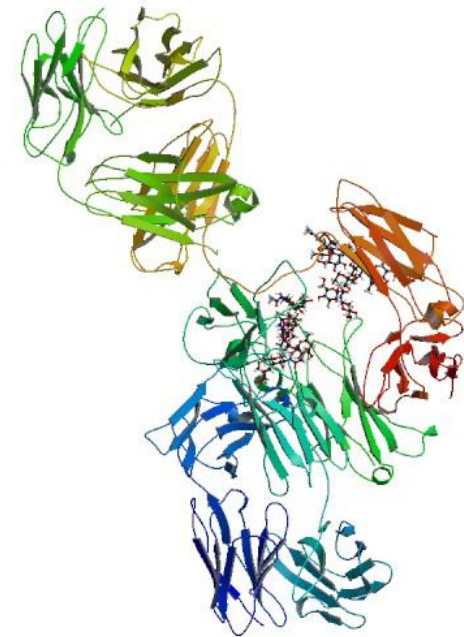
Innocent Bekard
(**CSL** Biotherapies)

Introduction

Proteins

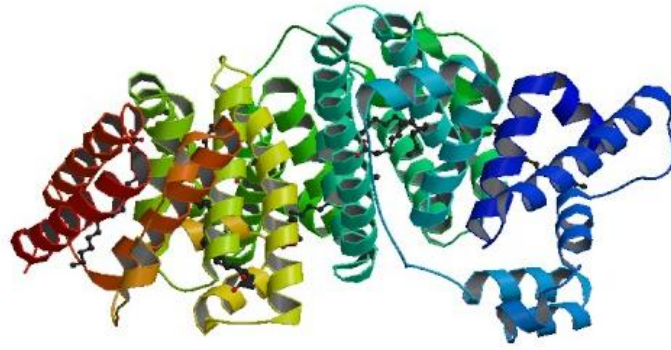
- polymer chains composed of amino acid residues
- assume a unique 3D structure in solution
- most abundant biological macromolecules
- exhibit enormous diversity in biological function

e.g. - enzymes
- hormones
- antibodies
- cytokines
- carrier proteins
- growth factors



Source: <http://www.pdb.org>

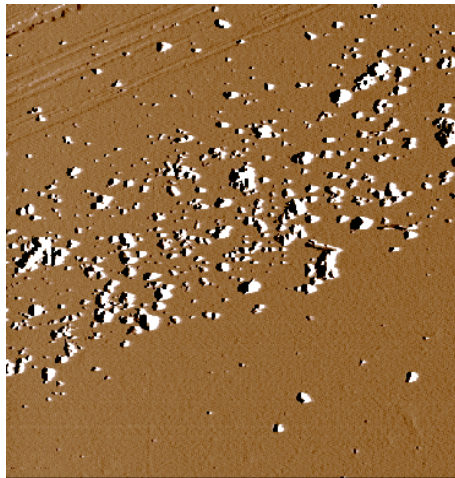
Protein aggregation



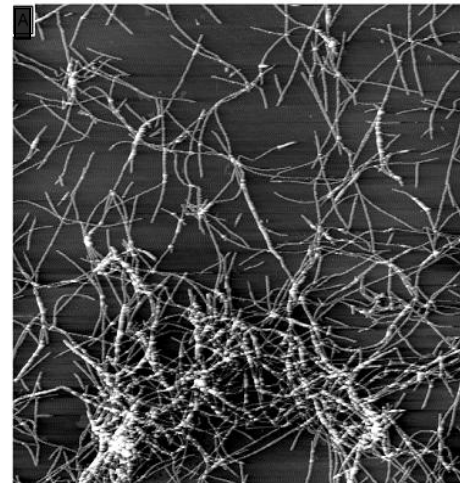
native conformation

Stress

- temperature
- pH
- denaturants
- shear



Amorphous aggregates



Fibrillar aggregates

Concerns about protein aggregation

Industry

- Aggregation during processing
 - *ultrafiltration*
 - *pumping*
 - *stirring*
- Aggregation during transportation and storage
- Therapeutic protein aggregates are:
 - *dysfunctional*
 - *reduce shelf life of product*
 - *trigger adverse reaction*
 - *elicit undesirable immune responses*

Physiology

- Protein misfolding diseases
 - *Alzheimer's disease*
 - *cataract*
- Vascular disorders
 - *atherosclerosis*

Techniques for detecting protein degradation, denaturation and aggregation

Protein structure

1°

- Mass spectrometry

2°

- Fourier transform IR spectroscopy
- Circular dichroism spectroscopy

3°

- Circular dichroism spectroscopy
- Fluorescence spectroscopy
- Differential scanning calorimetry

Protein aggregates

- Light scattering (SLS, DLS)
- Turbidimetry
- Analytical ultracentrifugation
- Particle counting
- Fluorescence spectroscopy

Aggregate morphology

- Electron microscopy
- Atomic force microscopy

Circular dichroism

Employs the differential absorption of left and right circularly polarised light to study the solution conformation of chiral molecules e.g. proteins

Far-UV CD

- **Range:** 180 – 240nm
- **Chromophore:** polypeptide backbone
- **Finger print region for protein secondary structure**

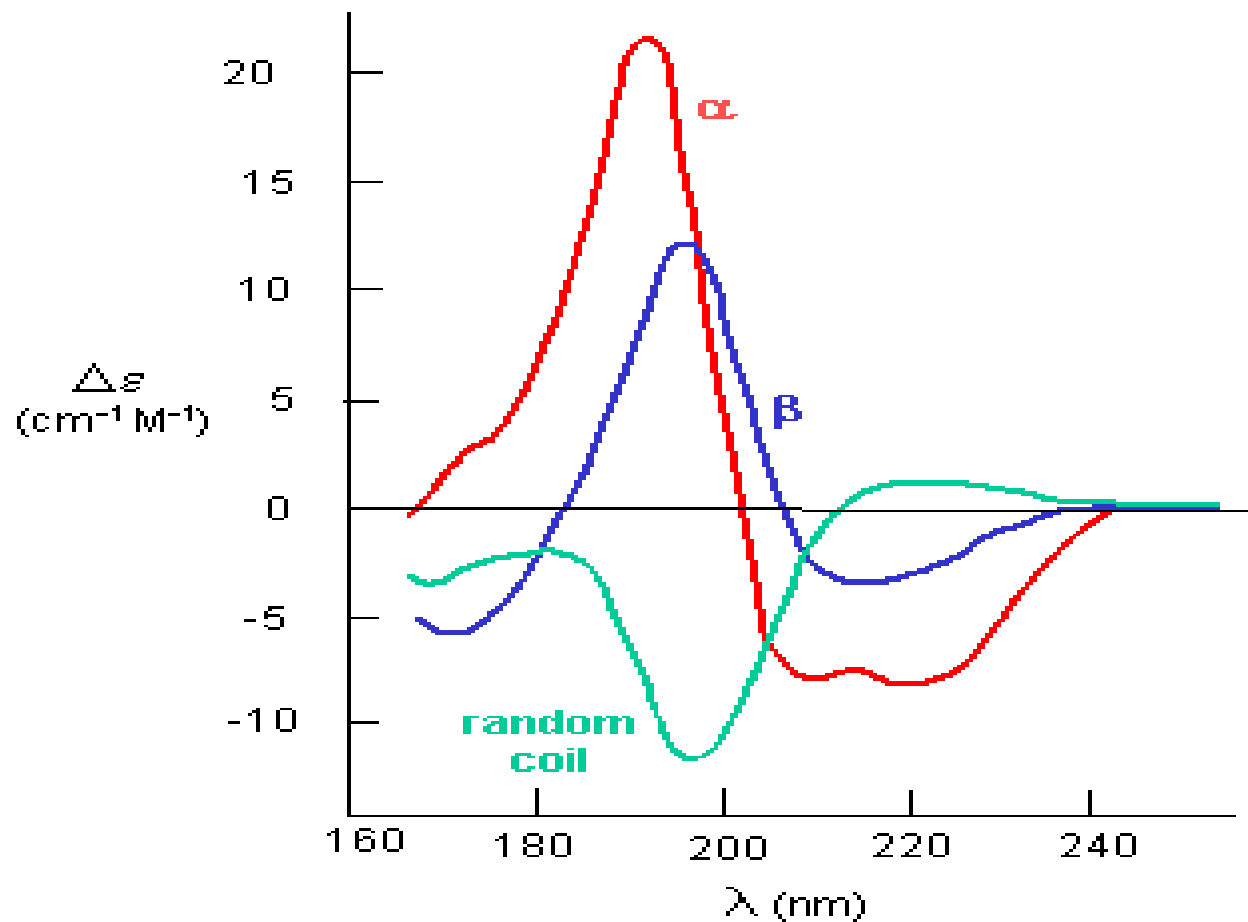
Near-UV CD

- **Range:** 250 – 350nm
- **Chromophore:** Trp, Tyr, Phe, His, disulfide bonds
- **Finger print region for protein tertiary structure**

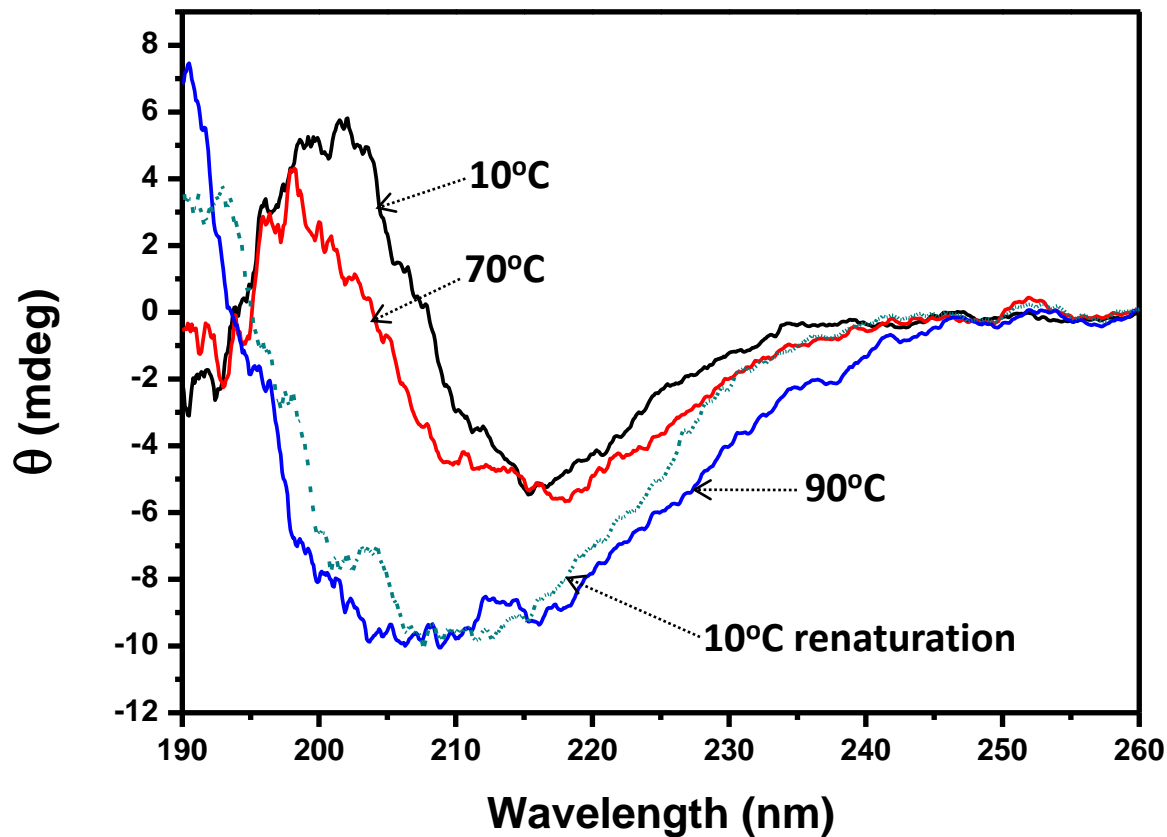


Circular dichroism spectropolarimeter

CD spectra of the basic protein secondary conformations

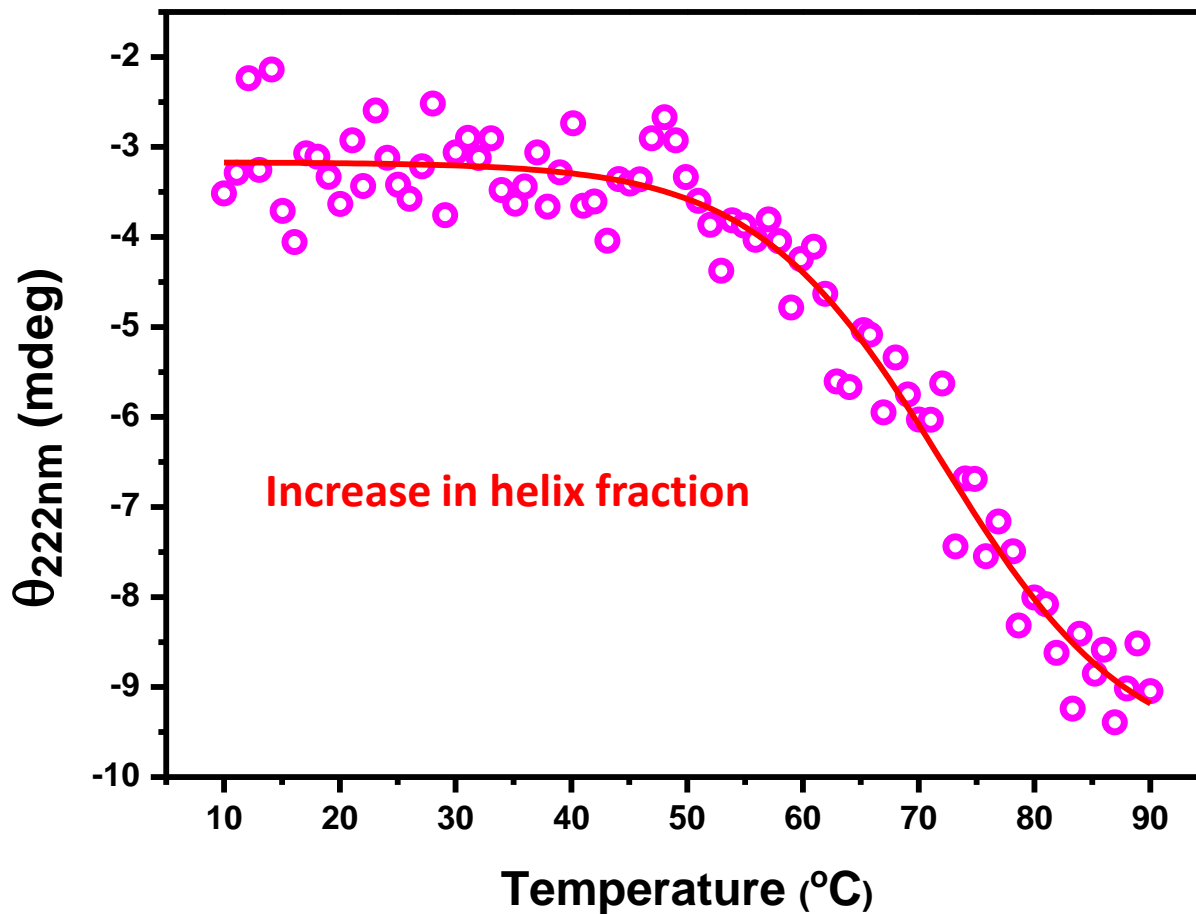


CD spectra of IgG during thermal treatment

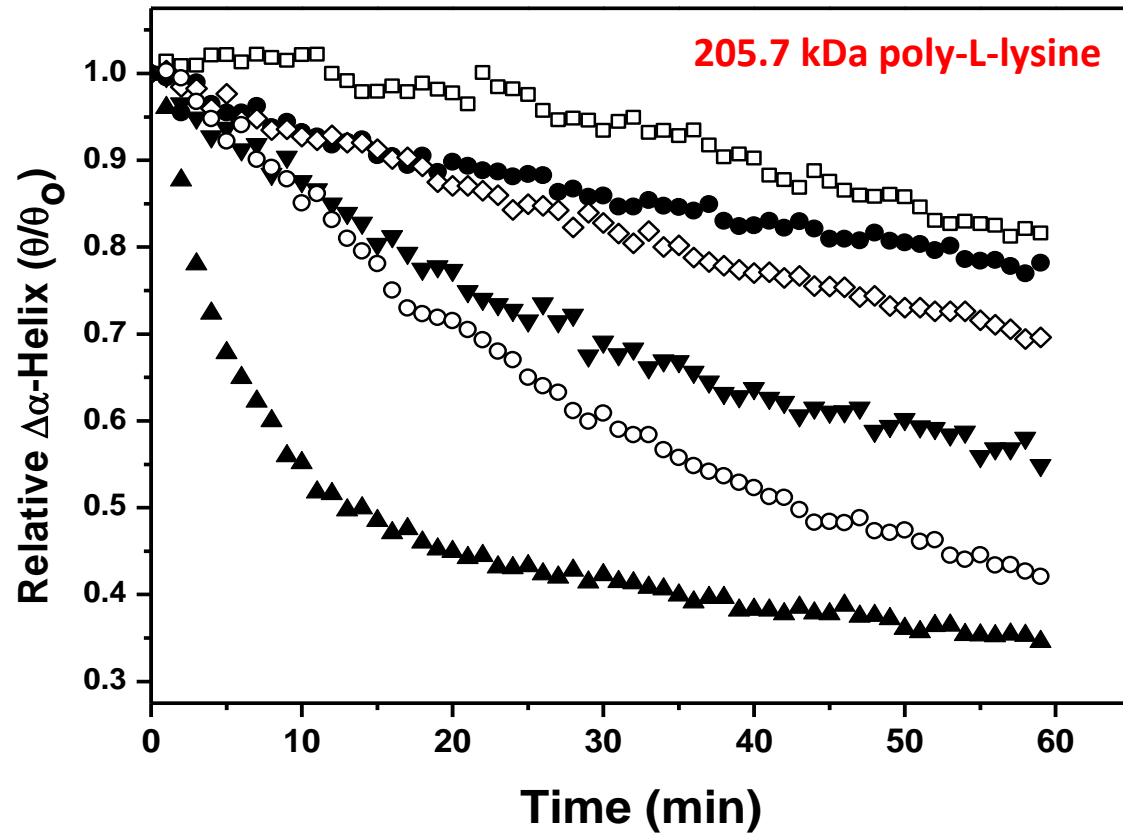


	Temperature (°C)	α -helix %	β -content %	Unordered%
Denaturation	10	3 \pm 1	63 \pm 2	34 \pm 1
	70	7 \pm 2	59 \pm 3	33 \pm 1
	90	12 \pm 2	53 \pm 3	35 \pm 1
Renaturation	10	10 \pm 1	57 \pm 2	34 \pm 1

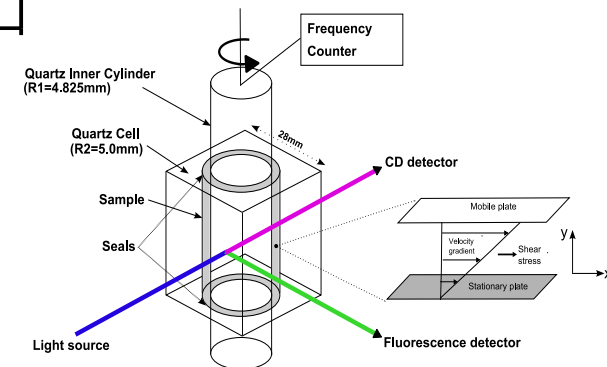
Thermal denaturation of IgG: Kinetics



Shear rate dependence of helix unfolding in poly-L-lysine



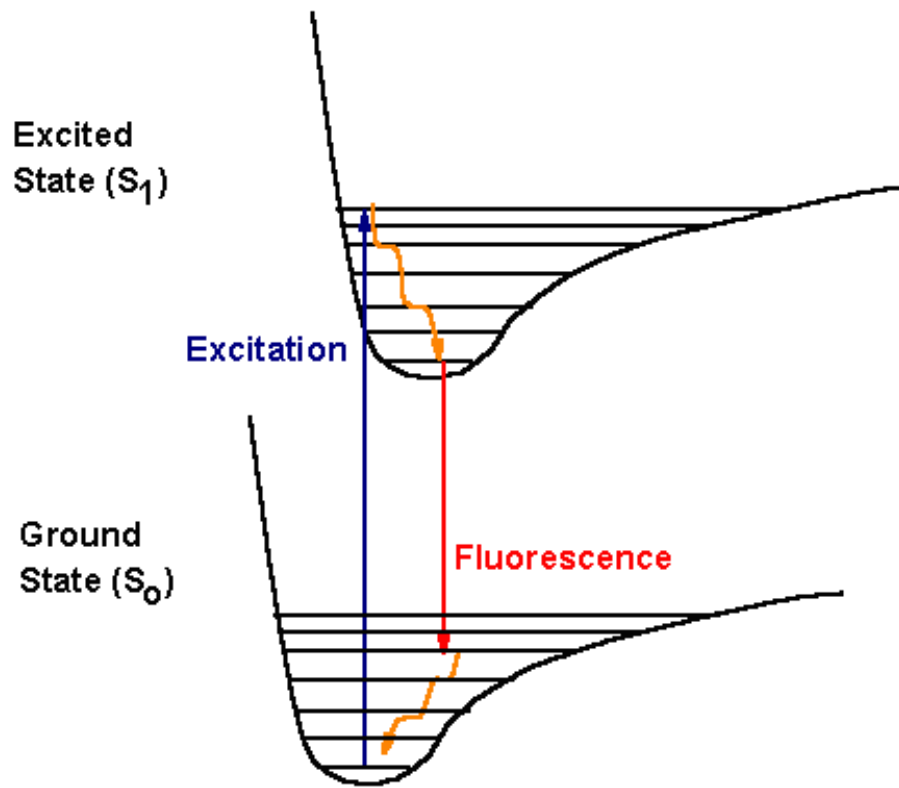
- (□) 74 s⁻¹
- (●) 117 s⁻¹
- (◇) 219 s⁻¹
- (▼) 302 s⁻¹
- (○) 518 s⁻¹
- (▲) 715 s⁻¹



Fluorescence spectroscopy

- **Fluorescence**

Sensitive to the conformational and structural transitions in protein molecules via change in the fluorescent properties of the **fluorophore** of interest.



Fluorescence spectrophotometer

Fluorescence: protein aggregation probes

Intrinsic fluorophores

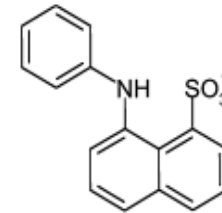
Tryptophan, Tyrosine, Phenylalanine

Extrinsic fluorophores

Solvent-exposed hydrophobic groups

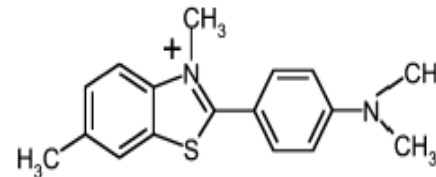
1-anilinonaphthalene-8-sulphonic acid (ANS)

(E_x 350nm, E_m 488nm)



Fibril formation

Thioflavin T (E_x 450nm, E_m 482nm)



Additionally

Oligomers (large aggregates)

Light scattering (E_x 600nm, E_m 605nm)

Mechanism of insulin aggregation

native insulin structure

60°C

pH 1.9

Stirring 120rpm



denaturation/unfolding

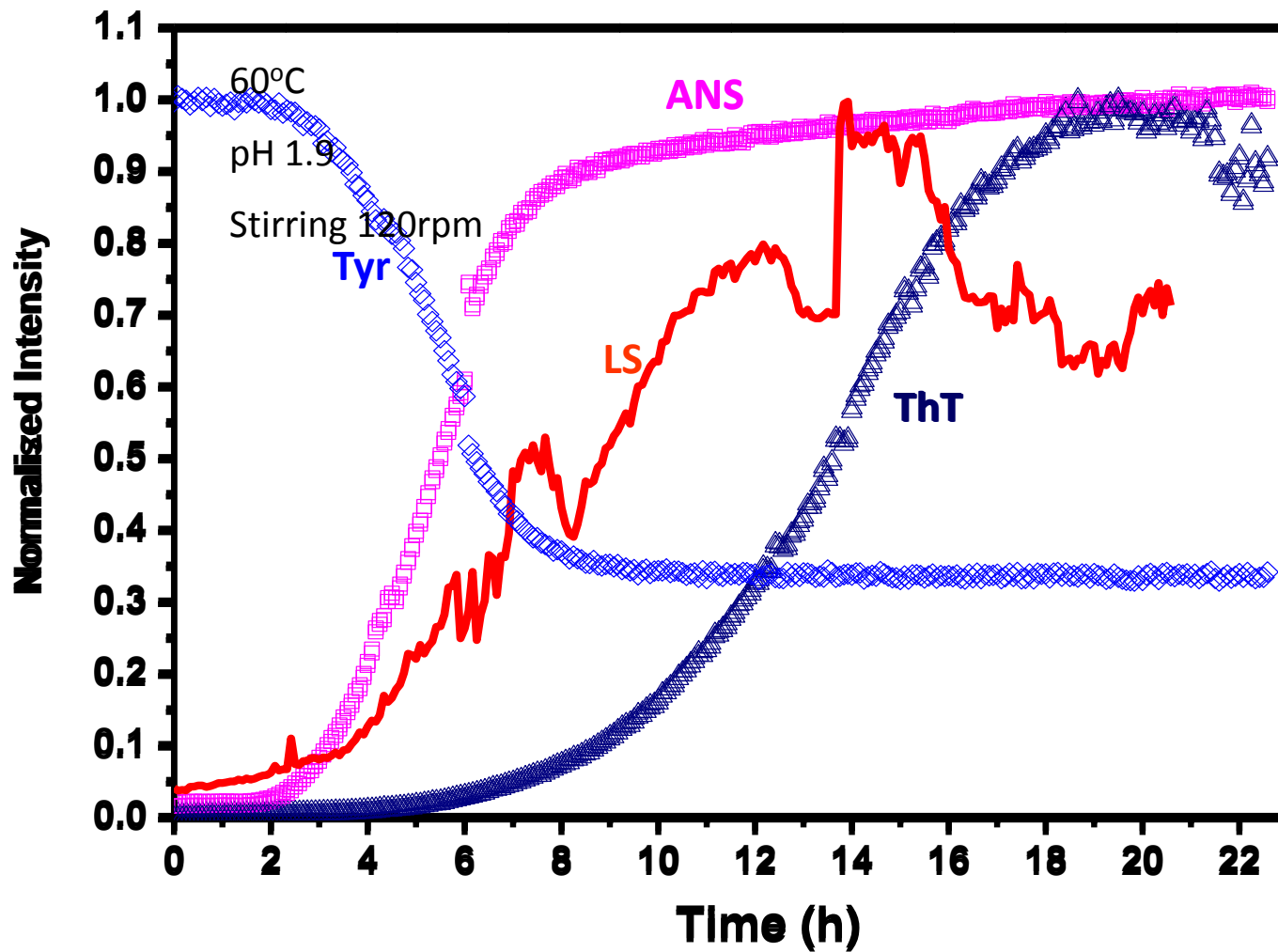
partially folded intermediate



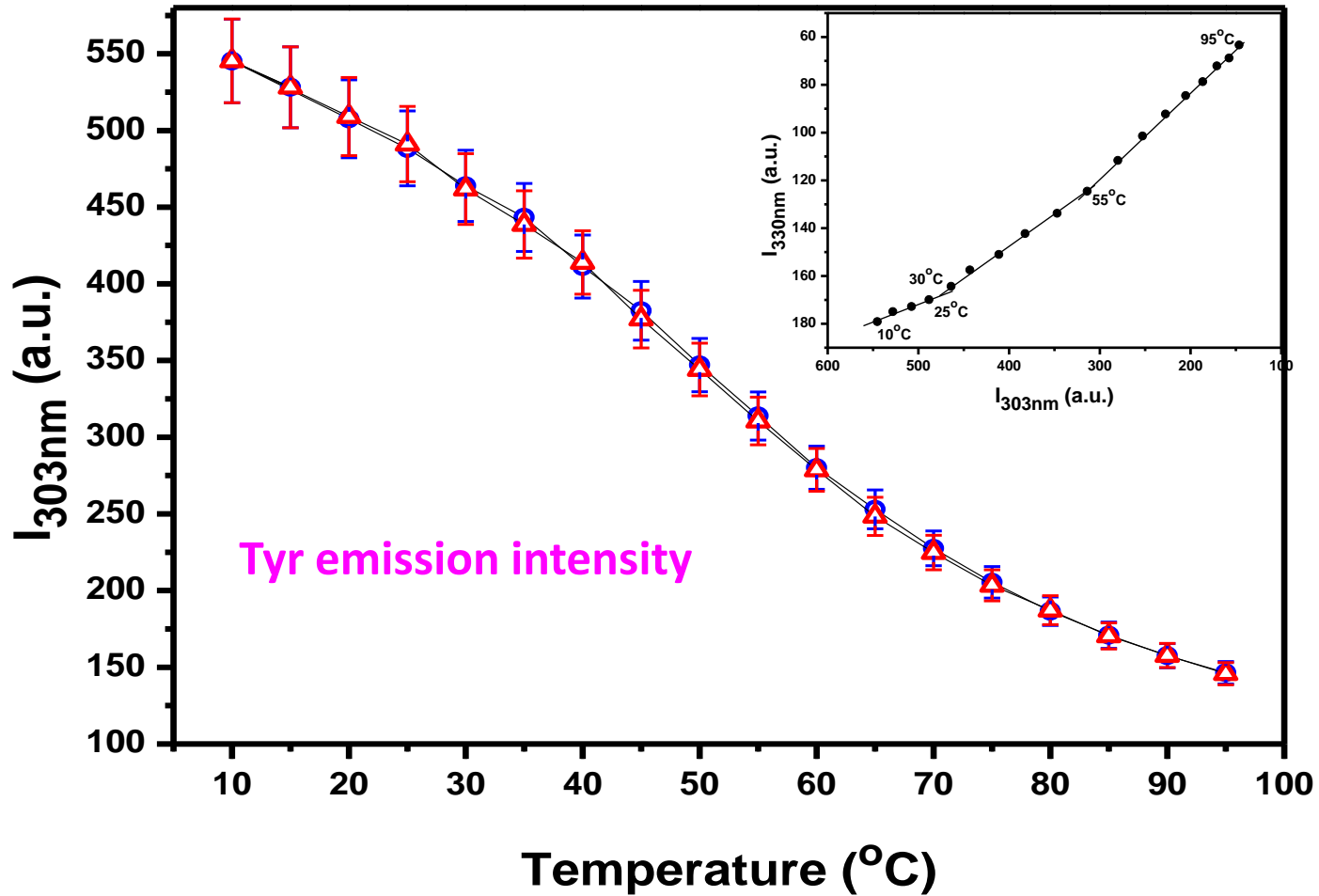
coalescence

aggregates/amyloid fibrils

Kinetics of insulin aggregation



Thermal denaturation and renaturation of bovine insulin

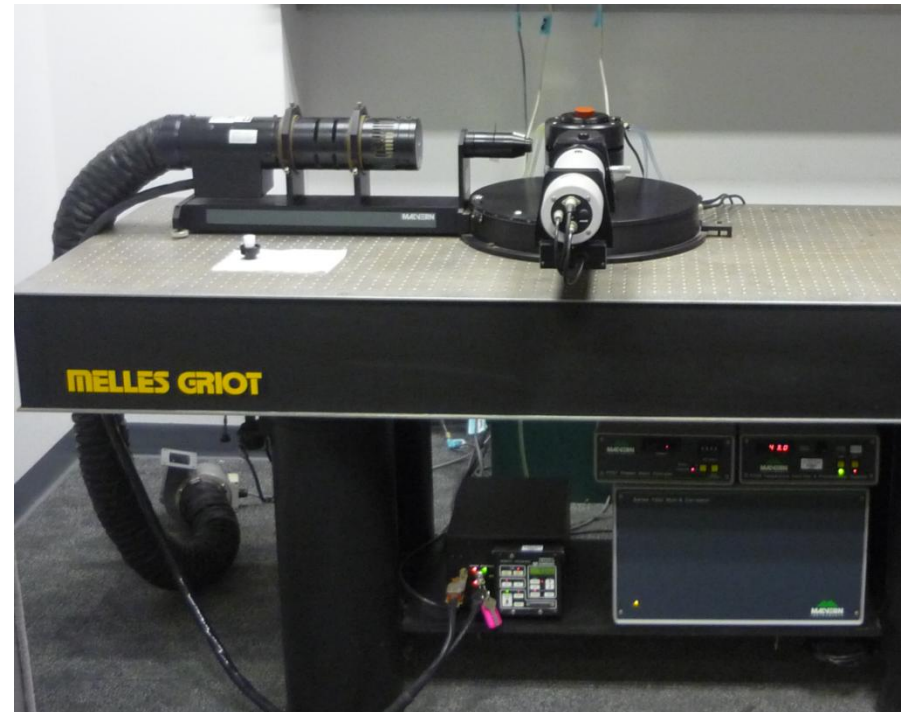


Light Scattering

Exploits the scattered intensity of incident light, measured at a particular angle, to detect change in the hydrodynamic size of protein molecules in solution.

Sensitivity

- Detects particle size <1 nm



Static and Dynamic Light Scattering

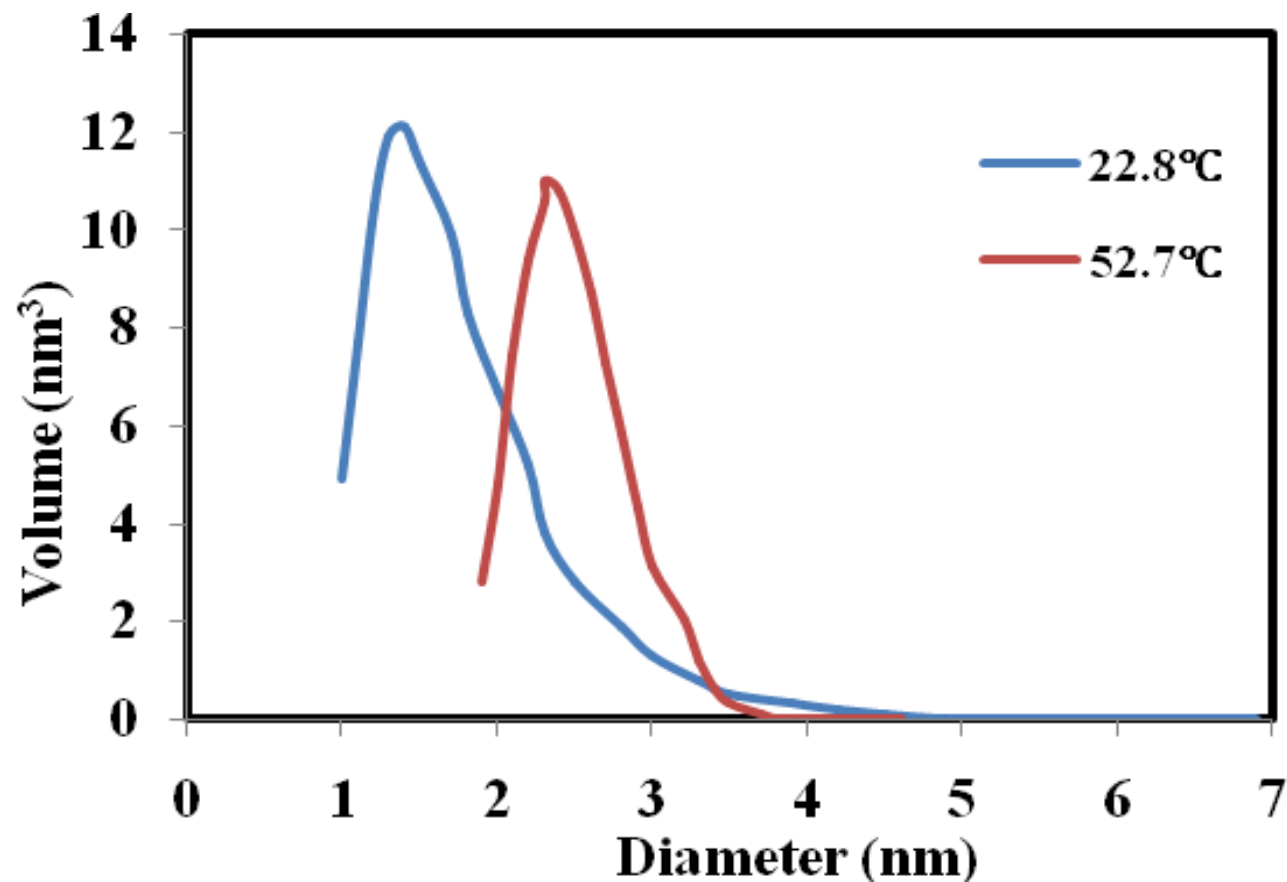
Static light scattering

- Measures scattered intensity of incident light
- Calculates **molecular weight** and **radius of gyration** of macromolecules
- Employed in multiple angle laser light scattering (MALLS) instruments

Dynamic light scattering

- Measures fluctuations in scattered intensity of incident light
- Calculates **hydrodynamic radius** of macromolecules in solution

DLS characterisation of thermally treated IgG



Atomic force microscopy

Employs a beam deflection system to:

- Measure forces at the molecular level
- Acquire high resolution images of nanoparticles
- Modify surfaces to produce nanostructures

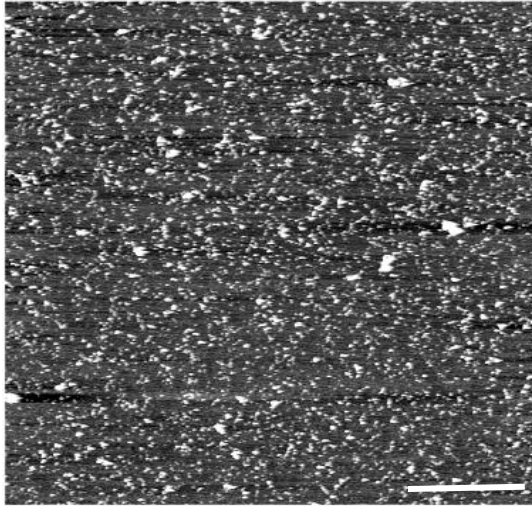
Operates in two main modes

- contact mode
- intermittent contact mode



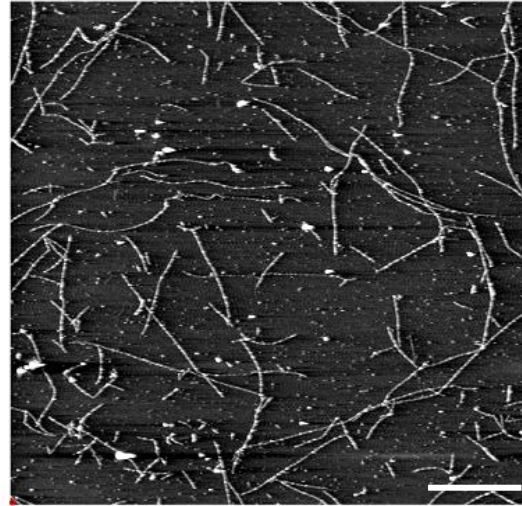
Atomic force microscope

Morphological transitions in amyloid- β fibril assembly



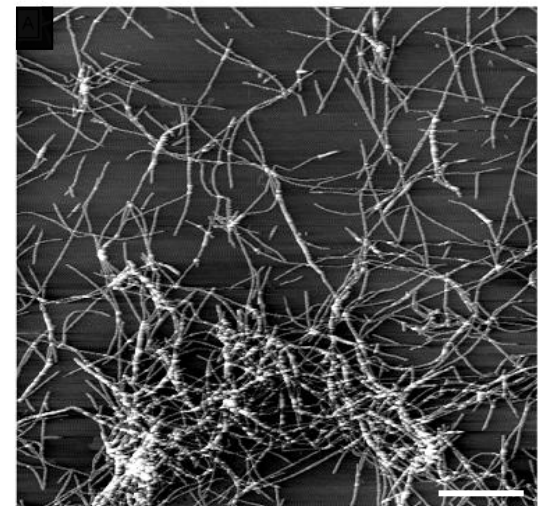
Oligomeric
aggregates

.....▶
37°C, stirring



Protofibrillar
aggregates

.....▶
37°C, stirring



Fibrillar
aggregates

Conclusion

- There are **many sensitive techniques** that allow the early detection of protein degradation, denaturation and subsequent aggregation during processing, formulation and storage
- Identification of processing conditions (physical and chemical) that initiate protein denaturation and aggregation is key to producing particle free formulations
- An **optimised** manufacturing environment will ensure the **safety** and **quality** of therapeutic protein products.
- Every **protein is unique**.