Refolding of Bovine Serum Albumin by Gemini Surfactants via Artificial Chaperone Protocol

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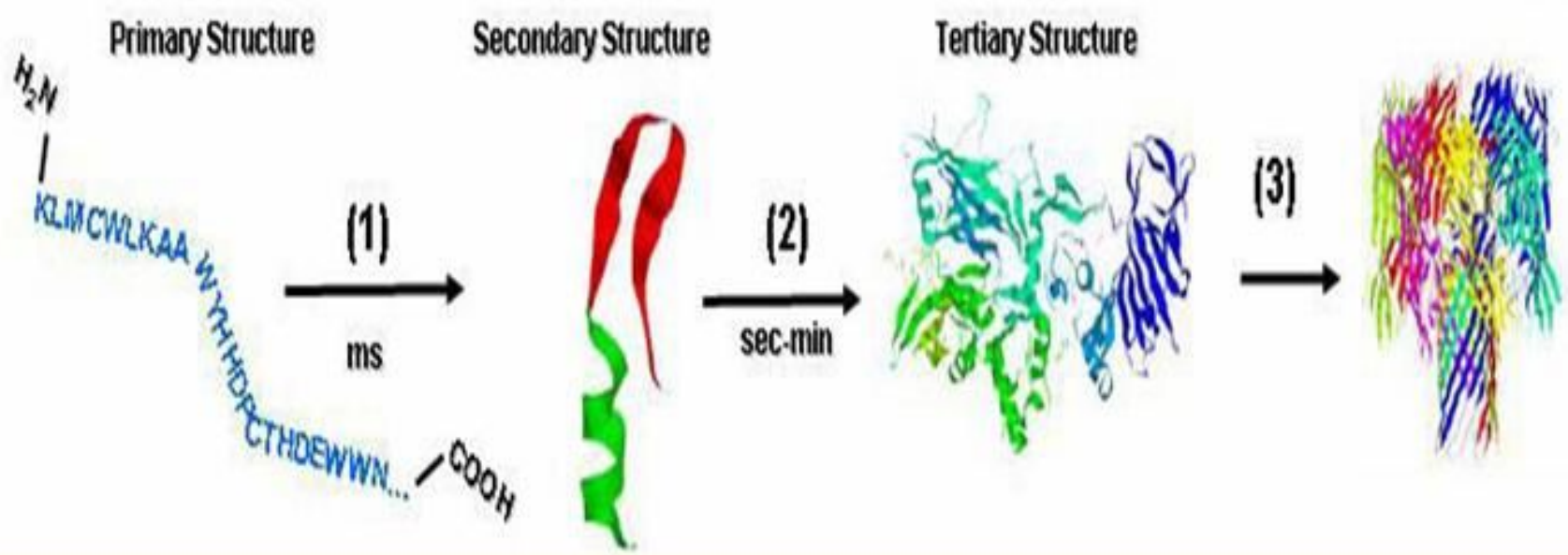
CSIR

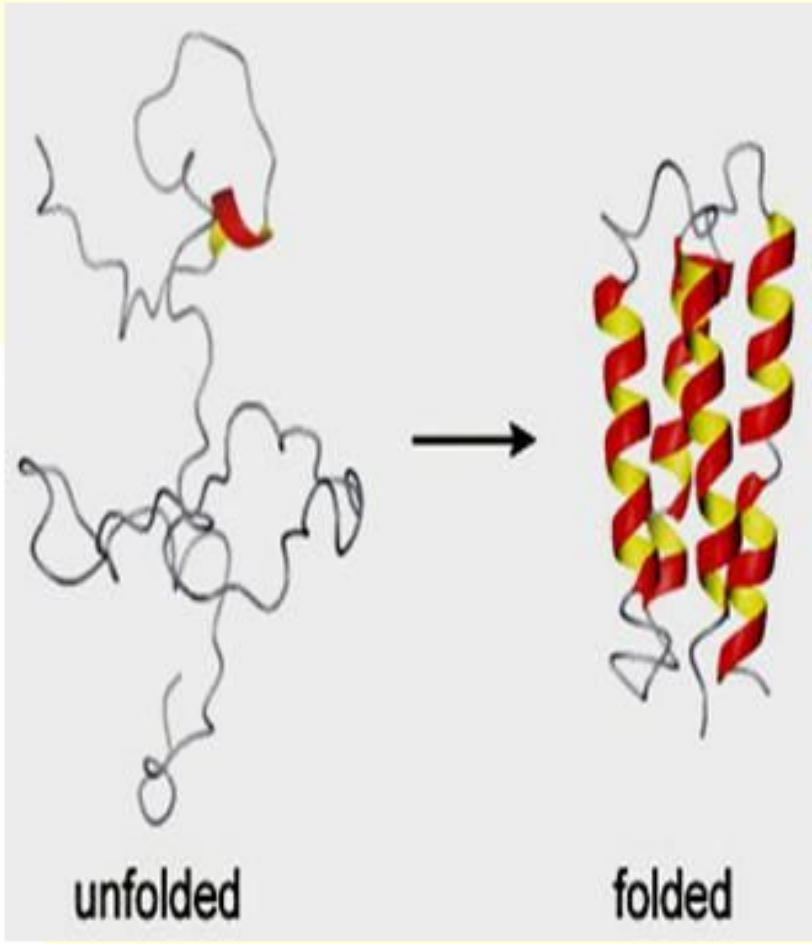
Plan of the talk

- ❖ **Protein folding**
- ❖ **Introduction to Artificial Chaperone Protocol for protein refolding**
- ❖ **Objectives of the present work**
- ❖ **Motivation**
- ❖ **Results**
- ❖ **Conclusions**

Protein Folding

Protein folding is the physical process by which a polypeptide folds into its characteristic and functional three-dimensional structure from random coil.

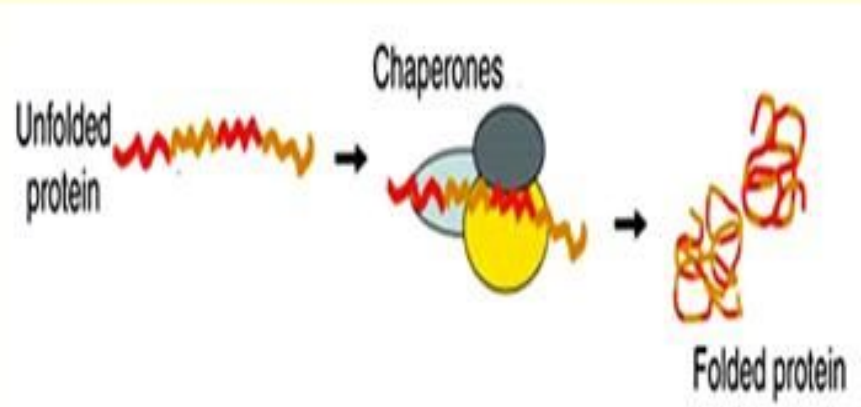




Protein folding is guided by Hydrophobic Interactions; Formation of intramolecular H-bonds and Vander Waal's forces

Protein Folding depends on:

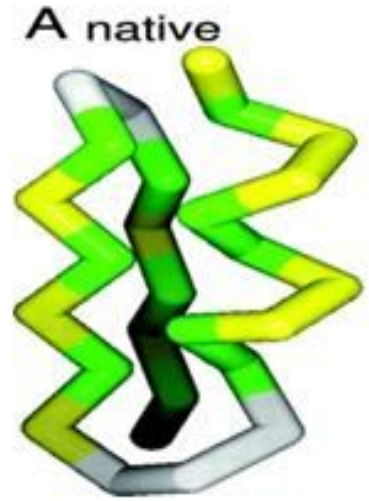
- ✓the solvent (water or lipid bilayer),
- ✓the concentration of salts
- ✓the temperature and
- ✓the presence of chaperones

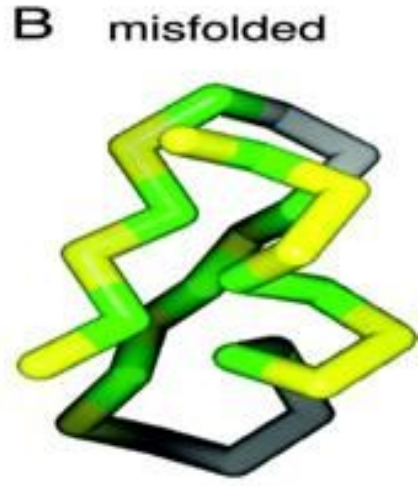


Misfolding and Aggregation of Proteins

Misfolding

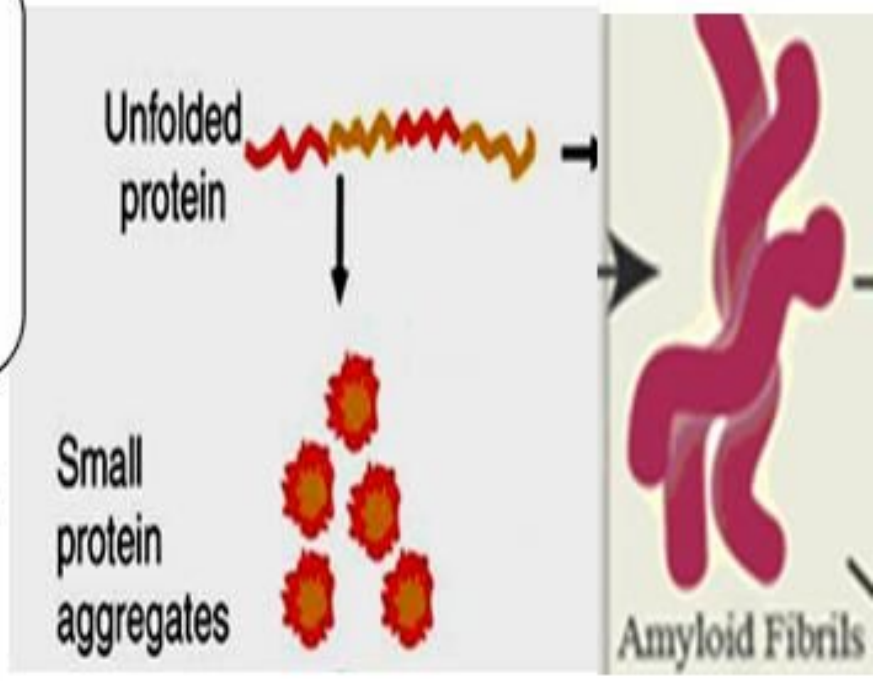
- **mutation**
- **reduced chaperone function**





Aggregation

- **partial unfolding during thermal or oxidative stress**
- **aggregation of fibrillar structures containing misfolded β-sheet rich proteins called Amyloids**



Consequences of Misfolding and Aggregation

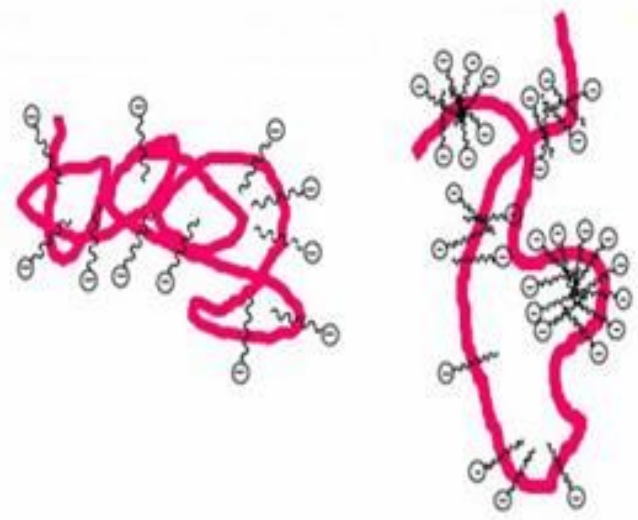
- ***In vivo:*** **Diseases like Alzheimer's disease, Parkinson's disease, Amyloid cardiomyopathy etc.**
- ***In Vitro:*** **Poses a serious problem in the industrial process of producing the recombinant proteins**

Artificial Chaperone Method

GdCl denatured Protein

Step-1
Capture step

Dilution + Detergent

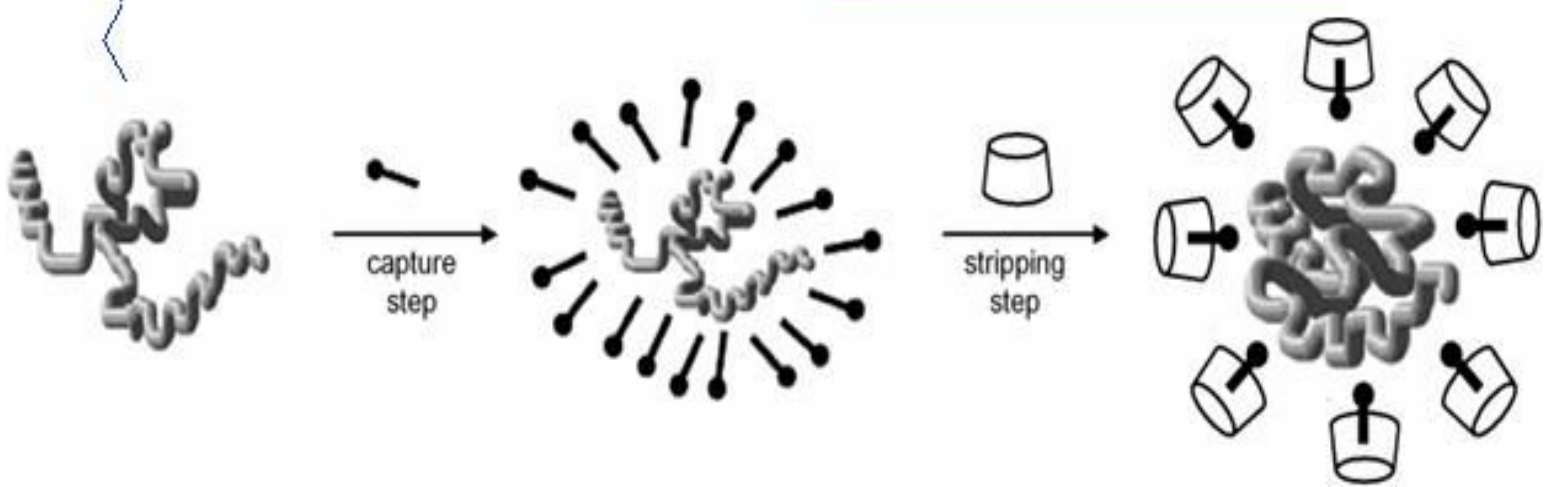


Protein Detergent Complex

Step-2
Stripping Step

Cyclodextrin

Native Protein



Rozema and Gellman

J. Am. Chem. Soc. 1995, *117*, 2373.
J. Biol. Chem. 1996, *271*, 3478.
Biochemistry 1996, *35*, 15760

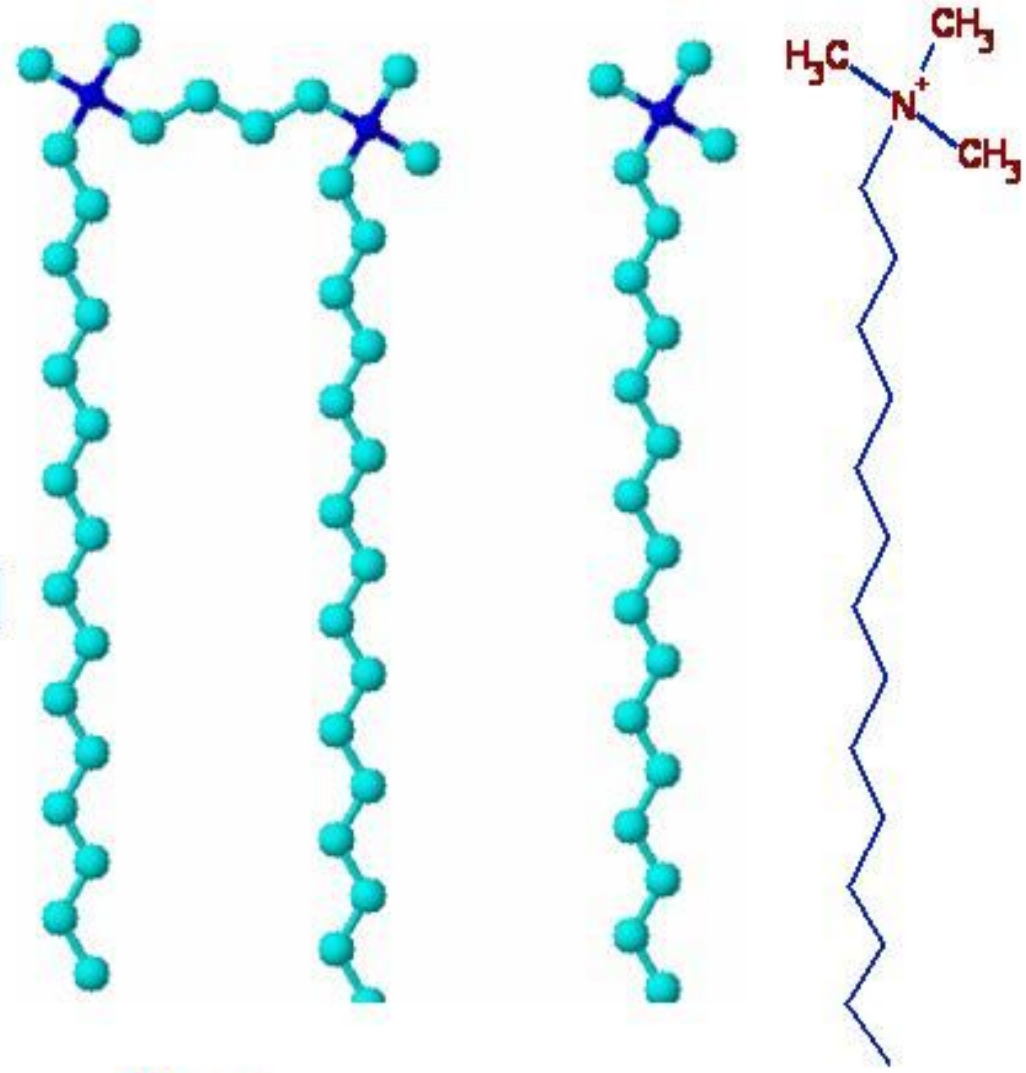
Single Chain ionic surfactants like SDS, CTAB etc. as capturing agents

Cyclodextrins; linear dextrins as stripping agents

Variety of proteins like Creatine Kinase, Lysozyme, Carbonic anhydrase

Objectives of the present work

Surfactants – As capturing agents



Gemini Surfactants

bis(cetyldimethylammonium)alkane dibromide

$(C_{16}H_{33}(CH_3)_2N^+–(CH_2)_n–N^+(CH_3)_2C_{16}H_{33}.2Br^-$

Spacer: $(CH_2)_4$ [G4]; $(CH_2)_5$ [G5]; $(CH_2)_6$ [G6]

Single chain counterpart

Cetyltrimethylammonium Bromide

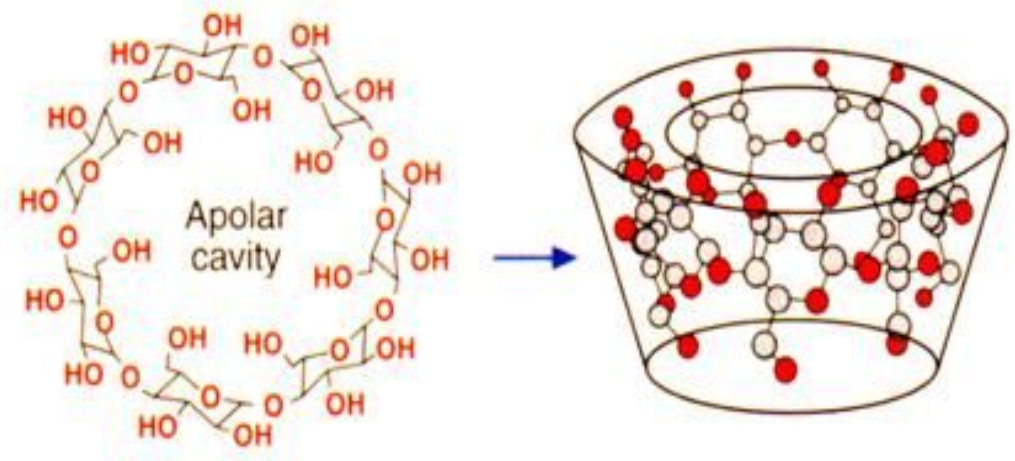
$(C_{16}H_{33}(CH_3)_2N^+–(CH_2)_3.Br^-$

Cyclodextrins – As Stripping agents

α-cyclodextrin (cyclohexamylose)

β-cyclodextrin (cyclohetaamylose)

Methylated-β-cyclodextrin



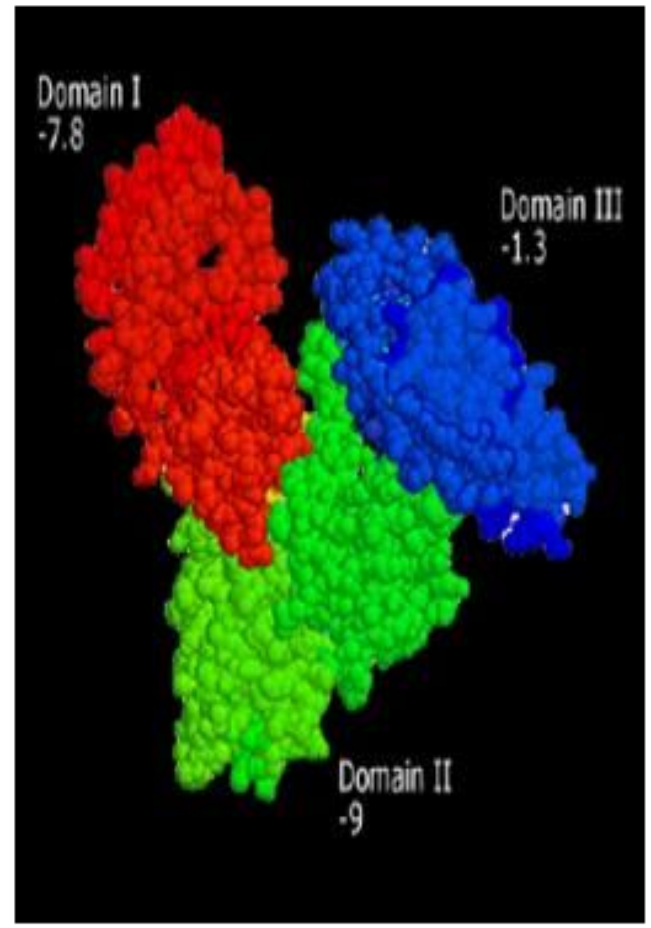
Objectives of the present work

Protein – Bovine Serum Albumin

- **Serum albumin is the most abundant protein in plasma contributing 60% of total globular protein and carrier of fatty acids.**

- **Its primary structure consists of 585 amino acids and the secondary structure consists of 67% α-helix with six turns and 17 disulfide bridges.**

- **The proposed BSA structure is heart shaped, consisting of three homologous domain which are divided into nine loops by disulfide bonds.**

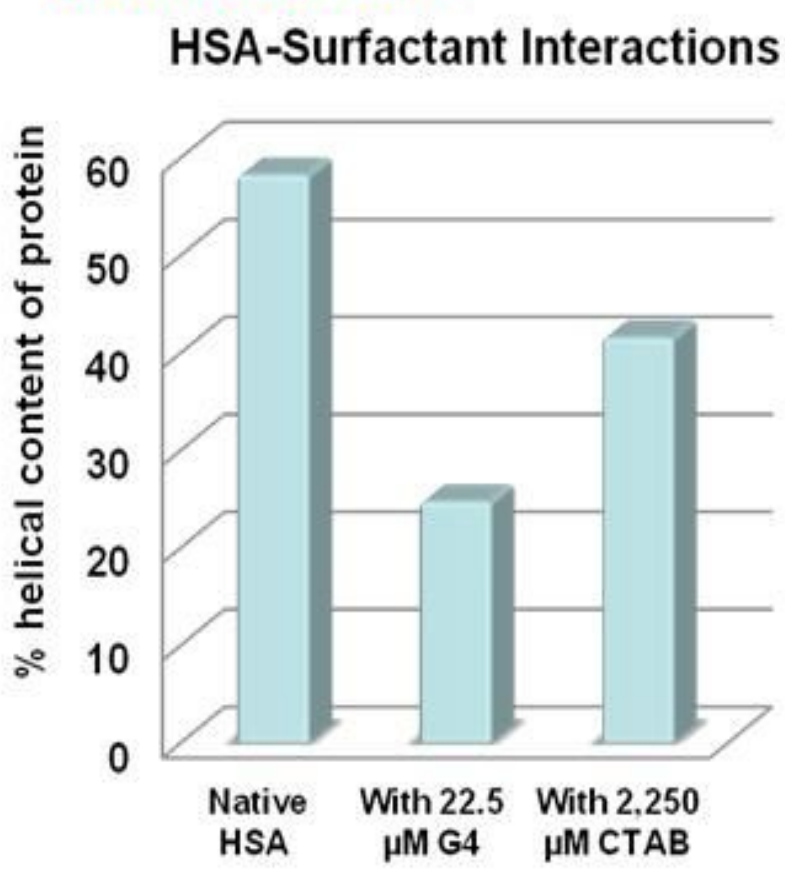


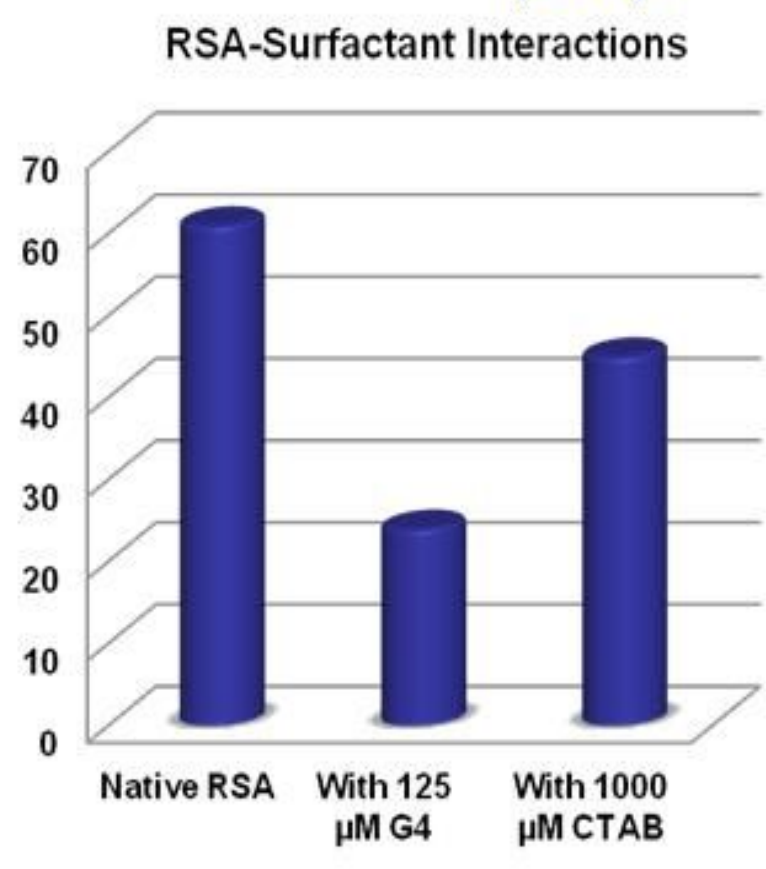
Motivation of the present work

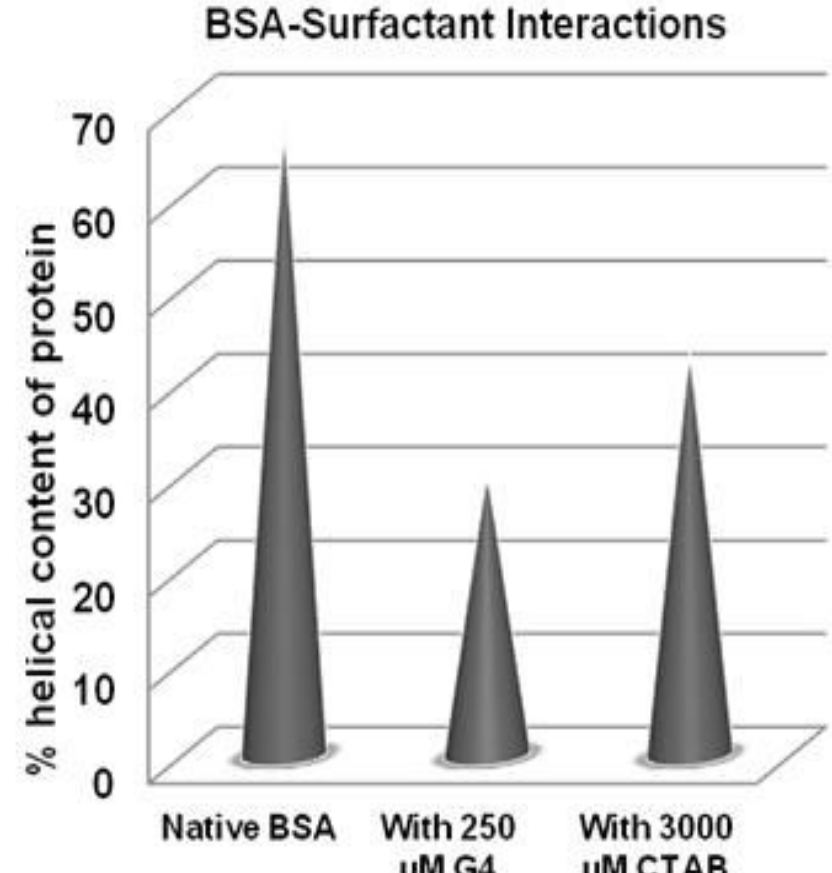
- **Comparative Studies on Interaction of *CTAB* and gemini *(G4)* with serum albumins**

Significantly *lower* concentrations of *G4* compared to CTAB *induce larger unfolding* of serum albumins

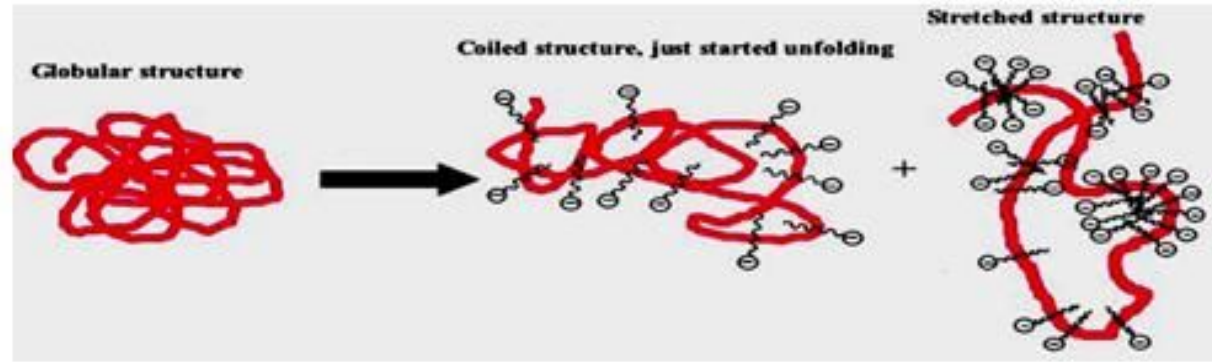
N. Gull et al. J. Biochem., 145 (1) 67–77 (2009)
N. Gull et al. Langmuir, 25 (19) 11686-11691 (2009)





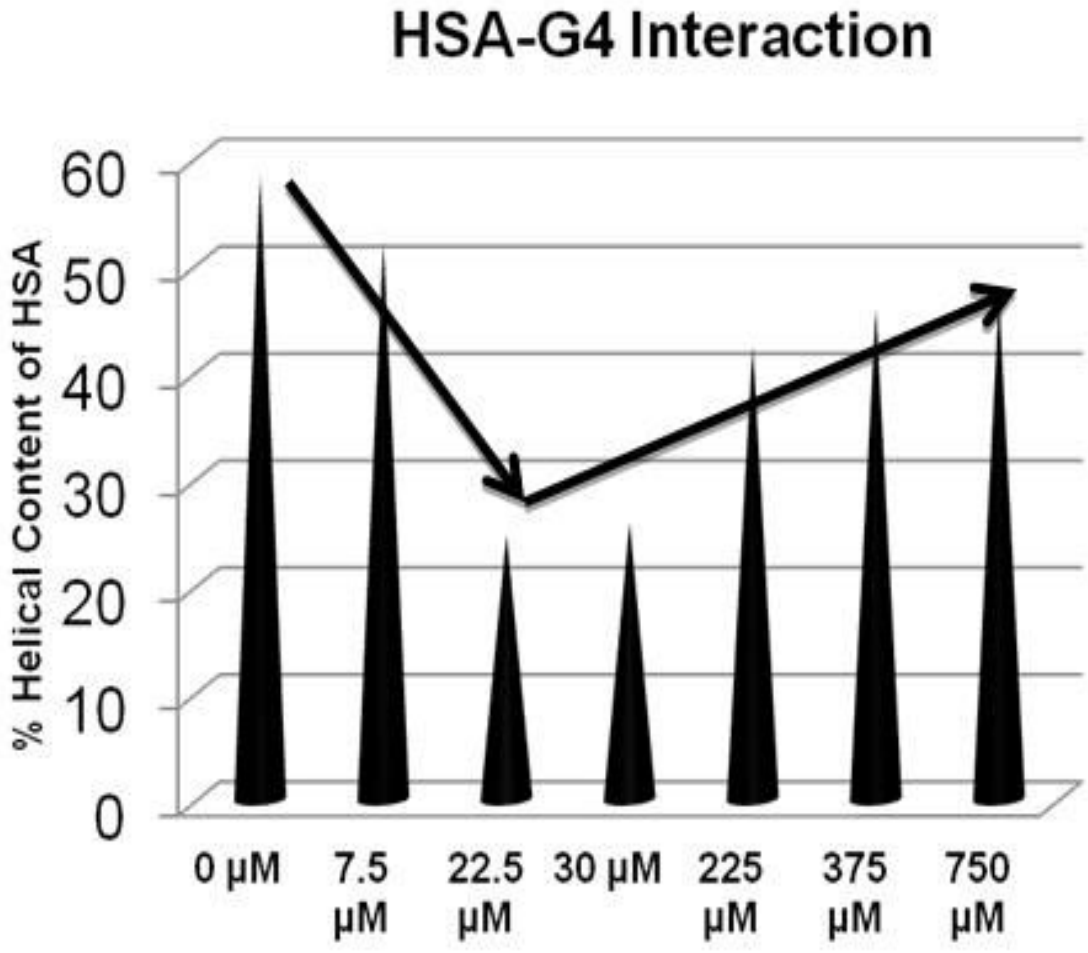


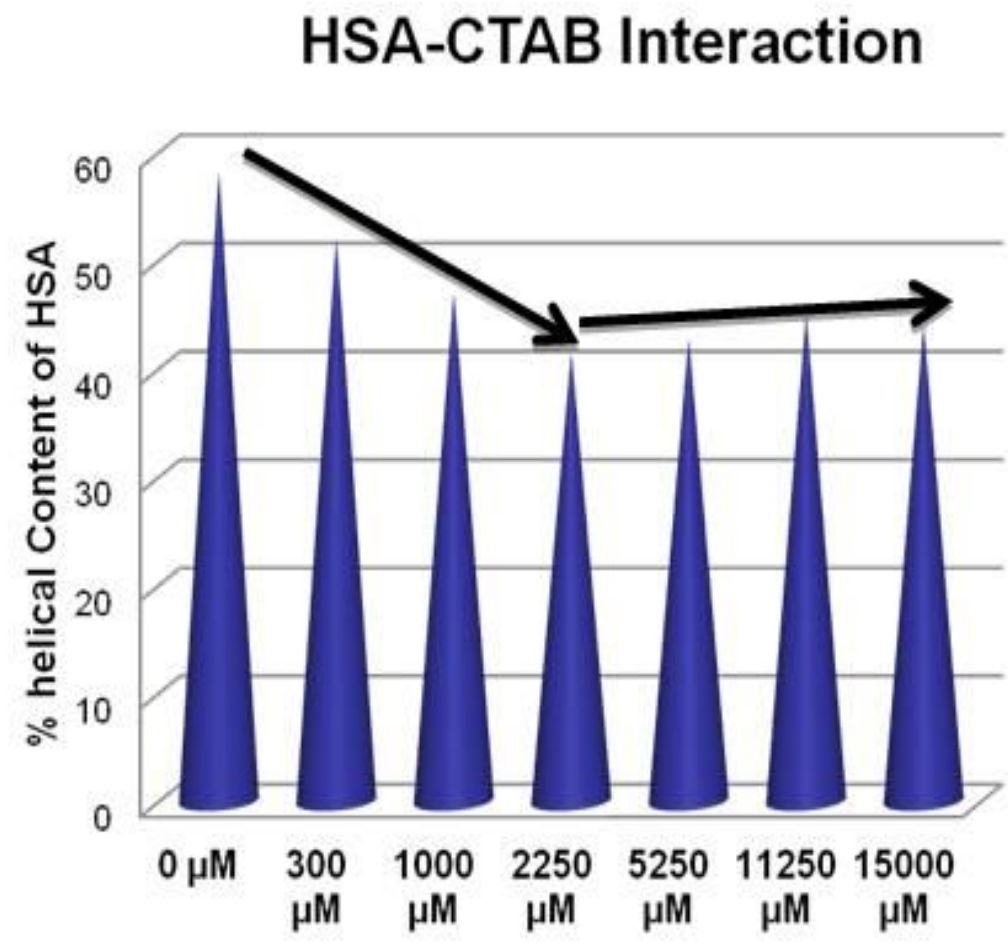
Gemini bind strongly to the protein compared to CTAB because of stronger electrostatic and hydrophobic forces, owing to the presence of two charged headgroups and two hydrophobic hydrocarbon tails, resulting in larger unfolding.



Motivation of the present work

Second important finding was that unlike CTAB *refolding* was observed at *higher* G4 concentrations





The stabilization at higher concentrations is attributed to the highly hydrophobic microdomain of the G4 aggregates formed at such concentrations.

CD-Spectroscopy (Results)

Circular dichroism (CD) Spectroscopy

It enables to predict changes in the secondary and tertiary structure of the protein by observing changes in the ellipticity in the far and near-UV region.

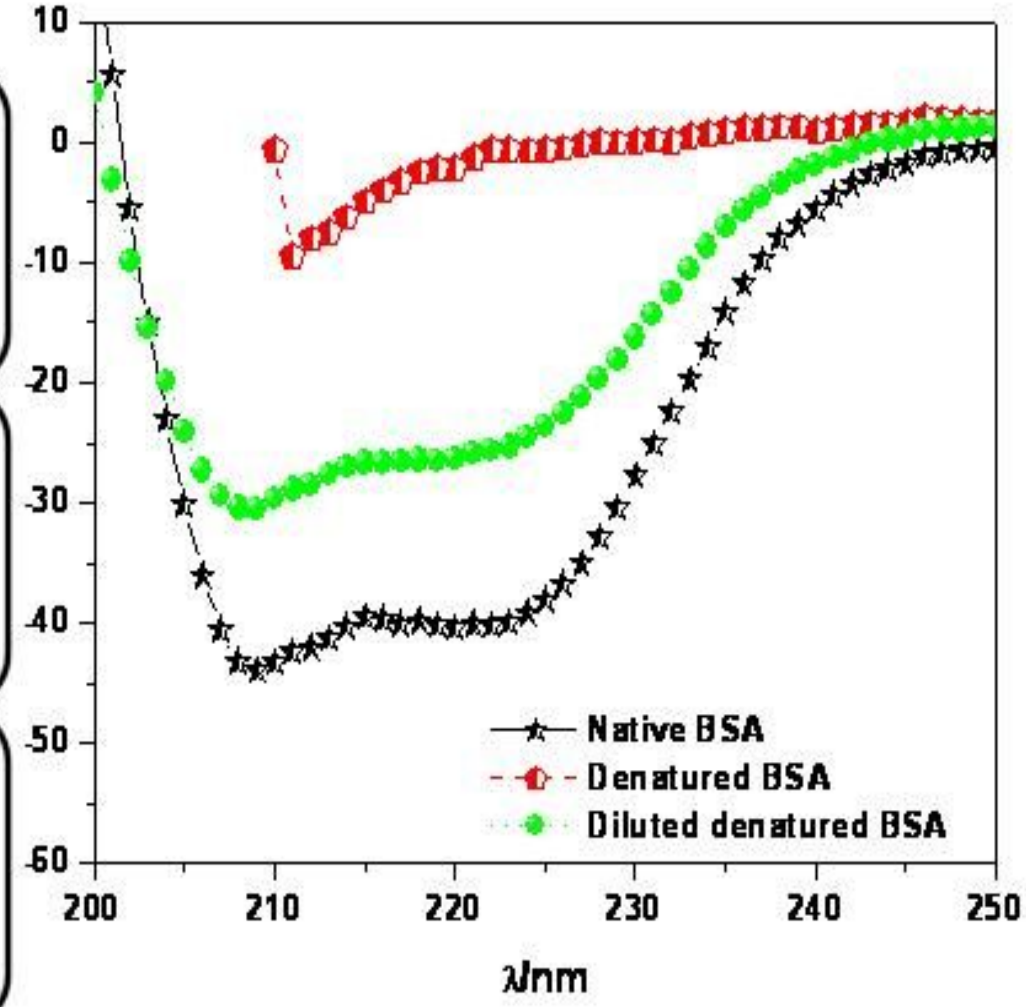
Jasco J-815 model spectropolarimeter

$$\Theta = \frac{2.303}{4\pi} \times 180\left(A_L - A_R\right)$$

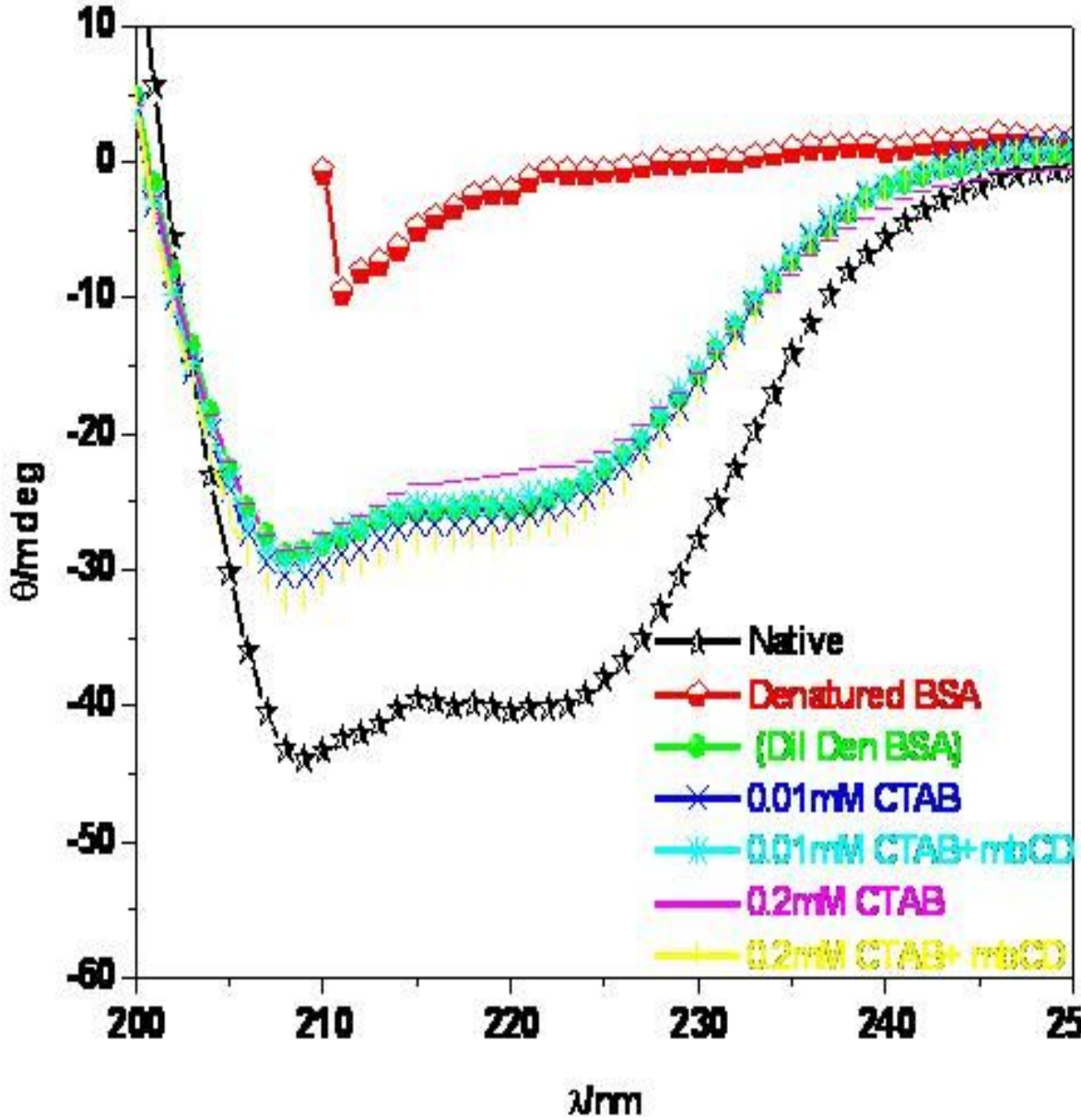
Characteristic negative bands at 208 and 222nm of a protein having an α-helical structure --- Native BSA (67% of α-helical content for the native protein)

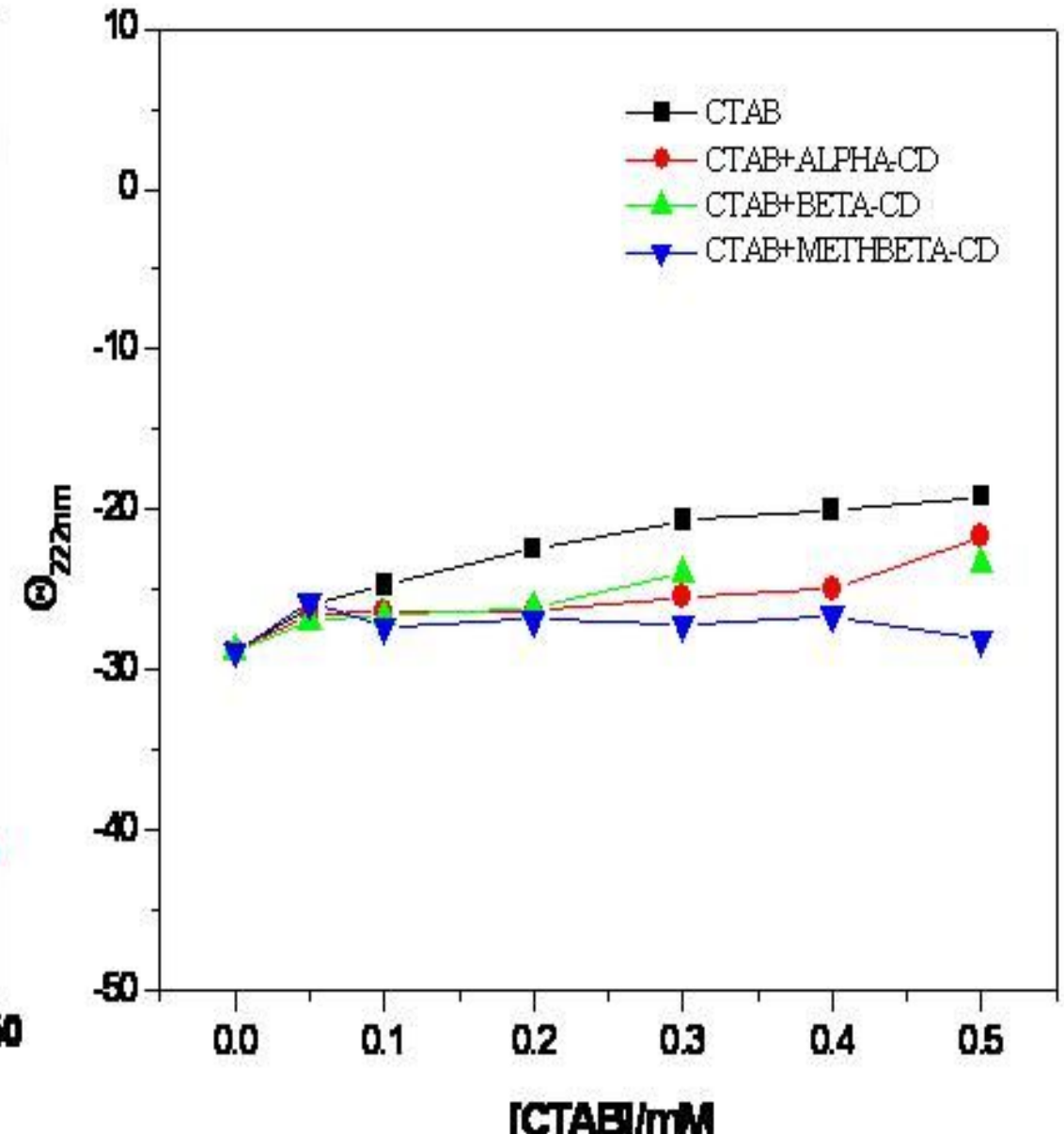
Denatured BSA (40mg/ml) with 6M GdCl --- complete loss of the secondary structure. (4% of α-helical content)

Diluted denatured sample 0.2 mg/ml protein concentration and 30 mM [GdCl] --- increase in the α-helical content. (46% of α-helical content)



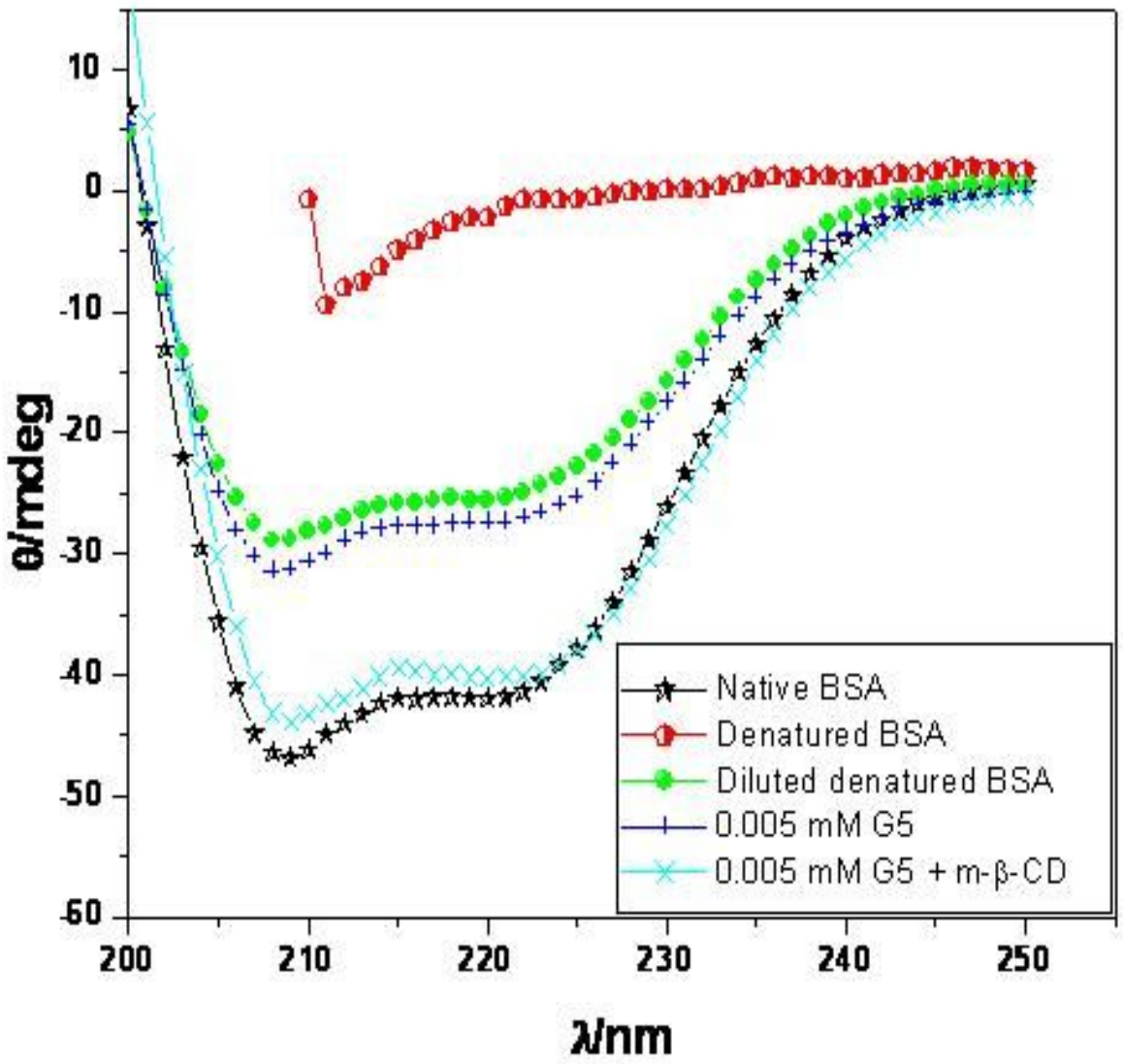
CD-Spectroscopy (CTAB + Cyclodextrins)

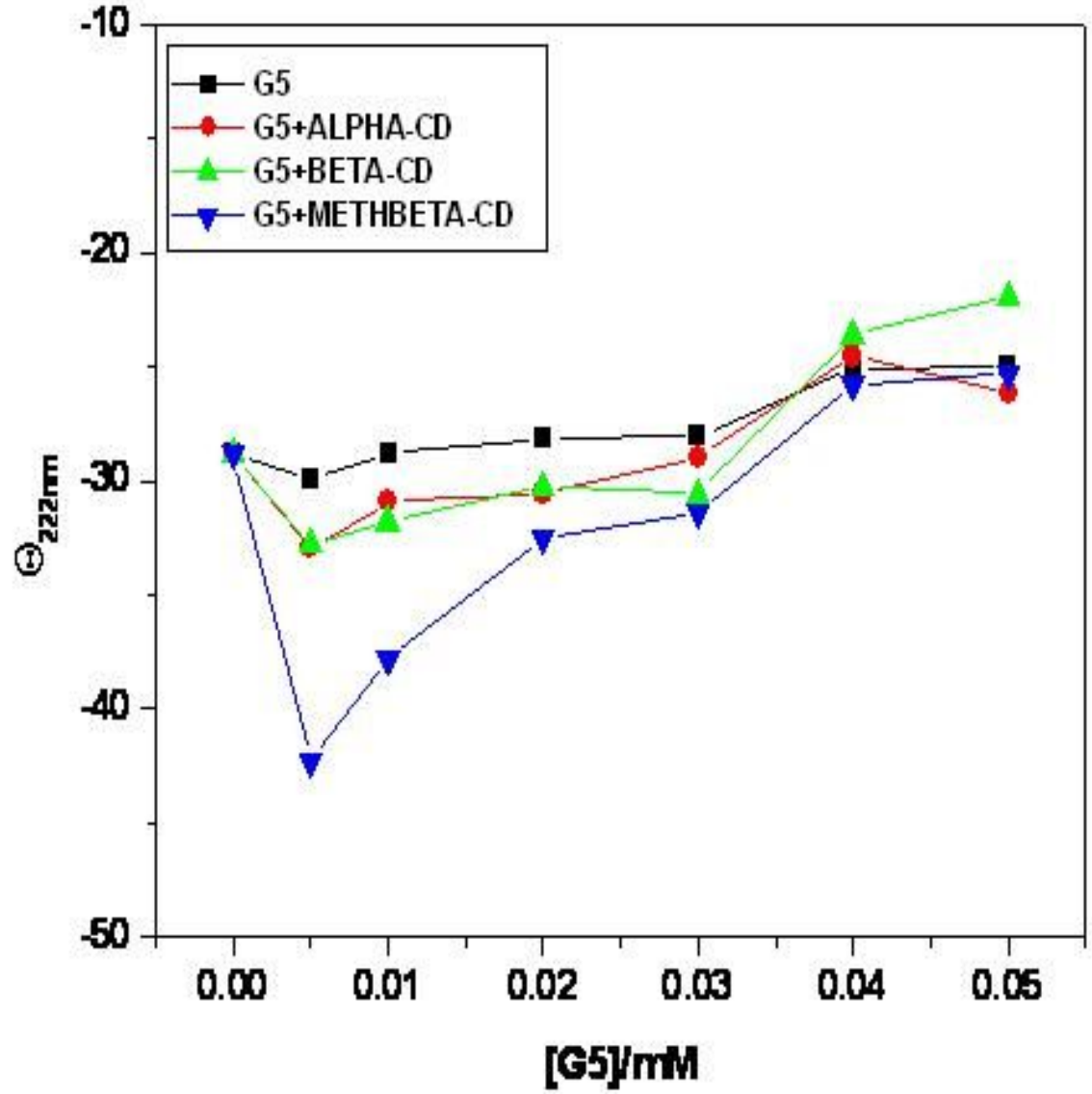




- Sequential addition of CTAB and stripping agent was not able to increase the helical content of the protein
- Also none of the three stripping agents was successful either

CD-Spectroscopy (Gemini (G5) + Cyclodextrins)



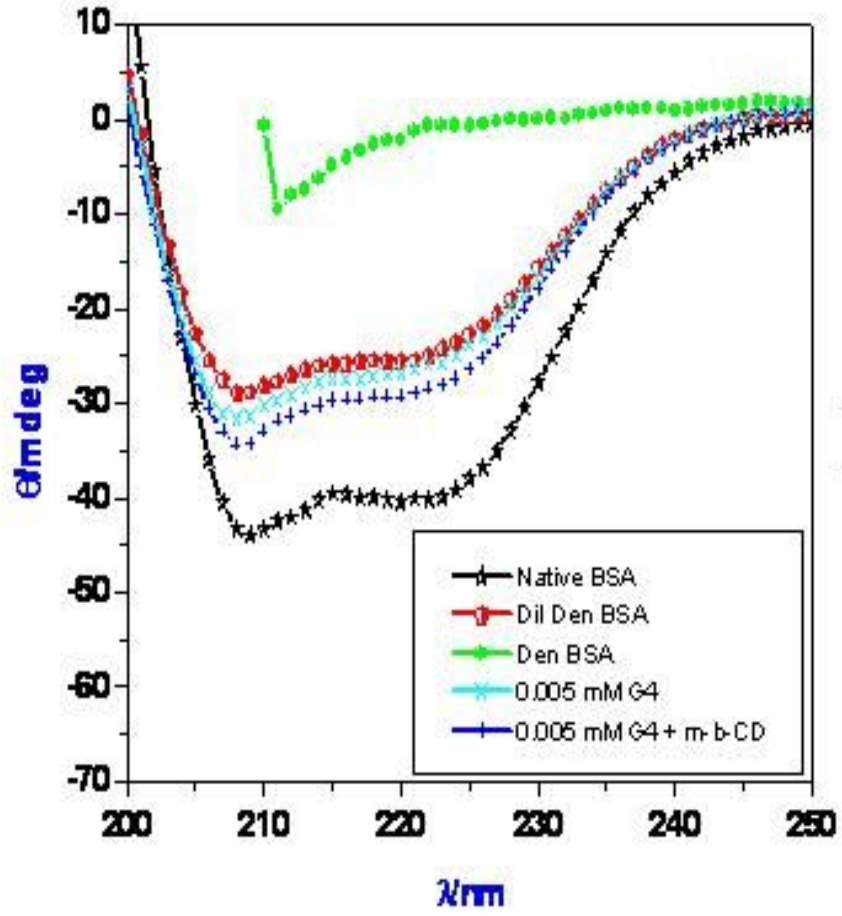


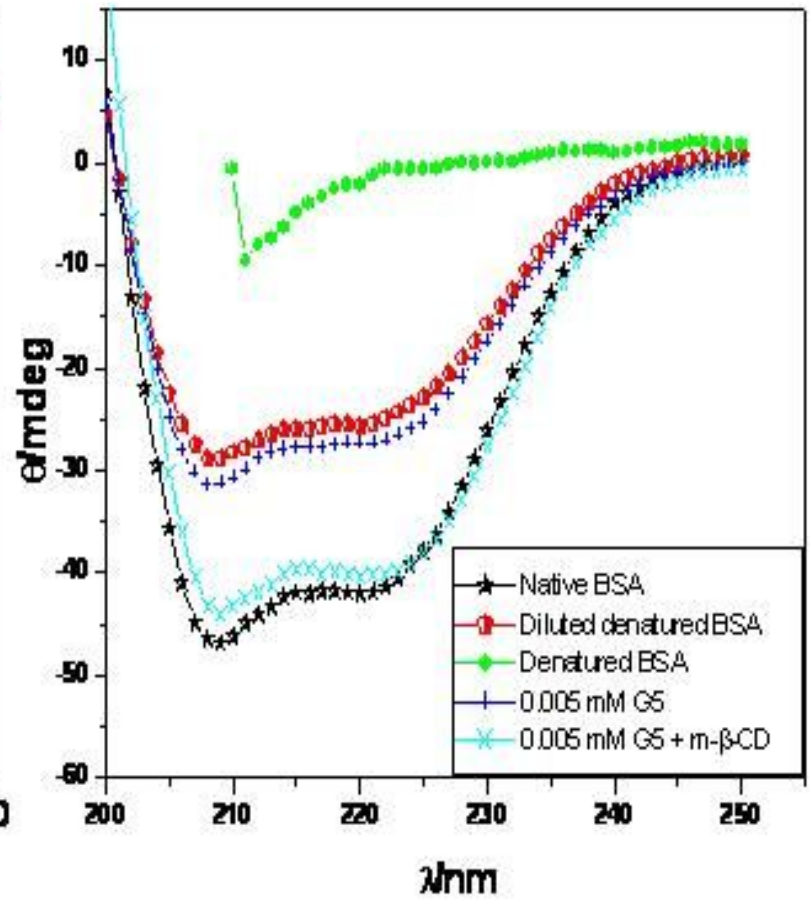
❖[G5] as low as 0.005 mM + methyl- β-cyloclodextrin bring the alpha helical content very much near to that of native protein.

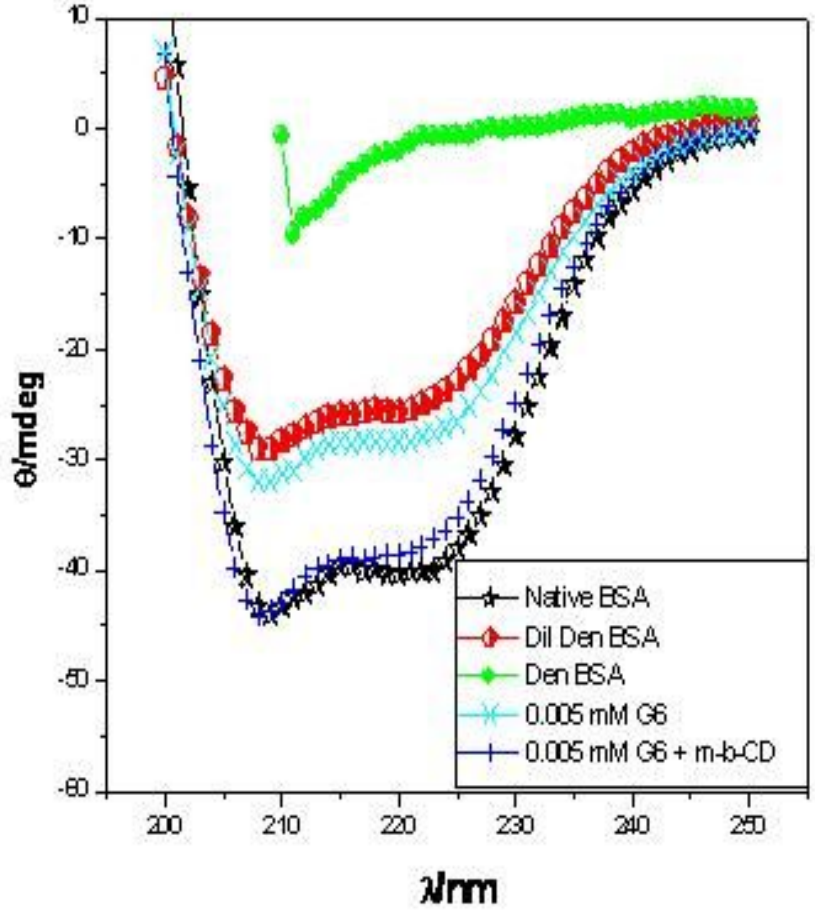
❖As the surfactant concentration is increased the helical content decreases owing to the decrease in the ratio of the [stripping agent] / [surfactant].

❖It is observed that the helical content is regained more on treatment with methyl-β-cyclodextrin compared to other stripping agents due to its greater hydrophobicity.

CD-Spectroscopy (Comparison of Gemini's + Cyclodextrins)







- Like G5, low concentrations of G6 (0.005 mM) followed by the stripping agent also renatures BSA.
- G4 like CTAB was not able to refold the protein to its native conformation as evident from the spectra but its effect was higher than that of CTAB. In fact at 0.01 mM G4 the effect was appreciable.
- Effectiveness related to their CAC values: 0.01 mM for G4; 0.005 mM for G5 and 0.004 mM for G6

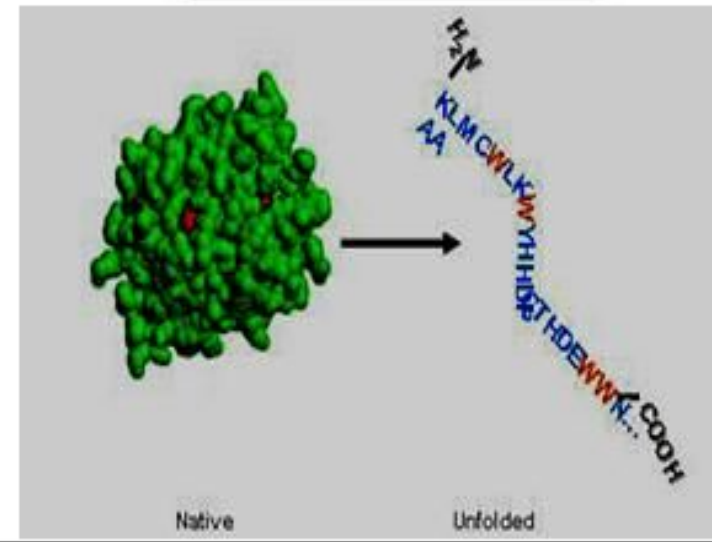
Intrinsic Fluorescence spectroscopy

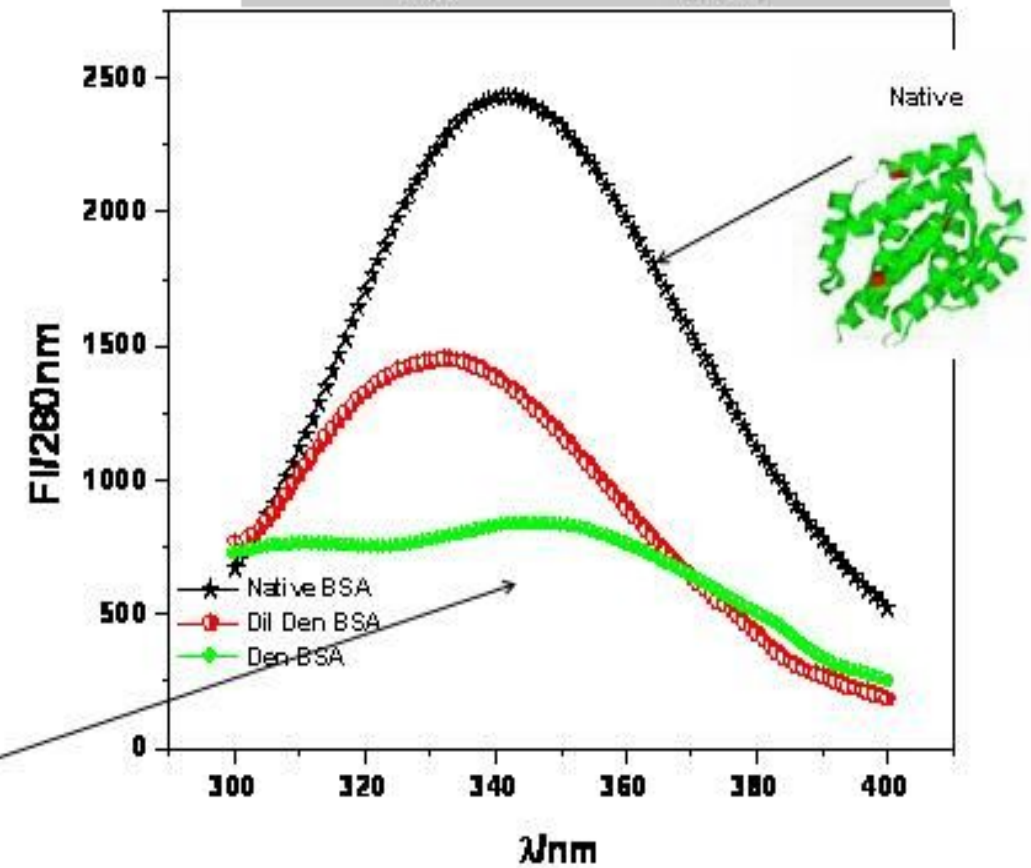
useful probe to study the change in the protein structure by observing the change in the fluorescence intensity of the protein.

Tyrosine and Tryptophan residues excite at 280 nm and fluoresce. The fluorescence intensity highly sensitive to environment

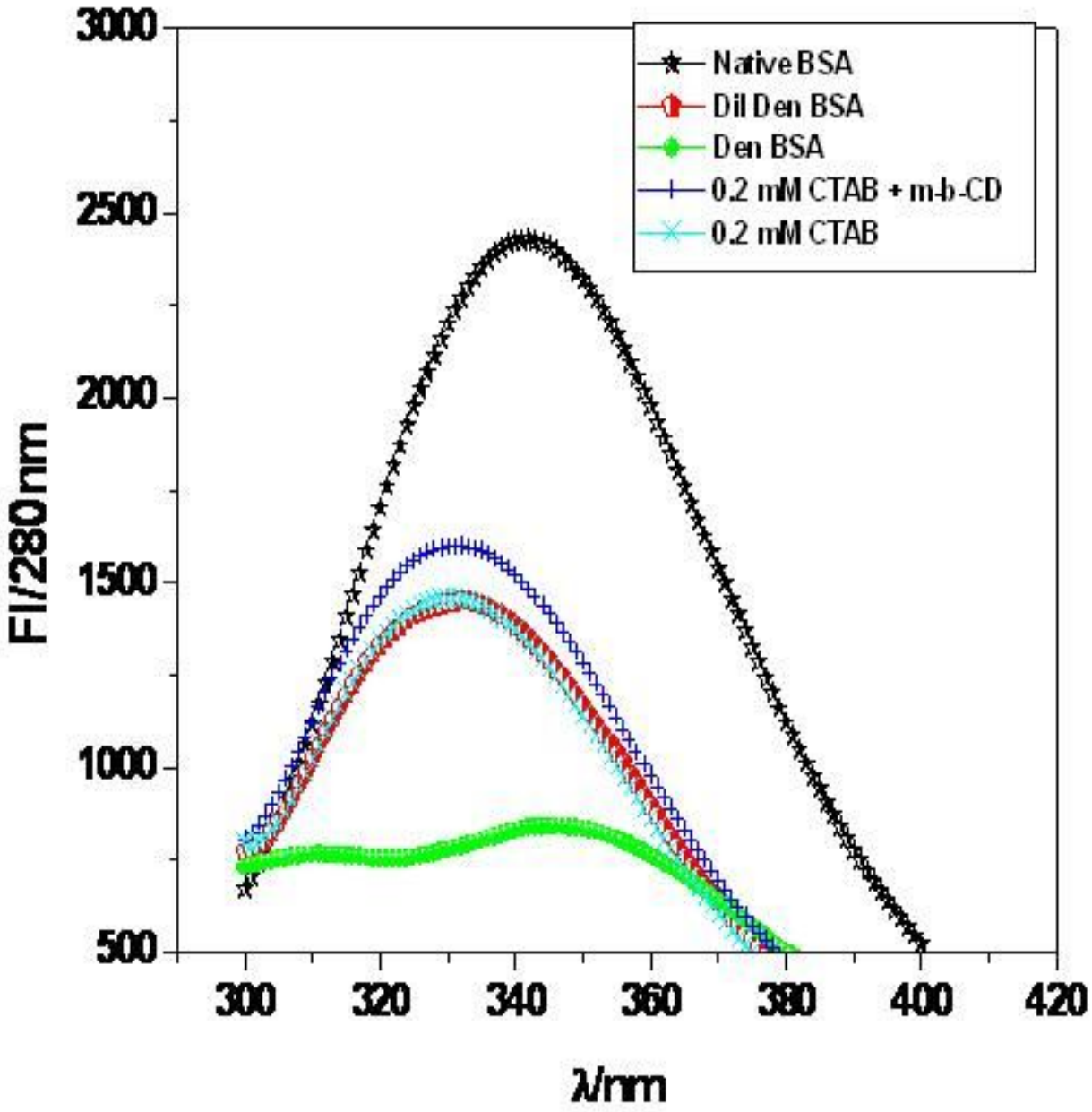
Folded protein present hydrophobic environment for residues and hence high intensity. However, unfolding of protein exposes them to hydrophillic environment and thus decreases their FI.. Intensity

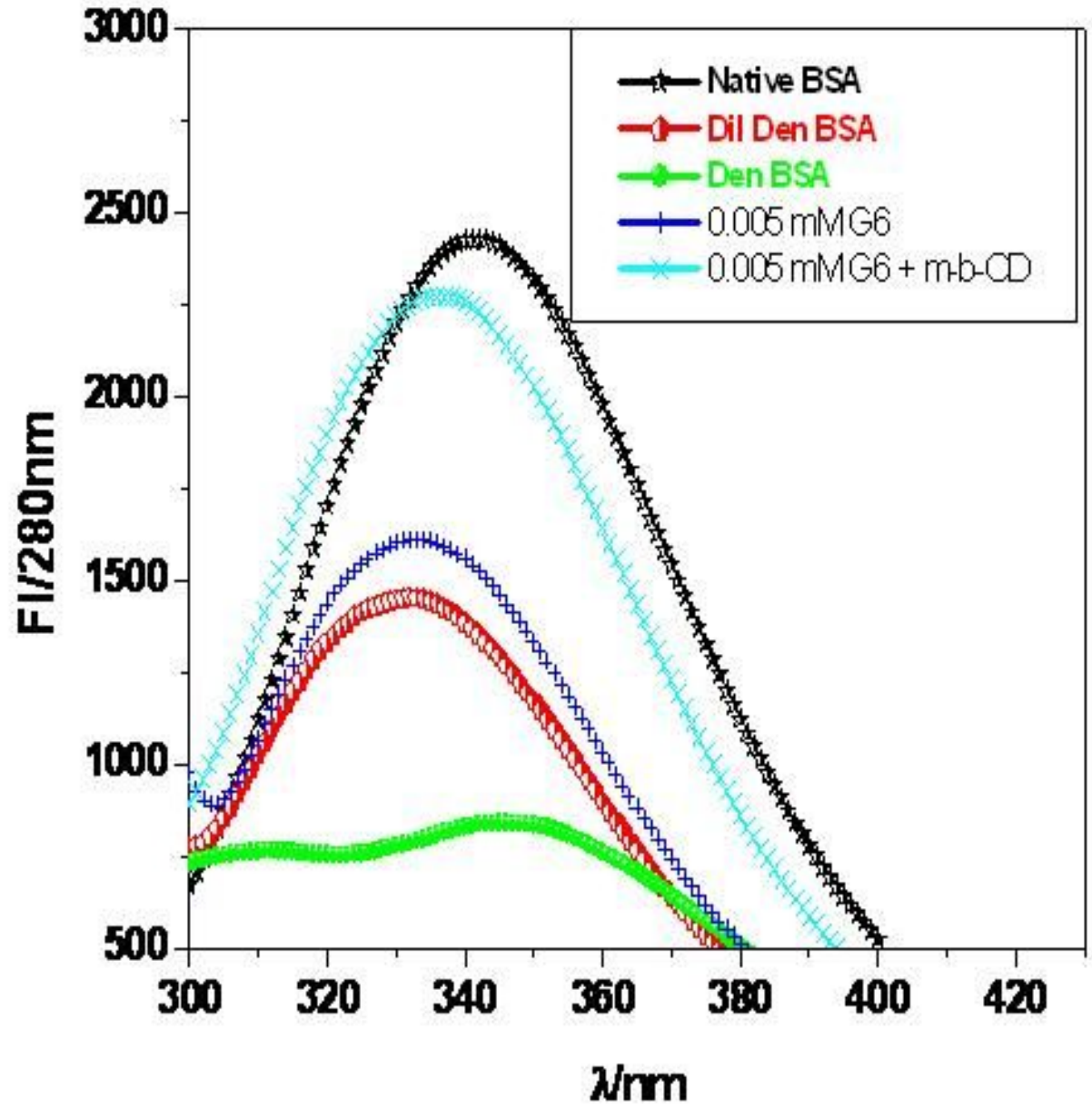
Hitachi spectrofluorimeter (model 2500)





Fluorescence spectroscopy (CTAB/Gemini + m-β-CD)



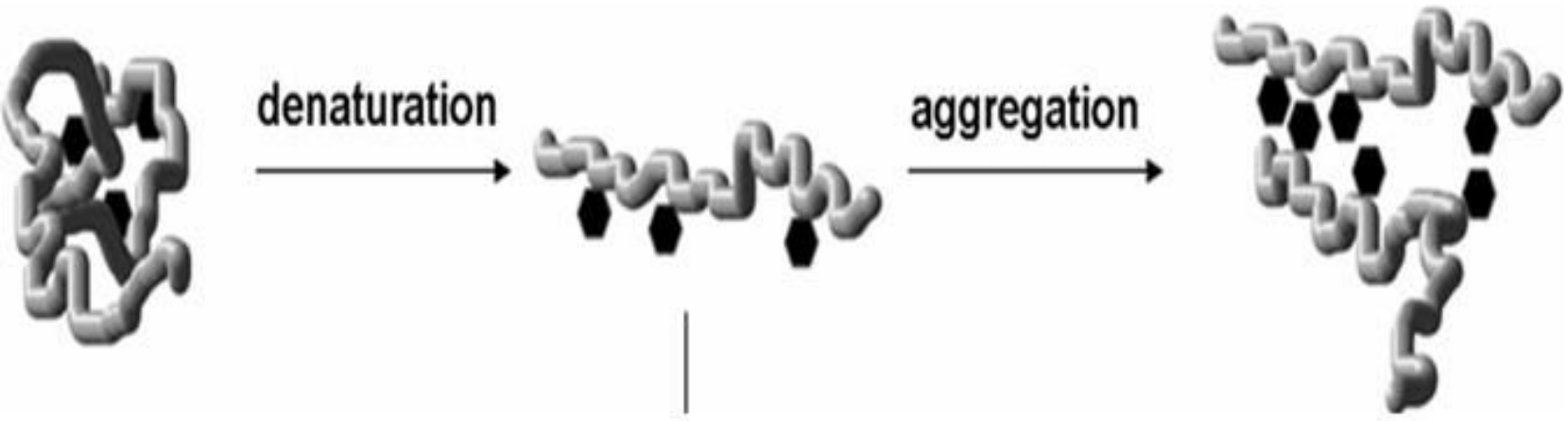


Dynamic Light Scattering (DLS)

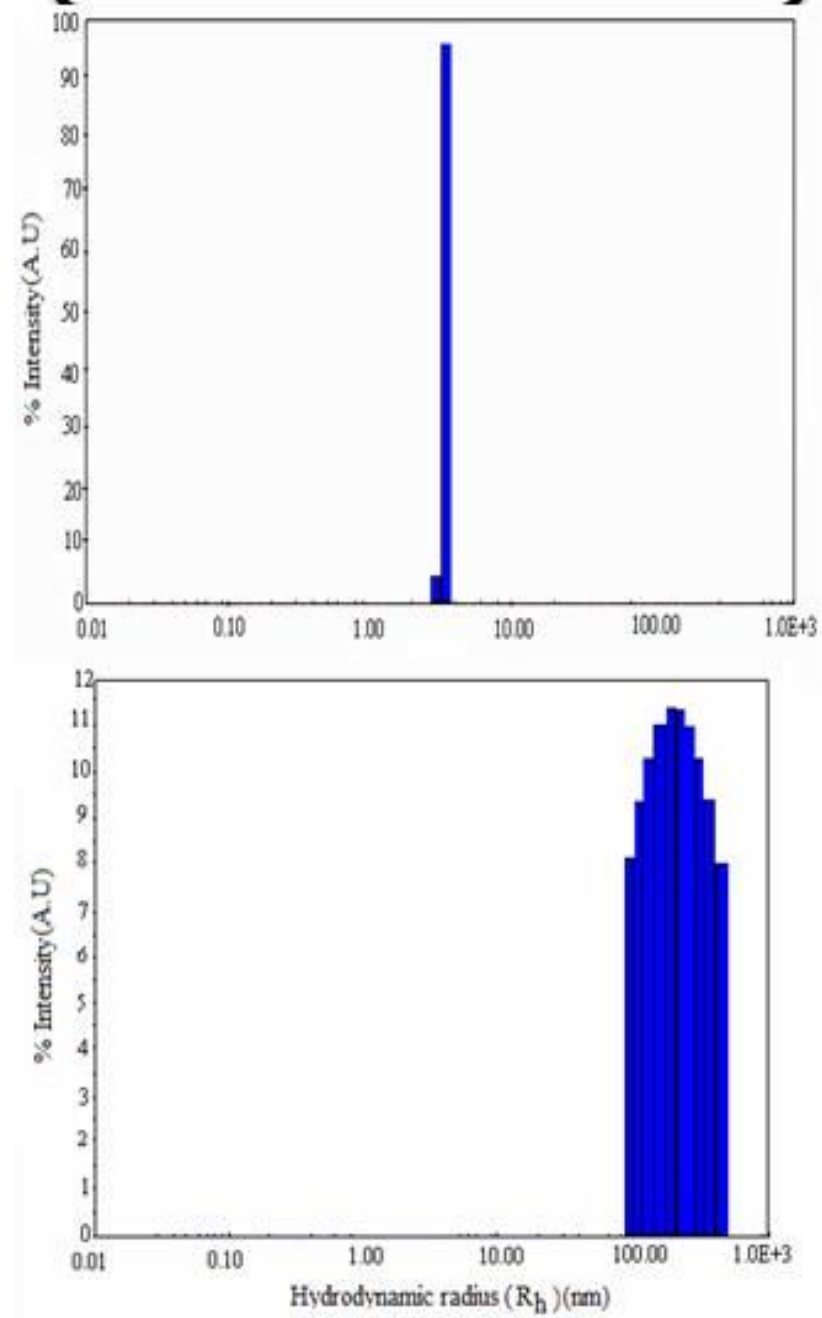
DLS has been used to monitor changes in the dimension of proteins during denaturation and renaturation.

The value of the hydrodynamic radius of native BSA (4.1nm), as illustrated in figure is in excellent agreement with literature

Dilution of GdCl denatured BSA to 30 mM GdCl + 0.2 mg/ml BSA led to increase in R (227 nm) due to the formation of aggregates as dilution is an aggregation prone pathway.



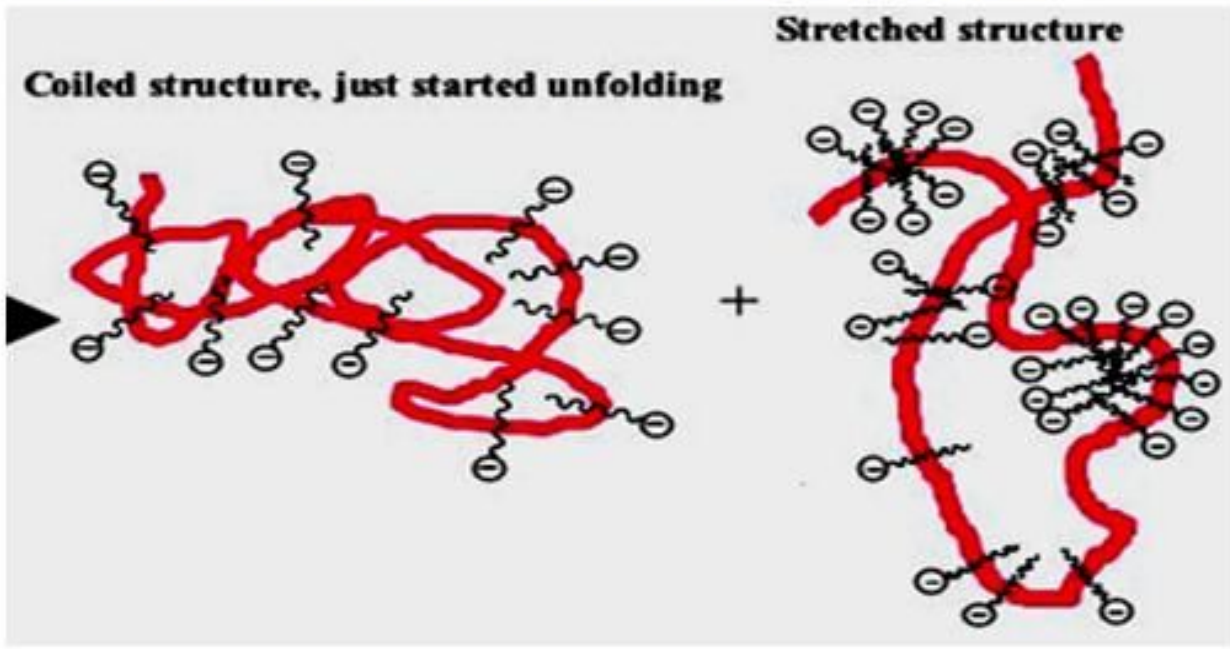
DynaPro-TC-04 dynamic light scattering equipment (Protein Solutions, Wyatt Technology, Santa Barbara, CA)



DLS studies

✓Addition of surfactant checks this aggregation as hydrophobic tail of the surfactant interacts with the exposed hydrophobic portions of the protein and leads to the formation of protein-surfactant complex.

✓After stripping step, the decrease in the hydrodynamic radii is attributed to the fact that methyl-β-cyclodextrin strips off the surfactant, breaks the protein-surfactant complexes and thus initiates refolding



Sample Name	(R_h) (nm)
Native BSA	4.1 ± 0.1
Diluted Denatured BSA	227 ± 1.2
BSA with 0.1mM CTAB	19.4 ± 0.8
BSA with 0.1mM CTAB +500 µM methyl-β-cyclodextrin	32.6 ± 0.6
BSA with 0.005 mM G4	17.4 ± 1
BSA with 0.005 mM G4 +500 µM methyl-β-cyclodextrin	10.3 ± 0.5
BSA with 0.005 mM G5	17.4 ± 0.6
BSA with 0.005 mM G5 +500 µM methyl-β-cyclodextrin	5.2 ± 0.4
BSA with 0.005mM G6	17.5 ± 0.9
BSA with 0.005mM G6 +500 µM methyl-β-cyclodextrin	4.7 ± 0.6

Conclusion

- **This mutitechqiue approach confirmed that the refolding of GdCl denatured BSA is achieved by very small concentrations of gemini surfactants at which the single chain surfactant is found to be ineffective during the artificial chaperone assisted two step method.**

- **This is also suggested that use of gemini surfactants for refolding of proteins via the artificial chaperone protocol might address one of the most pressing demands of the biotechnological industry for the development of inexpensive and efficient folding agents.**