

1-21-2015

# Structural and Thermal Stability Characterization of Protein-Fatty acid Adducts

Dhurga Dharmaraj  
dhurgadharmaraj88@gmail.com

---

## Recommended Citation

Dharmaraj, Dhurga, "Structural and Thermal Stability Characterization of Protein-Fatty acid Adducts" (2015). *Master's Theses*. 713.  
[https://opencommons.uconn.edu/gs\\_theses/713](https://opencommons.uconn.edu/gs_theses/713)

This work is brought to you for free and open access by the University of Connecticut Graduate School at OpenCommons@UConn. It has been accepted for inclusion in Master's Theses by an authorized administrator of OpenCommons@UConn. For more information, please contact [opencommons@uconn.edu](mailto:opencommons@uconn.edu).

**Structural and Thermal Stability Characterization of  
Protein- Fatty Acid Adducts**

**Dhurga Dharmaraj**

**B.Tech (Biotechnology), Anna University, 2011**

**A Thesis**

**Submitted in Partial Fulfillment of the**

**Requirements for the Degree of**

**Master of Science**

**At the**

**University of Connecticut**

**2015**

## APPROVAL PAGE

Masters of Science Thesis

# Structural and Thermal Stability Characterization of Protein-Fatty acid Adducts

Presented by

Dhurga Dharmaraj, B.Tech

Major Advisor Dr. Challa Vijaya Kumar

Associate Advisor Dr. Rajeswari M. Kasi

Associate Advisor Dr. Jing Zhao

University of Connecticut

2015

## Table of Contents

Approval Page	ii
Table of Contents	iii
List of Tables	v
List of Figures	vi
List of Schemes	ix
Chapter1:Protective effect of Capric acid on secondary structure of protein1	
1.1Abstract	2
1.2Introduction	3
1.3Experimental	6
1.3.1 Materials	6
1.3.2 Protein/capric acid sodium salt (CA) complex	6
1.3.3 Steam sterilization and Heat studies	6
1.3.4 Circular Dichroism (CD) studies	6
1.3.5 Activity studies	6
1.3.6Agarose Gel Electrophoresis	7
1.3.7 SDS-PAGE Gel Electrophoresis	8
1.3.8 Energy transfer complex	8
1.4Results and Discussion	9
1.4.1 Agarose and SDS-Gel Electrophoresis	9
1.4.2 Stability towards Steam sterilization and Heat	19
1.4.5 Activity study	36
1.4.4 Energy transfer complex	42
1.5Conclusion	44
Chapter 2: Chemical modification of proteins with octadecenyl succinic anhydride	45
2.1 Abstract	46
2.1Introduction	47



<b>2.2 Experimental</b>	<b>50</b>
2.2.1 Materials	50
2.2.2 Method	50
2.2.3 Agarose Gel Electrophoresis	50
2.2.4 Circular Dichroism (CD) studies	50
2.2.5 Dynamic light scattering experiment	51
2.2.6 Activity Study	51
2.3 Results and Discussion	51
2.3.1 Agarose Gel Electrophoresis to study chemical modification	51
2.3.2 Circular Dichroism study	56
2.3.3 Activity studies	56
2.3.4 Dynamic light scattering	56
2.3.5 Solubility test in organic solvents	60
2.4 Conclusion	62
References	63

## List of Tables

**Table.2.2.**Shows the diameter of the C18SA modified hemoglobin (Hb).It was observed that buffer showed a particle size >150nm which was consistently found in all the samples that interfered with the actual particle measurement.Column.3 shows an increasing trend in the size of the C18SA modified hemoglobin with increase in the ratio of C18SA.

**Table.2.3.**Showing the solubility of C18SA modified hemoglobin in different organic solvents.200ul of 50uM C18SA modified BSA was mixed with 1mL of each organic solvent to test the solubility

## List of Figures

**Figure.1.1)** Steam sterilized hemoglobin in the presence of varying concentration of capric acid. No precipitate of hemoglobin was observed above the CMC of CA.

**Figure.1.2)** Agarose gel of Hemoglobin (Hb) in varying concentration of CA. The steam sterilized (SS) Hb in CA became more negative charge.

**Figure.1.3)** Agarose gel of Catalase (Cat) in varying concentration of CA. The steam sterilized (SS) Cat in CA became more negative charge.

**Figure.1.4)** Agarose gel of Lysozyme (Lyz) in varying concentration of CA. The steam sterilized (SS) Lyz in CA became more negative charge.

**Figure.1.5)** SDS-PAGE gel of hemoglobin(Hb) in varying concentration of CA. The Steam sterilized (SS) Hb in CA showed a smeared band.

**Figure.1.6)** SDS-PAGE gel of Catalase (Cat) in varying concentration of CA. The Steam sterilized (SS) Cat in CA showed a smeared band.

**Figure.1.7)** SDS-PAGE gel of Lysozyme (lyz) in varying concentration of CA. The Steam sterilized (SS) Lyz in CA showed a smeared band.

**Figure.1.8)** Agarose gel of BSA in varying concentration of CA. The BSA-CA adducts formed after heated became more positive charge.

**Figure.1.9.** A) UV-CD of hemoglobin in varying concentration of capric acid before steam sterilization. B) UV-CD of hemoglobin in varying concentration of capric acid after steam sterilization

**Figure.1.10.** A) UV-CD of myoglobin in varying concentration of capric acid before steam sterilization. B) UV-CD of myoglobin in varying concentration of capric acid after steam sterilization

**Figure.1.11.** A) UV-CD of catalase in varying concentration of capric acid before steam sterilization B) UV-CD of catalase in varying concentration of capric acid after steam sterilization

**Figure.1.12.** A) UV-CD of lysozyme in varying concentration of capric acid before steam sterilization B) UV-CD of lysozyme in varying concentration of capric acid after steam sterilization

**Figure.1.13.** % of structure change relative to protein in 50mM sodium phosphate buffer plotted against the varying concentration of CA

**Figure.1.14** % of structure change relative to un heated protein in 50mM sodium phosphate buffer plotted against the varying concentration of CA

**Figure.1.15.** % of structure change relative to steam sterilized protein in 50mM sodium phosphate buffer plotted against the varying concentration of CA

**Figure.1.16.**% of total hydrophobic residues in the protein with respect to structure retention

**Figure.1.17.**% of surface hydrophobic residues in the protein with respect to structure retention

**Figure.1.18.**% of melting point of the protein with respect to structure retention

**Figure.1.20** A) UV CD of BSA-CA adducts in varying concentration of CA unheated. B) UV CD of BSA-CA adducts in varying concentration of CA (heated to 90C for 6 minutes and cooled to room temperature)

**Figure.1.21.**% of specific activity of hemoglobin after steam sterilization in varying concentration of capric acid

**Figure.1.22.**% of specific activity of myoglobin after steam sterilization in varying concentration of capric acid

**Figure.1.23.**% of specific activity of catalase after steam sterilization in varying concentration of capric acid

**Figure.1.24.**% of specific activity of Lysozyme after steam sterilization (SS)in varying concentration of capric acid

**Figure.25.A)** Emission spectra of BSA-CA adducts that measured at excitation wavelength of hoeschst (350nm).The energy was transferred in the dye cascade system and the lowest energy acceptor emission (rhodamine B) was observed as emission spectra. B) The emission spectra obtained after exciting at 650nm.

**Figure.2.1** A) C18SA modified Hemoglobin (Hb) with increasing ration of Hb:C18SA. Color change was observed with increase in the C18SA concentration. B) C18SA modified BSA with increasing ratio of BSA: C18SA. The BSA: C18SA ratio of 1:20 and 1:30 became turbid after the reaction.

**Figure.2.2.A)**Shows the Agarose gel ran for C18SA modified Hemoglobin (Hb) .As the Hb:C18SA ratio was increased the protein became more negative.Maximum change in charge was observed for 1:50. B) Shows the Agarose gel ran for C18SA modified hemoglobin (Hb) with a control sample of Hb in the presence of corresponding % of THF

**Figure.2.3** Shows the Agarose gel ran for C18SA modified BSA. It was observed that as the ratio of BSA: C18SA was increased the protein became more negative. For BSA:C18SA ratio of 1:40 maximum change in the charge was observed.

**Figure.2.4.**A) Shows the change in the UV-CD (secondary structure) of hemoglobin with increasing C18SA ratio. B) Shows the Soret CD (heme environment) of hemoglobin with increasing concentration of C18SA

**Figure.2.5.**A) Shows the change in the activity of C18SA modified hemoglobin (Hb)  
B) Hb activity in different % of THF

## List of Schemes

**Scheme.1.** Showing the reversible thermal denaturation of protein in the presence of capric acid micelles

**Scheme.2.1.** Chemical modification of protein with octadecenyl succinic anhydride

## **Chapter.1.**

### **Protective effect of capric acid on secondary structure of protein**

## 1.1ABSTRACT

Proteins are biological molecules that have an important application as a catalyst for various reactions. The sensitivity of protein to denature at high temperature limits its industrial application where a catalyst that works at high temperature is required. Some protein denature even at optimal room temperature. The primary interest of this study is to protect the protein for thermal denaturation by stabilizing proteins within the capric acid (a ten-carbon chain fatty acid) micelle. The protein-Capric acid adducts showed reversible thermal denaturation when the Capric acid (CA) concentration was present above the critical micellar concentration (CMC). Although the secondary structure was protected for thermal denaturation the activity was lost for most of the proteins studied and SDS-PAGE gel showed that covalent modification of protein with capric acid resulted in high temperature heating. Thus a new lipoprotein material was synthesized by heating protein with capric acid (CA). Bovine serum albumin (BSA)-capric acid adduct was used in this study to build an efficient simple artificial energy transfer complex.

**Keywords:** *Capric acid, SDS-PAGE gel, Bovine serum albumin, Antenna complex*



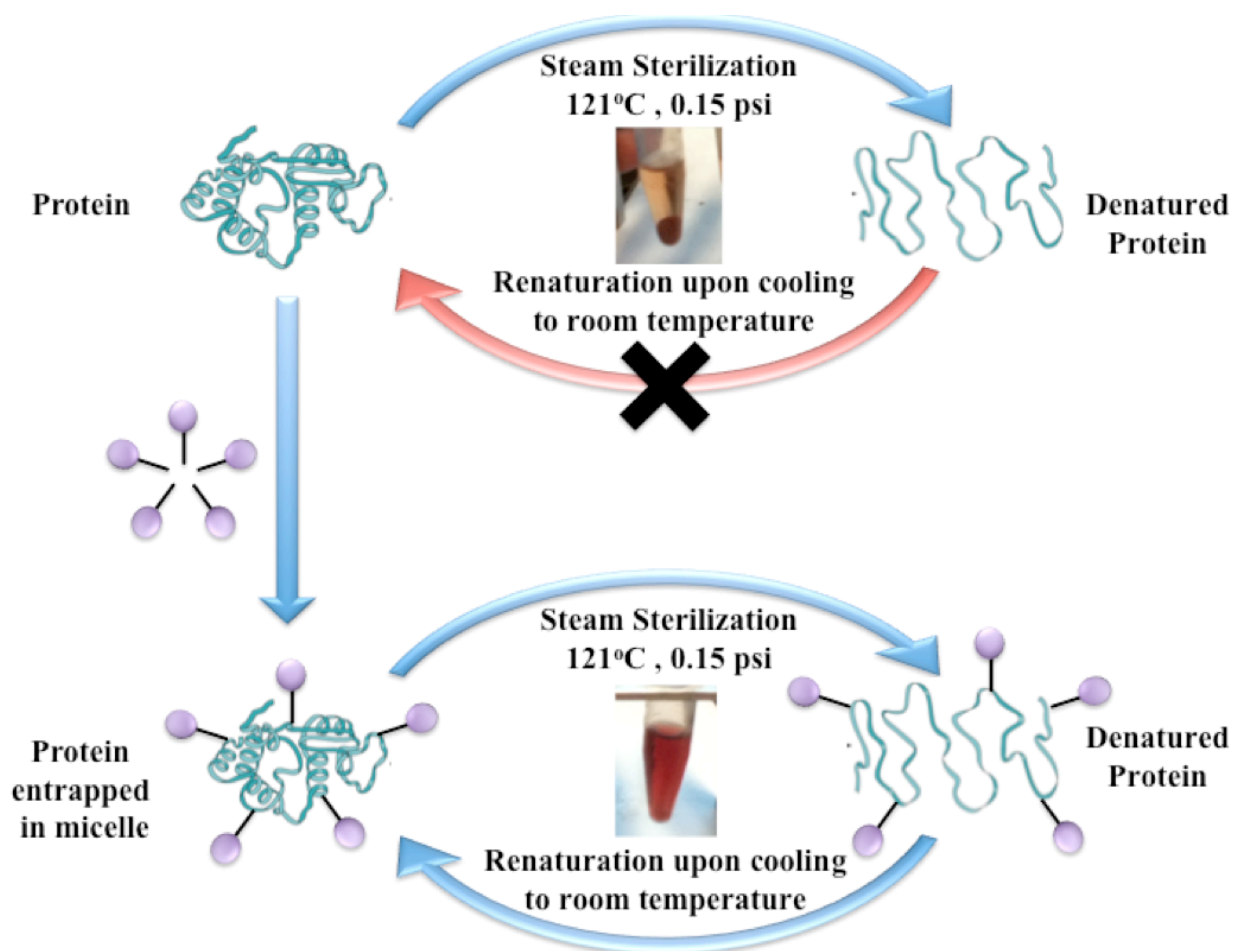
## 1.2 INTRODUCTION:

Proteins are very sensitive to their environment change. Most proteins denature at high temperature and even the thermostable proteins are completely denatured upon steam sterilization. Making the proteins stable for heat is an important issue to be addressed.<sup>1, 2</sup> Protein surfactant interactions are known for a long time and most of the studies focus on investigating the denaturation of protein in the presence of surfactant like sodium dodecyl sulfate (SDS).<sup>3</sup> The binding of surfactant was analyzed extensively based on the possible hydrophobic and ionic interactions of surfactant to the protein. Most studies on protein denaturation support the theory of pearl-necklace structure of protein with surfactant, where SDS forms a micellar structures on the protein backbone.

Kunio Takeda et al studied protective effect of SDS on secondary structure of protein. They showed that adding small amount of SDS to Bovine Serum Albumin (BSA) could protect the thermal denaturation of BSA below 85<sup>0</sup>C. This shows that SDS at a low concentration can prevent aggregation of protein.<sup>4</sup> Prevention of protein aggregation at low concentration of SDS was also characterized by Marcel Tabak et al showing that oxy-hemoglobin (oxy-HbGp ) in the presence of small amount of SDS can prevent the precipitation of protein upon heating suggesting that separating proteins from contact with other protein can prevent protein precipitation and refolding upon thermal denaturation of protein.<sup>5</sup>

In the present study we observed that capric acid (CA), which is made up of 10-carbon hydrophobic tail, could stabilize the secondary structure of proteins for steam sterilization. In our previous study of hemoglobin-PAA system we showed that the protein could be stabilized for steam sterilization by encapsulating proteins within the polymer shell.<sup>6</sup> In this

study CA concentration above its critical micellar concentration (CMC) was used to protect the secondary structure of protein by forming a protective layer around protein. Proteins with different pI were studied to understand the interaction of proteins with surfactant and the influence of charge on protein stability. The results show that proteins can refold upon cooling to room temperature after steam sterilization in the presence of CA above its CMC. The protein-CA adducts were made by heating at high temperature and was used in making the energy transfer complex. A highly efficient artificial energy transfer complex like that of naturally occurring photosystem<sup>7</sup> to transfer energy between dyes was made by self-assembly of dyes on the BSA-CA adducts.



**Scheme.1. Showing the reversible thermal denaturation of protein in the presence of capric acid micelles**

### 1.3. EXPERIMENTAL SECTION:

**1.3.1 Materials.** Hemoglobin and Catalase were purchased from MP biomedical and Worthington respectively. Lysozyme, Myoglobin, Sodium Dodecyl Sulfate and Capric acid were purchased from sigma-aldrich. A sodium phosphate buffer of 50mM Concentration was used with a pH 8.0. Different concentration of capric acid solution (50, 90,100,120,150,200,300 mM) was made in sodium phosphate buffer, pH8.0.

**1.3.2 Protein/capric acid sodium salt (CA) complex.**100uM Protein (Hemoglobin, Myoglobin, Catalase, Lysozyme) was dissolved in different concentration of capric acid sodium salt solution.

**1.3.3 Steam Sterilization and Heat studies** Proteins in varying concentration of capric acid was steam sterilized in autoclave for 20 minutes at 121 C, 0.15 psi (Yamato, JW-500). The bovine serum albumin was heated in a water bath at 80 and 90 C to make BSA/CA adduct to use in energy transfer complex.

**1.3.4 Circular Dichroism (CD) studies.** Jasco J-710 CD spectrophotometer (Easton, MD) was used to measure the CD. A path length of 0.05cm quartz cuvettes was used in the wavelength range of 260- 190 nm. The spectra obtained were normalized per micro molar bound enzyme per 1 cm path length and the structure retention was calculated for the steam sterilized protein samples.

#### 1.3.5 Activity studies.

**Hemoglobin and Myoglobin.** The peroxidase-like activity of Hb and Myo (pH 8) was used

to study the % of activity retention after steam sterilization. Substrate O-methoxyphenol and oxidant  $\text{H}_2\text{O}_2$  were added to the protein solution. Oxidized product formation was monitored as a function of time in the kinetics mode reading the absorbance at 470nm using an HP 8453 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA).<sup>8</sup> From the plot of absorbance versus time, initial specific activities were calculated.

**Catalase.** Decomposition of  $\text{H}_2\text{O}_2$  by catalase was measured at 240 nm to compare the activity of steam-sterilized catalase to that of the unheated protein.<sup>9</sup> The absorbance at 240 nm was monitored continuously with respect to time, using an HP 8453 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) and from the plot of absorbance versus time the initial specific activities were calculated.

**Lysozyme.** Lysis of *Micrococcus luteus* cells by lysozyme was monitored at 450nm using an HP 8453 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). The drop in the absorbance at 450nm with the cell lysis was plotted against time. The specific activity was calculated from the plot and the activity of lysozyme before and after steam-sterilization was compared.<sup>10</sup>

**1.3.6 Agarose Gel Electrophoresis.** Agarose (0.5% w/v) was dissolved in a hot Tris acetate buffer (40mM, pH 8) to prepare native agarose gels. Tris acetate (40 mM pH 8) was used as the running buffer to run the gel in a horizontal gel electrophoresis apparatus (Gibco model 200, Life Technologies Inc., MD) Samples were made with loading buffer (50% v/v glycerol and 0.01% m/m bromophenol blue), and loaded into the gel, and the gel electrophoresis was carried out at room temperature for 30 mins at 100mV. The gel was then removed and stained overnight with 10% v/v acetic acid and 0.02% m/m Coomassie

blue followed by destaining with 10% v/v acetic acid for 4 hours.

**1.3.7 SDS-PAGE gel electrophoresis.** Covalent modification of protein with capric acid at high temperature is analyzed using a sodium dodecyl sulfate gel with 12 % separating and 5 % stacking gel recipe. Samples were made in 2% SDS and 10% BME and it was loaded and ran at 200 V in a Bio Rad Mini protein electrophoresis apparatus. The gel was stained in 10% v/v acetic acid and 0.02% m/m Coomassie blue followed by destaining with 10% v/v acetic acid for 4 hours.

**1.3.8. *Dynamic light scattering experiment.*** Samples were filtered with 0.2 micron filtered and particle size were determined by Dynamic light scattering instrument (Precision Detectors, Varian Inc) using a 1cm square plastic cuvette at 658nm excitation laser source that was placed at 90° geometry for the purpose of excitation and monitoring.

#### **1.3.9. Energy Transfer Complex.**

A mix containing coumarin (125uM), fluorescein (100uM), rhodamine (40uM), BSA-CA (0.5mM) was added to Hoechst donor (50uM) and was loaded on a slide and dried overnight and then the fluorescence spectra was measured by exciting at two different excitation wavelengths (350 nm and 650 nm)

## **1.4. RESULTS AND DISCUSSION**

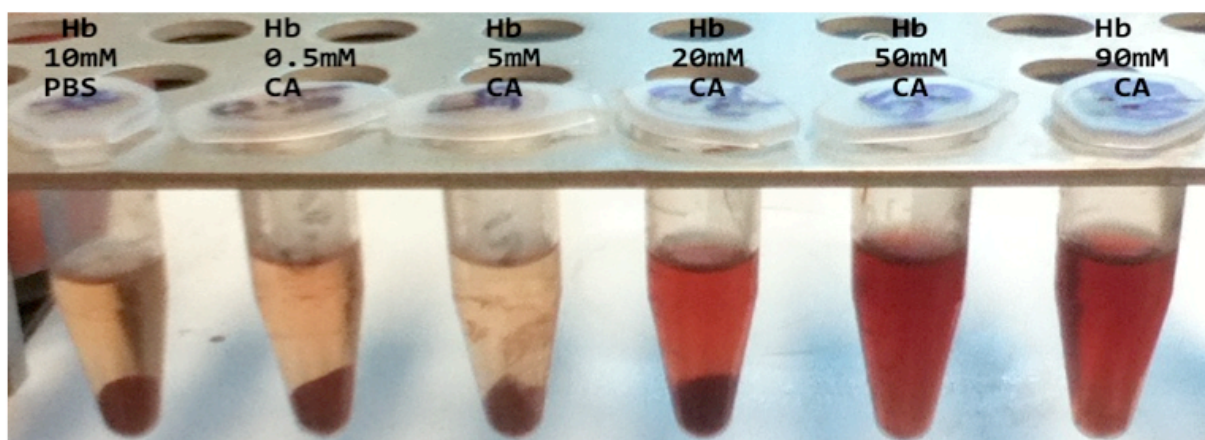
### **1.4.1. Agarose and SDS-PAGE Gel electrophoresis**

Agarose gel electrophoresis was run on the samples of protein in varying concentration of capric acid before and after the steam sterilization done. It was observed that the hemoglobin, catalase and lysozyme became more negative after steam sterilization (Figure 1.2-1.4). The possible explanation could be the covalent modification of proteins with capric acid at high temperature neutralizing the positive amine group of the protein and making it more negative. SDS PAGE gel electrophoresis which separates the protein based on the molecular weight was run to analyze the covalent modification of the protein with CA upon steam sterilization. The SDS-PAGE gel of all three proteins studied in this experiment showed a smeared band after steam sterilization of the proteins with CA which suggests that the covalent modification of proteins with CA after steam sterilization resulting in a non uniform modification of the protein and thus resulted in migration of proteins at different rate as a smeared band (Figure 1.5-1.7).

As an application of the protein/CA adduct in constructing an artificial energy transfer antenna complex BSA/CA adduct was made. A high concentration of BSA (100mg/mL) was needed for the making the energy transfer complex and steam sterilization of BSA at that high concentration resulted in forming a gel and hence the temperature was optimized to keep the BSA/CA adducts in the liquid phase. Time controlled heating was done at 80 and 90 °C. BSA in 90mM CA stayed as liquid for 7 minutes of heating at 90 °C with slight change in the viscosity and formed a gel after 8 minutes of heating at 90 °C. The agarose gel showed that after 6 minutes of heating at 90 °C the protein became more positive than

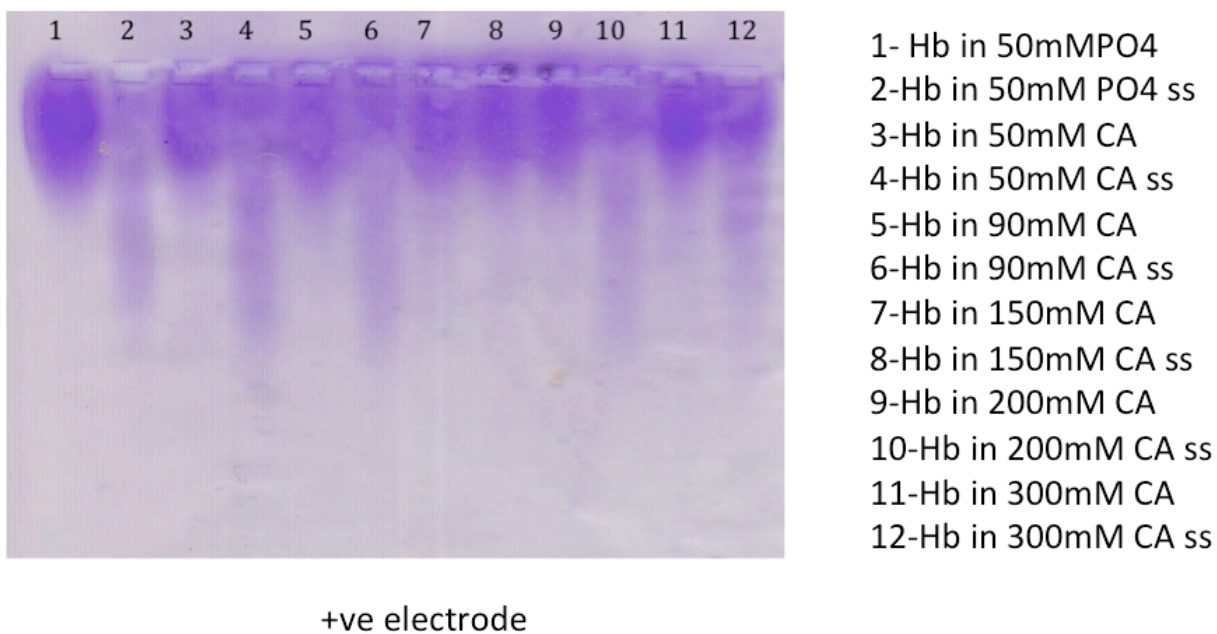
the unmodified BSA in phosphate buffer and also the rate of change in the charge was slower (18 minutes) when heated at 80 °C. This shows that BSA/CA adducts can be formed even at lower temperature (Figure 1.8).



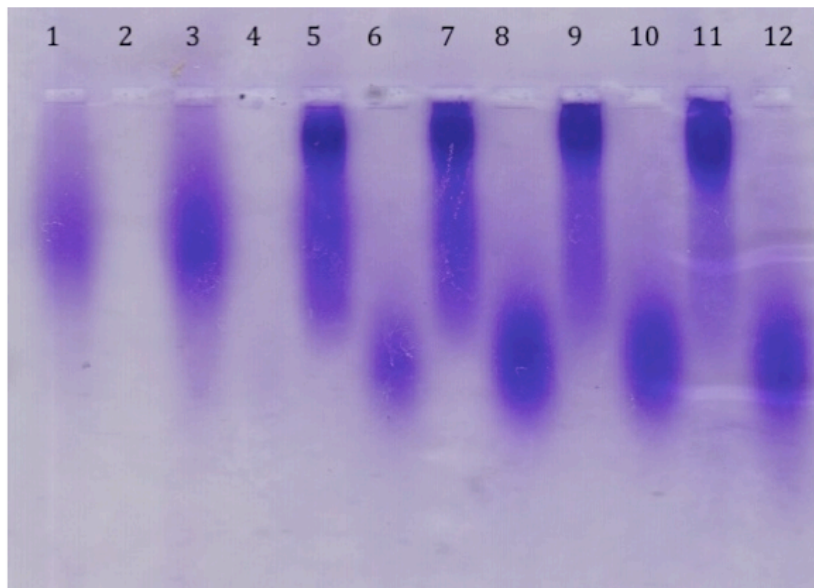


*Steam sterilized hemoglobin in the presence of varying concentration of capric acid.*

*No precipitate of hemoglobin was observed above the CMC of CA.*



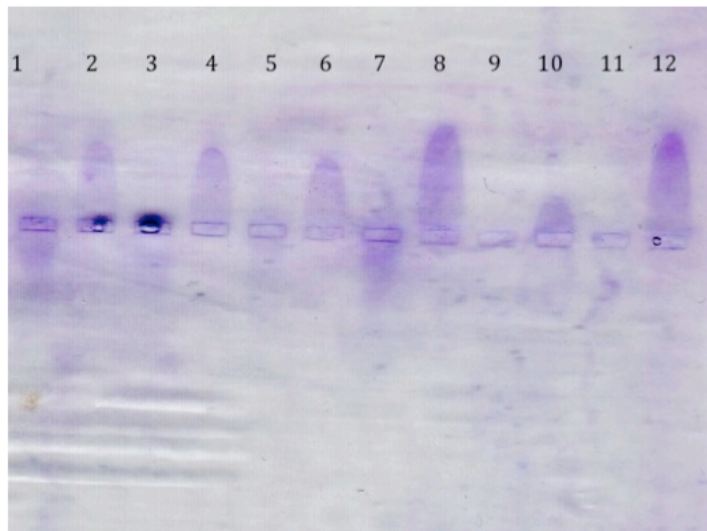
**Figure.1.2) Agarose gel of Hemoglobin (Hb) in varying concentration of CA. The steam sterilized (SS) Hb in CA became more negative charge.**



- 1- Cat in 50mMPO4
- 2-Cat in 50mM PO4 ss
- 3-Cat in 50mM CA
- 4-Cat in 50mM CA ss
- 5-Cat in 90mM CA
- 6-Cat in 90mM CA ss
- 7-Cat in 150mM CA
- 8-Cat in 150mM CA ss
- 9-Cat in 200mM CA
- 10-Cat in 200mM CA ss
- 11-Cat in 300mM CA
- 12-Cat in 300mM CA ss

**+ve electrode**

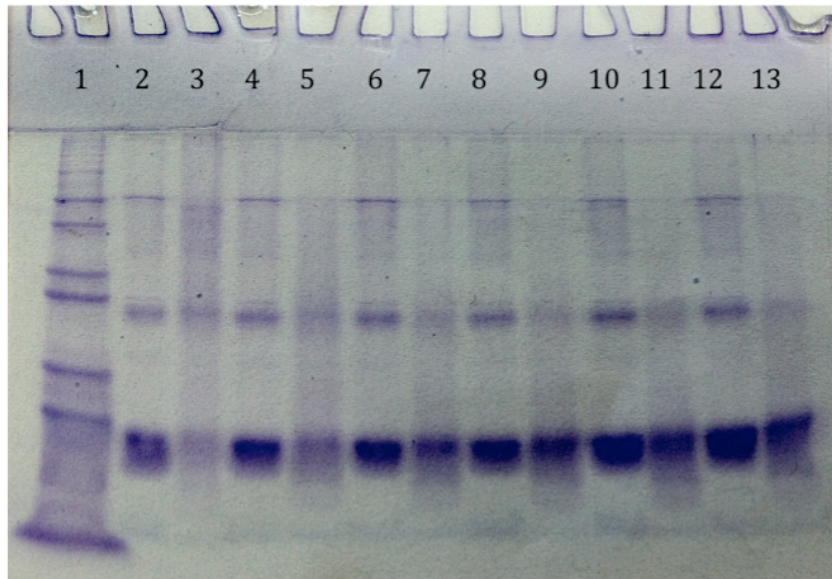
**Figure.1.3) Agarose gel of Catalase (Cat) in varying concentration of CA. The steam sterilized (SS) Cat in CA became more negative charge.**



- 1- lyz in 50mMPO4
- 2-lyz in 50mM PO4 ss
- 3- in 50mM CA
- 4-lyz in 50mM CA ss
- 5-lyz in 90mM CA
- 6-lyz in 90mM CA ss
- 7-lyz in 150mM CA
- 8-lyz in 150mM CA ss
- 9-lyz in 200mM CA
- 10-lyz in 200mM CA ss
- 11-lyz in 300mM CA
- 12-lyz in 300mM CA ss

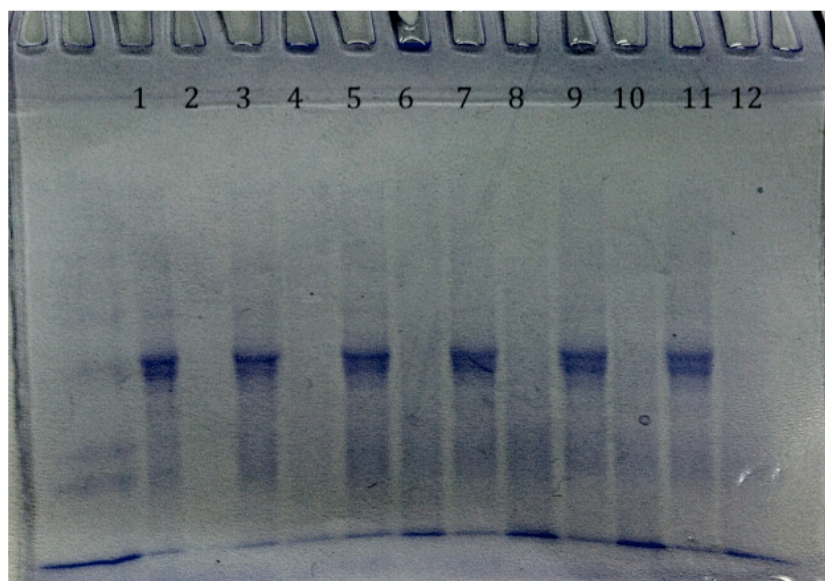
+ve electrode

**Figure.1.4) Agarose gel of Lysozyme (Lyz) in varying concentration of CA. The steam sterilized (SS) Lyz in CA became more negative charge.**



- 1-Marker
- 2- Hb in 50mMPO4
- 3-Hb in 50mM PO4 ss
- 4-Hb in 50mM CA
- 5-Hb in 50mM CA ss
- 6-Hb in 90mM CA
- 7-Hb in 90mM CA ss
- 8-Hb in 150mM CA
- 9-Hb in 150mM CA ss
- 10-Hb in 200mM CA
- 11-Hb in 200mM CA ss
- 12-Hb in 300mM CA
- 13-Hb in 300mM CA ss

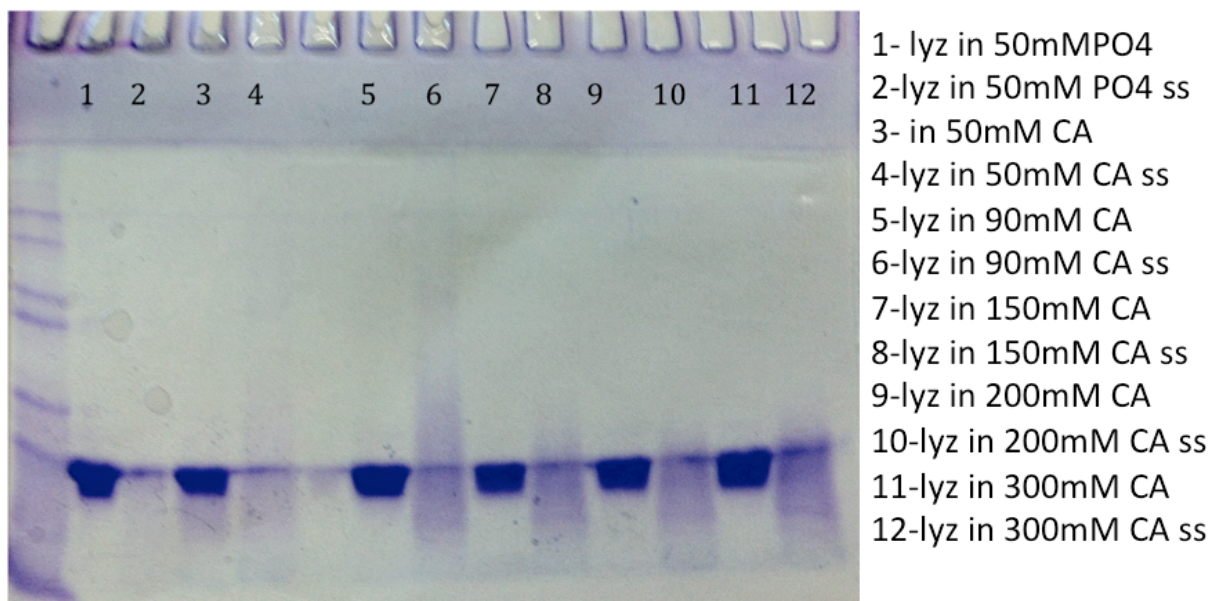
**Figure.1.5) SDS-PAGE gel of hemoglobin(Hb) in varying concentration of CA.The Steam sterilized (SS) Hb in CA showed a smeared band.**



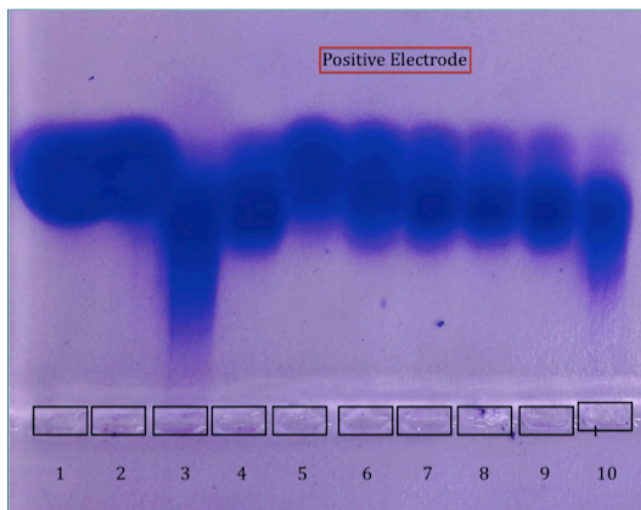
- 1- Cat in 50mMPO4
- 2-Cat in 50mM PO4 ss
- 3-Cat in 50mM CA
- 4-Cat in 50mM CA ss
- 5-Cat in 90mM CA
- 6-Cat in 90mM CA ss
- 7-Cat in 150mM CA
- 8-Cat in 150mM CA ss
- 9-Cat in 200mM CA
- 10-Cat in 200mM CA ss
- 11-Cat in 300mM CA
- 12-Cat in 300mM CA ss

**Figure.1.6) SDS-PAGE gel of Catalase (Cat) in varying concentration of CA. The Steam sterilized (SS) Cat in CA showed a smeared band.**





**Figure.1.7) SDS-PAGE gel of Lysozyme (lyz) in varying concentration of CA. The Steam sterilized (SS) Lyz in CA showed a smeared band.**



- 1) BSA in 50mM PO<sub>4</sub> buffer
- 2) BSA in 90mM CA Before heating
- 3) BSA in 90 mM CA (heated in 90<sup>0</sup>C water bath for 6 mins)
- 4) BSA in 90 mM CA (heated in 90<sup>0</sup>C water bath for 3 mins)
- 5) BSA in 90 mM CA (heated in 80<sup>0</sup>C water bath for 3 mins)
- 6) BSA in 90 mM CA (heated in 80<sup>0</sup>C water bath for 6 mins)
- 7) BSA in 90 mM CA (heated in 80<sup>0</sup>C water bath for 9 mins)
- 8) BSA in 90 mM CA (heated in 80<sup>0</sup>C water bath for 12 mins)
- 9) BSA in 90 mM CA (heated in 80<sup>0</sup>C water bath for 15 mins)
- 10) BSA in 90 mM CA (heated in 80<sup>0</sup>C water bath for 18 mins)

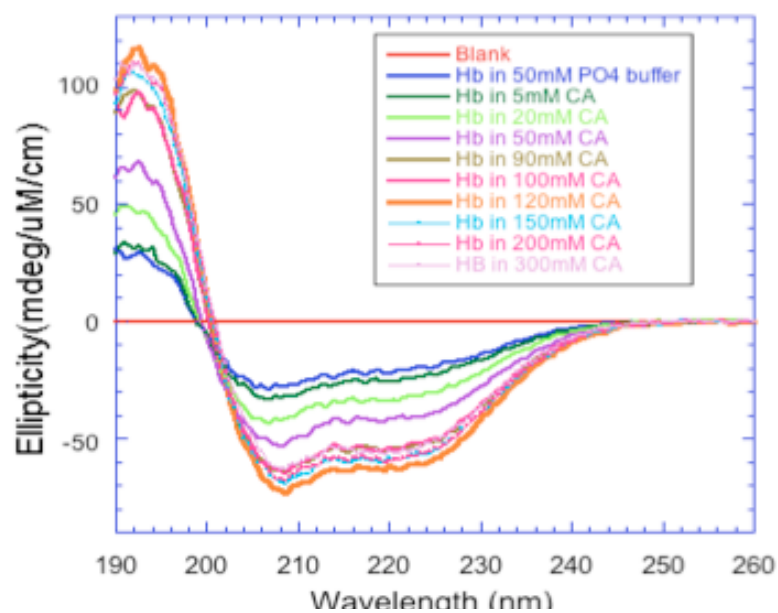
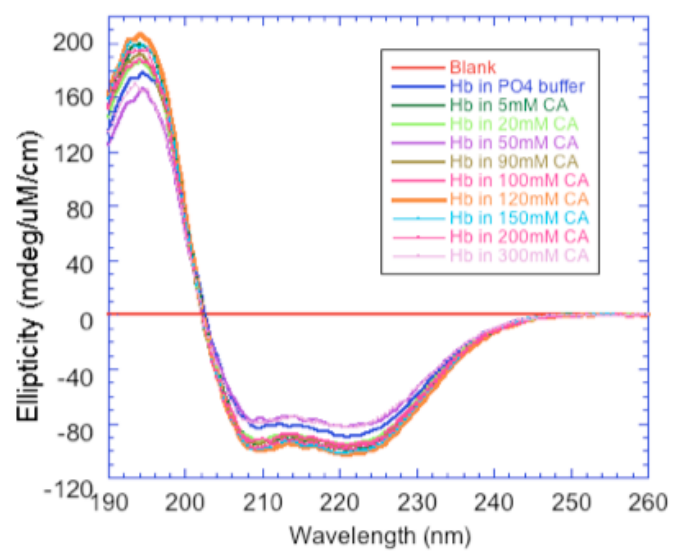
**Figure.1.8) Agarose gel of BSA in varying concentration of CA. The BSA-CA adducts formed after heated became more positive charge.**



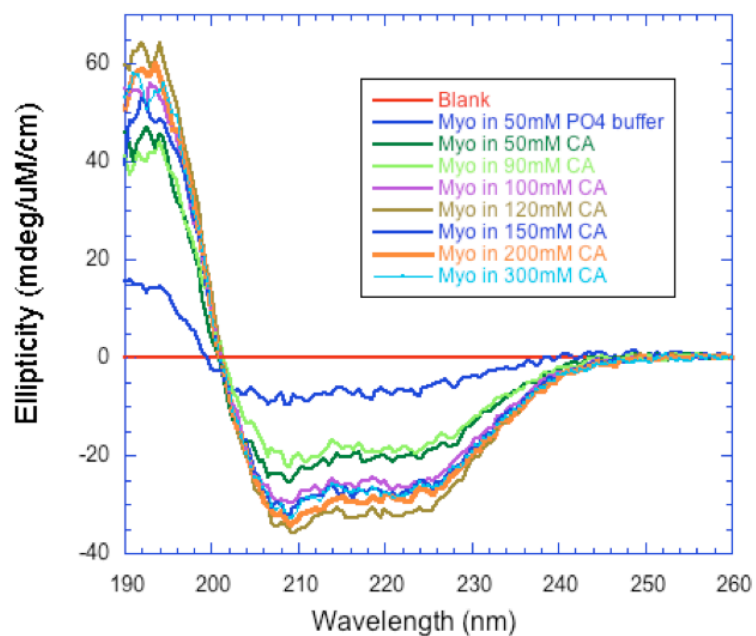
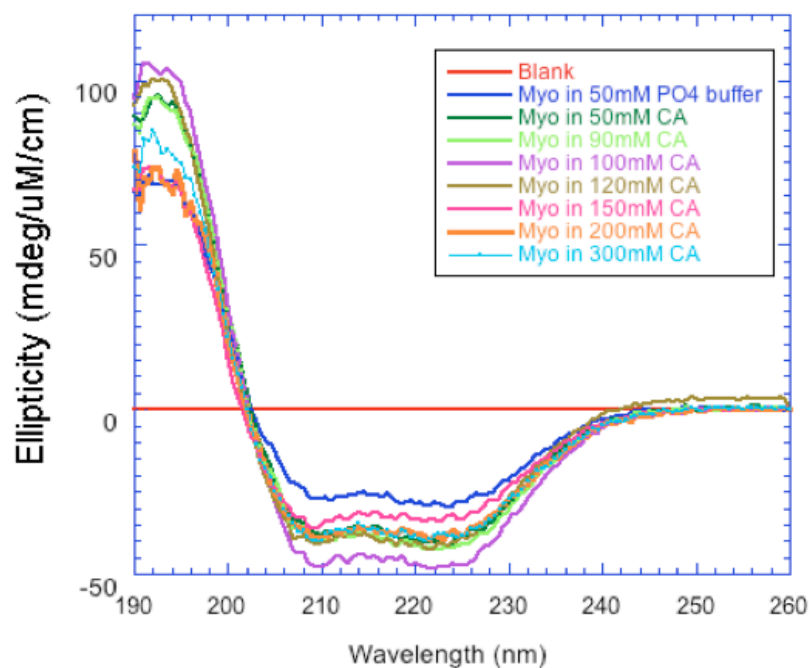
### 1.4.2 Stability towards steam sterilization and heat

In the present study the secondary structure of four different proteins with different pI range was studied in the presence of CA for thermal denaturation. The proteins were steam sterilized at 121<sup>0</sup>C for 20 minutes and then cooled to room temperature and the proteins were characterized before and after steam sterilization. Proteins without CA precipitated out completely upon steam sterilization. In the presence of CA when the concentration of CA was increased above the CMC of CA the protein didn't precipitate and the secondary structure (alpha and beta sheets) of the protein was protected. The CD was measured to calculate the percentage of secondary structure retention, which was observed to vary with different protein. The structural change of protein was studied in the presence of varying concentration of CA before steam sterilization (Figure 1.9-1.12).

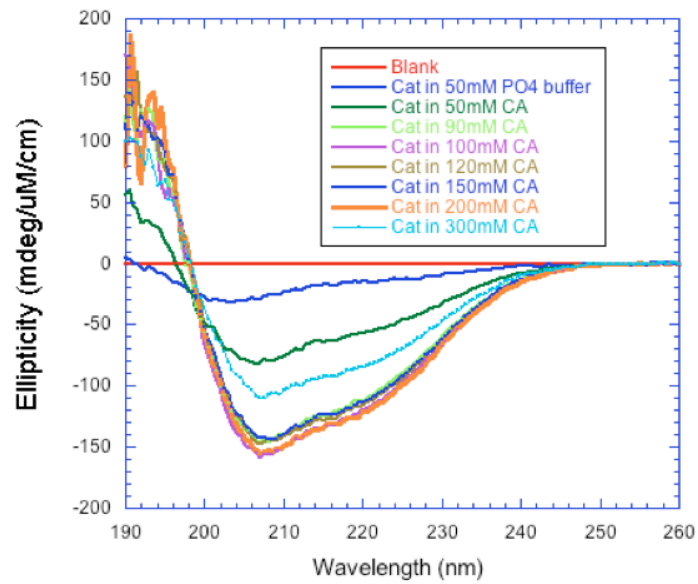
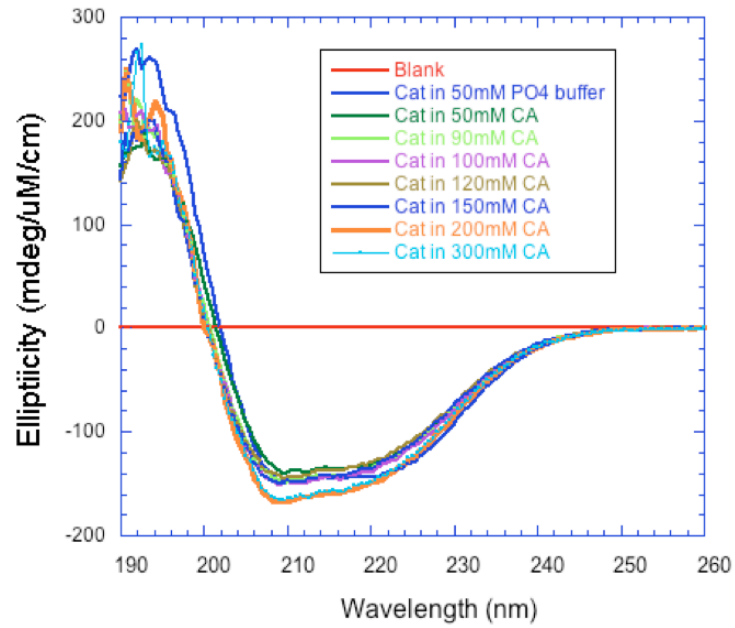
The percentage of structure change of protein relative to protein in 50mM phosphate buffer calculated based on the CD obtained plotted against varying concentration of CA (Figure 1.13). Hemoglobin showed a maximum of 15% structural change at 120mM CA and decreased to a negative change of 7.4 % with increasing the concentration of CA to 300mM CA. Myoglobin showed a maximum structural change among the proteins studied with a structural change of 70 % at 100mM CA and again a decreasing trend of structural change was observed. Catalase in 120mM CA showed a maximum change with a negative structural change of 12% compared to the catalase in 50mM phosphate buffer. Lysozyme in 50mM CA didn't dissolve completely and hence showed 70% negative structure retention relative to lysozyme in 50mM phosphate buffer and a structural change of 15% was observed at 300mM CA.



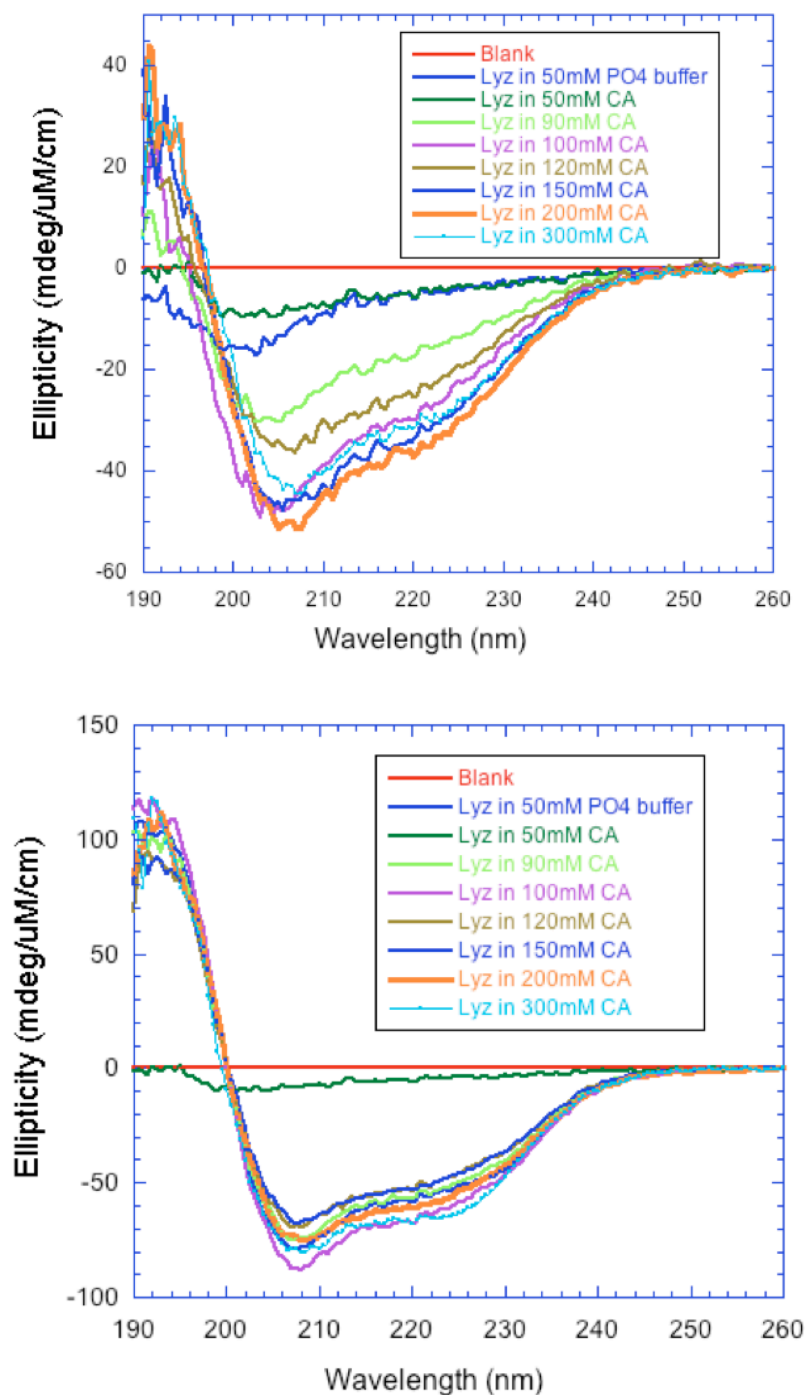
**Figure.1.9. A) UV-CD of hemoglobin in varying concentration of capric acid before steam sterilization.B) UV-CD of hemoglobin in varyind concentration of capric acid after steam sterilization**



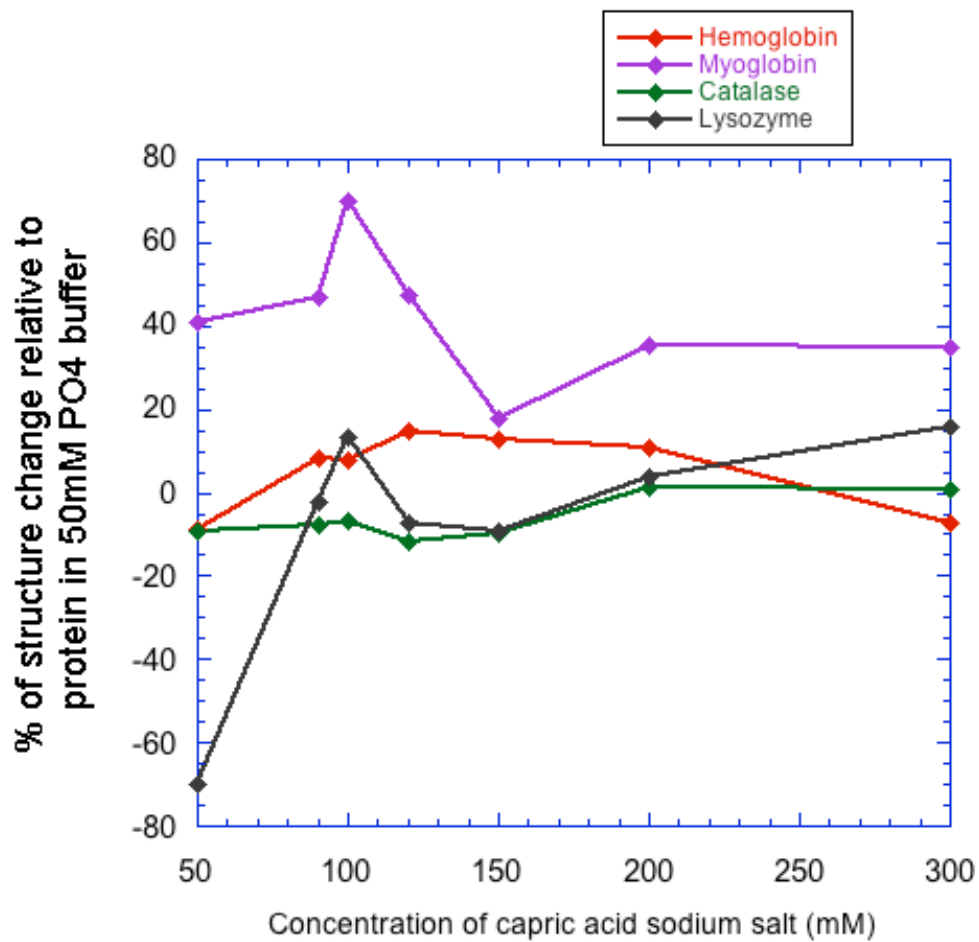
**Figure.1.10. A) UV-CD of myoglobin in varying concentration of capric acid before steam sterilization.B) UV-CD of myoglobin in varying concentration of capric acid after steam sterilization**



**Figure.1.11. A) UV-CD of catalase in varying concentration of capric acid before steam sterilization B) UV-CD of catalase in varying concentration of capric acid after steam sterilization**



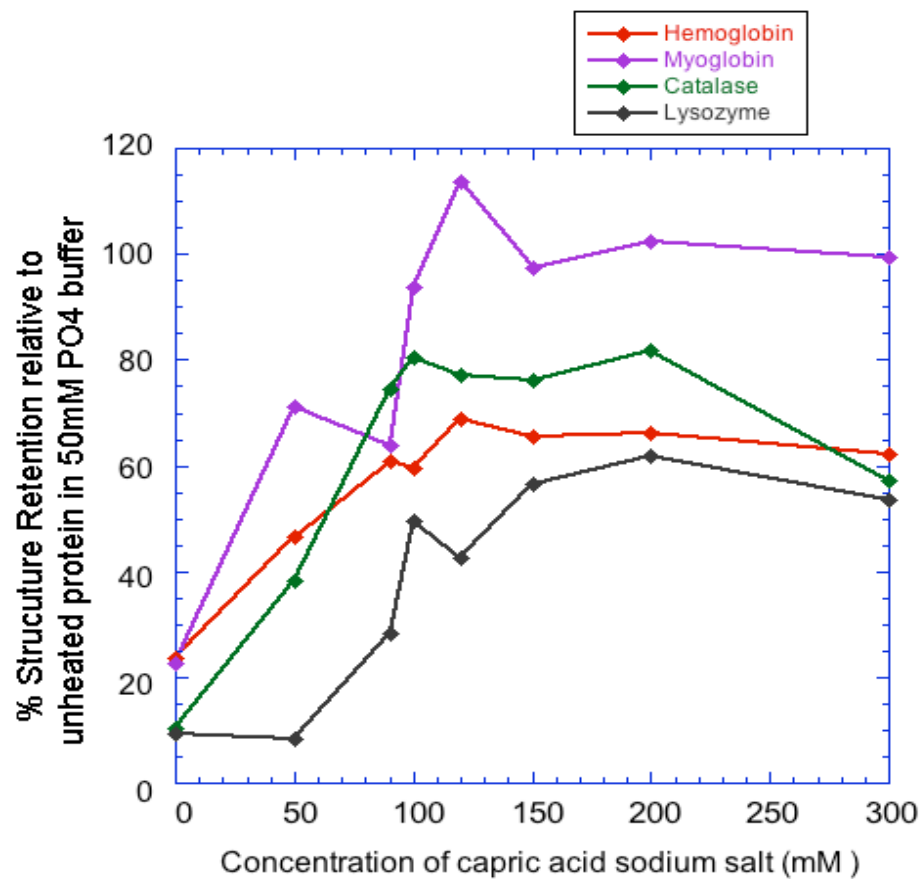
**Figure.1.12. A) UV-CD of lysozyme in varying concentration of capric acid before steam sterilization B) UV-CD of lysozyme in varying concentration of capric acid after steam sterilization**



**Figure.1.13. % of structure change relative to protein in 50mM sodium phosphate buffer plotted against the varying concentration of CA**

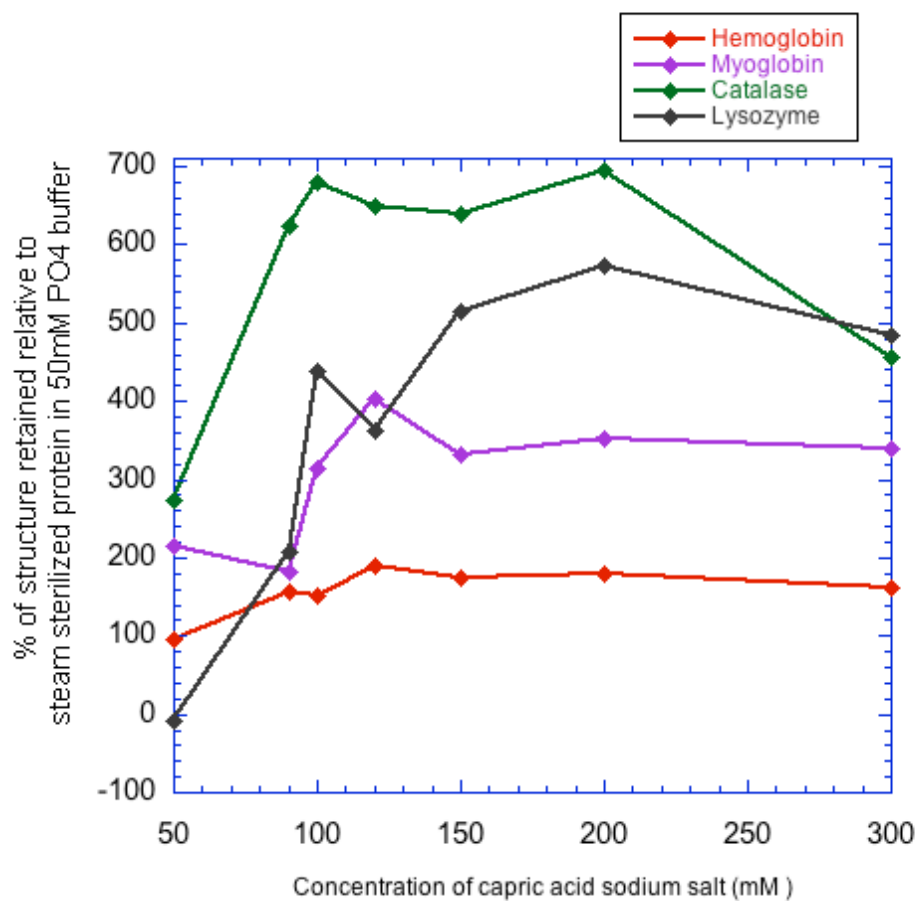
The percentage of structure retention of the proteins after steam sterilization was compared to that of the unheated protein. Myoglobin a smaller protein with a molecular weight of 16.95KDa, with a near neutral pI-6.8 showed a maximum of 135% percentage of structure retention at 300mM CA concentration compared to that of the unheated protein in buffer. Hemoglobin with a molecular weight of 64.5KDa with pI-6.8, which is larger in size compared to Myoglobin showed a maximum of 69% of structure retention at 120mM CA. Catalase which is the largest protein studied with a molecular weight of 240 KDa and with a pI -5.8 showed a maximum of 81% structure retention at 200mM CA. Lysozyme (MWt-14.3KDa) with a pI-11.3 showed a maximum of 61% structure retention at 200mM CA (Figure 1.14).

The CD of the steam-sterilized sample was also compared to that of the steam-sterilized protein in 50mM phosphate buffer to understand the relative percentage of efficiency of CA to refold the protein to its secondary structure. Among the proteins studied catalase showed maximum efficiency of refolding with 695% at 200mM CA as most of catalase in 50mM phosphate buffer precipitated out upon steam sterilization. Followed by catalase lysozyme showed a higher efficiency of refolding with a maximum of 573% at 200mM CA. Myoglobin and hemoglobin showed a maximum of 403% and 189% efficiency respectively at 120mM CA respectively (Figure1.15).



**Figure.1.14 % of structure change relative to un heated protein in 50mM sodium phosphate buffer plotted against the varying concentration of CA**





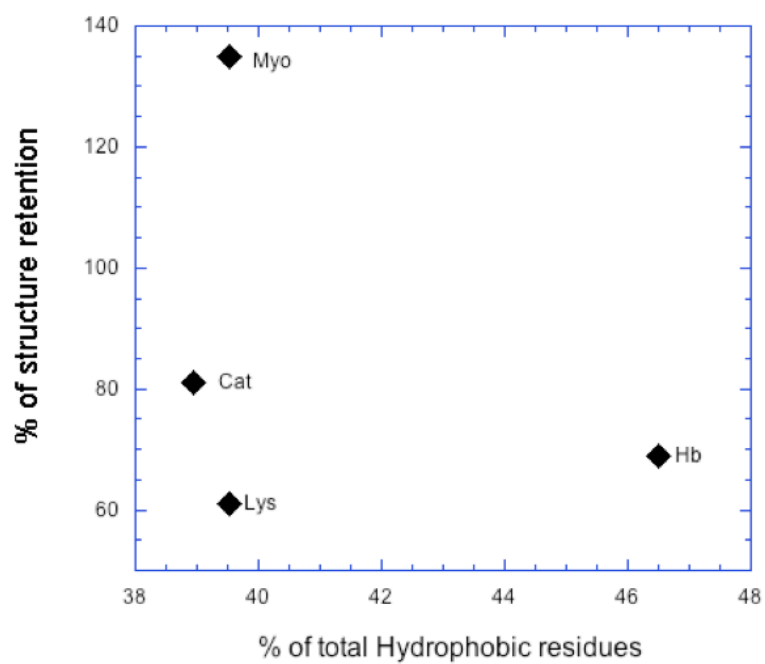
**Figure.1.15. % of structure change relative to steam sterilized protein in 50mM sodium phosphate buffer plotted against the varying concentration of CA**

As mentioned earlier, it is already known that surfactant like SDS shows protective effect on secondary structure of protein well below its CMC and it denatures protein at its CMC. Interestingly in our study it was observed that CA stabilizes protein and prevents aggregation and s

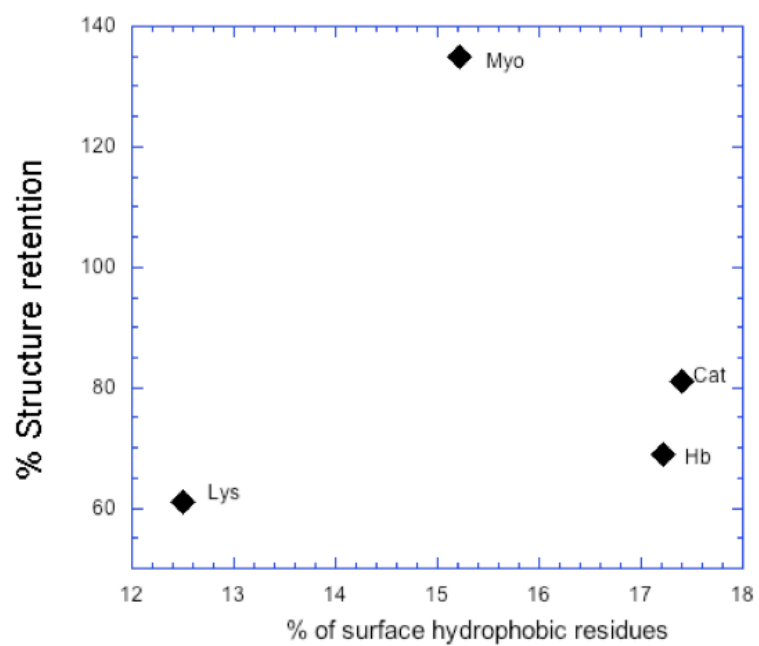
hows maximum structure retention at a concentration above its CMC which is contradictory to the classical pearl – necklace model proposed for protein denaturation by SDS at its CMC. The interaction of CA with protein well above its CMC shows a maximum improvement in the secondary structure upon steam sterilization. The structural improvement was also observed upon addition of CA to protein is an evidence of strong interaction of CA with protein. Myoglobin showed maximum structural change upon adding CA, which corresponds to the maximum structure retention showed by myoglobin after steam-sterilization. There are no definite correlations with the pI or size to that of the protective effect by CA on protein for steam sterilization. Hydrophobic and ionic interactions are the two possible ways for CA to form complex with protein and thus preventing aggregation of protein upon steam sterilization.

Hydrophobic residues of protein were thus analyzed to understand the role of hydrophobic interaction of CA in the protective effect of protein for steam sterilization. Myoglobin showed structural change to a greater degree even before steam sterilization though it has a lower % of hydrophobic residues with 35.29% compared to hemoglobin with 46.5% (Figure 1.16). Hence the surface residues of the protein were

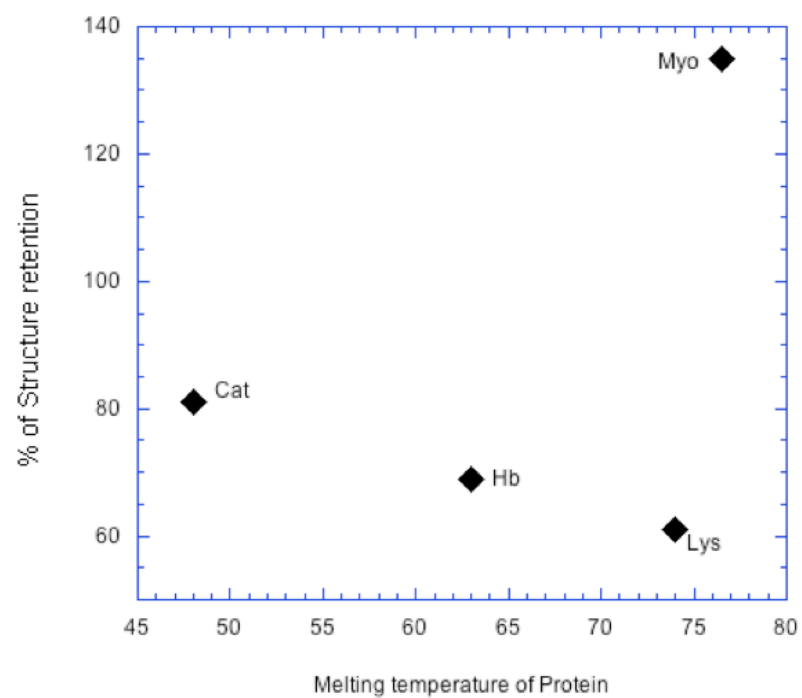
considered and analyzed for the % of surface residues to that of the CA protective effect on protein structure (Figure 1.17). There was no distinct difference in the % of hydrophobic residues for the proteins studied. Myoglobin has surface hydrophobic residues of 15.21% while hemoglobin with 17.21%. Lysozyme and catalase have 12.5% and 15.21% of surface hydrophobic residues respectively. These suggest that % of distribution of hydrophobic and ionic residues don't influence the protective effect of CA on protein for steam sterilization.



**Figure.1.16.% of total hydrophobic residues in the protein with respect to structure retention**



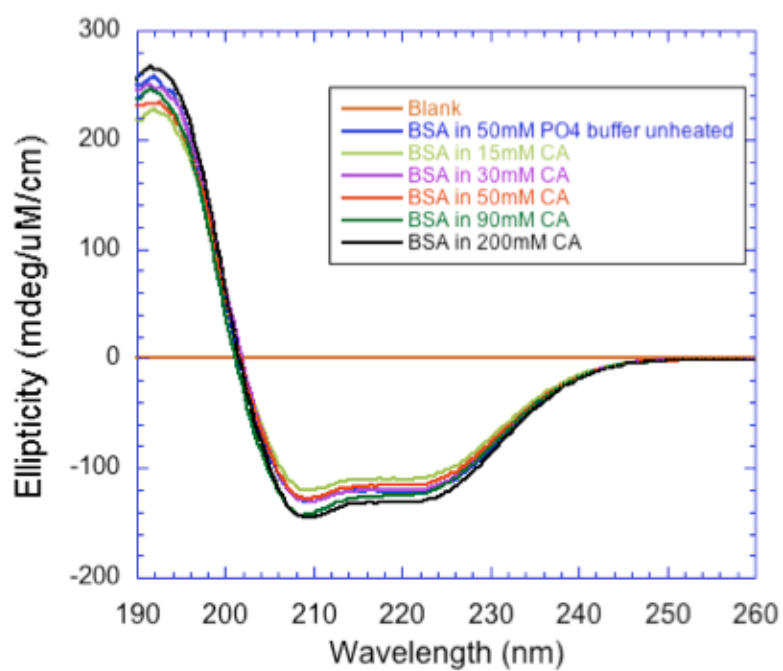
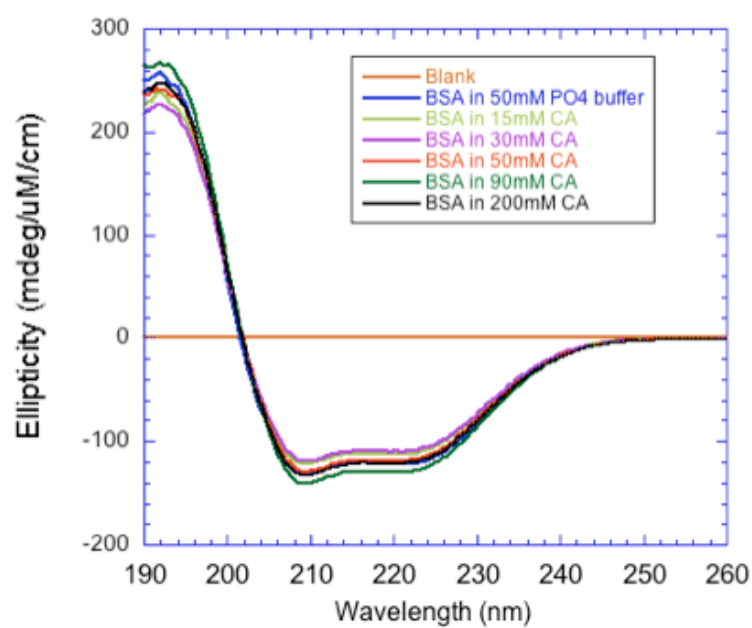
**Figure.1.17.% of surface hydrophobic residues in the protein with respect to structure retention**



**Figure.1.18.% of melting point of the protein with respect to structure retention**

The melting temperature of the protein was further analyzed to understand the stability of protein in CA. Myoglobin has a higher melting temperature of 63<sup>o</sup>C compared to that of hemoglobin with a melting temperature of 76.5<sup>o</sup>C. This corresponds to the higher % of structure retention by myoglobin compared to that of the hemoglobin but Lysozyme with a melting point of 74<sup>o</sup>C showed a lowest % of structure retention (61%) compared to all the proteins studied. Also Catalase with a melting point of 48<sup>o</sup>C showed 81% of structure retention. The intrinsic stability of protein also does not explain the stability of protein in CA as no correlation can be made based on the melting temperature of protein for the varying degree of structure retention showed by different protein. (Figure.1.18)

The agarose gel electrophoresis showed that the protein capric acid adducts can be formed even at temperature below its boiling point. The structural changes of BSA thus measured after heating to 90 °C to analyze structural changes. The BSA in capric acid showed good structure retention after heating for 6 minutes at 90 °C (Figure.1.20).



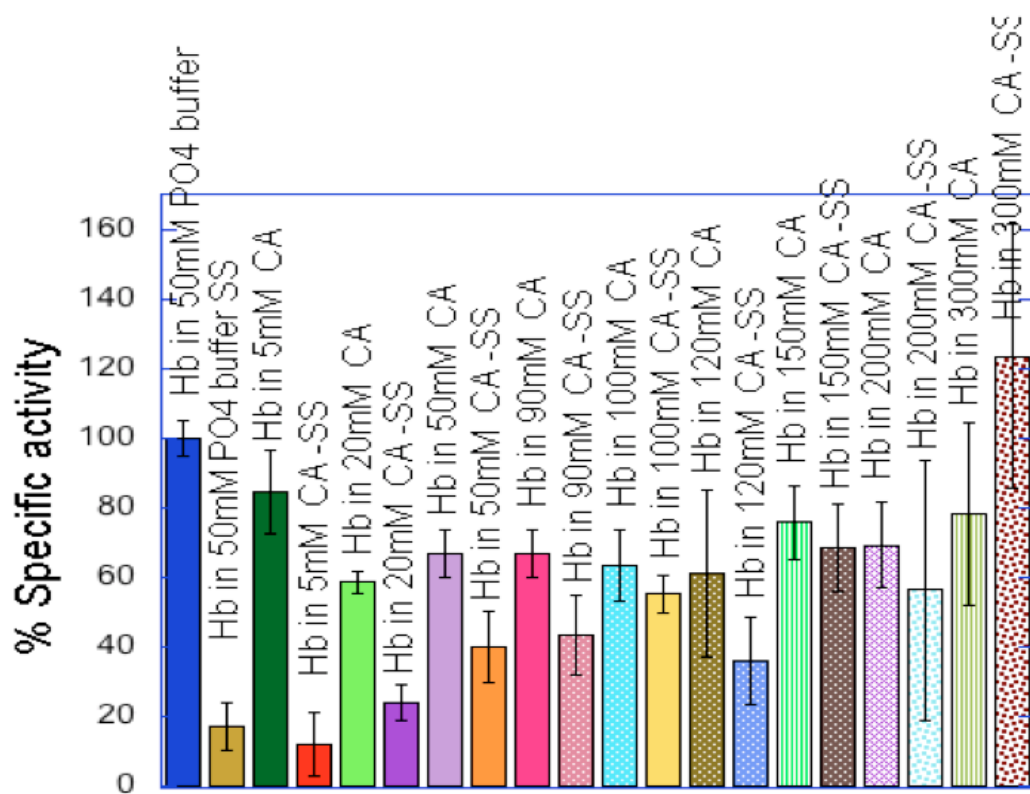
**Figure.1.20 A) UV CD of BSA-CA adducts in varying concentration of CA unheated. B) UV CD of BSA-CA adducts in varying concentration of CA (heated to 90C for 6 minutes and cooled to room temperature)**



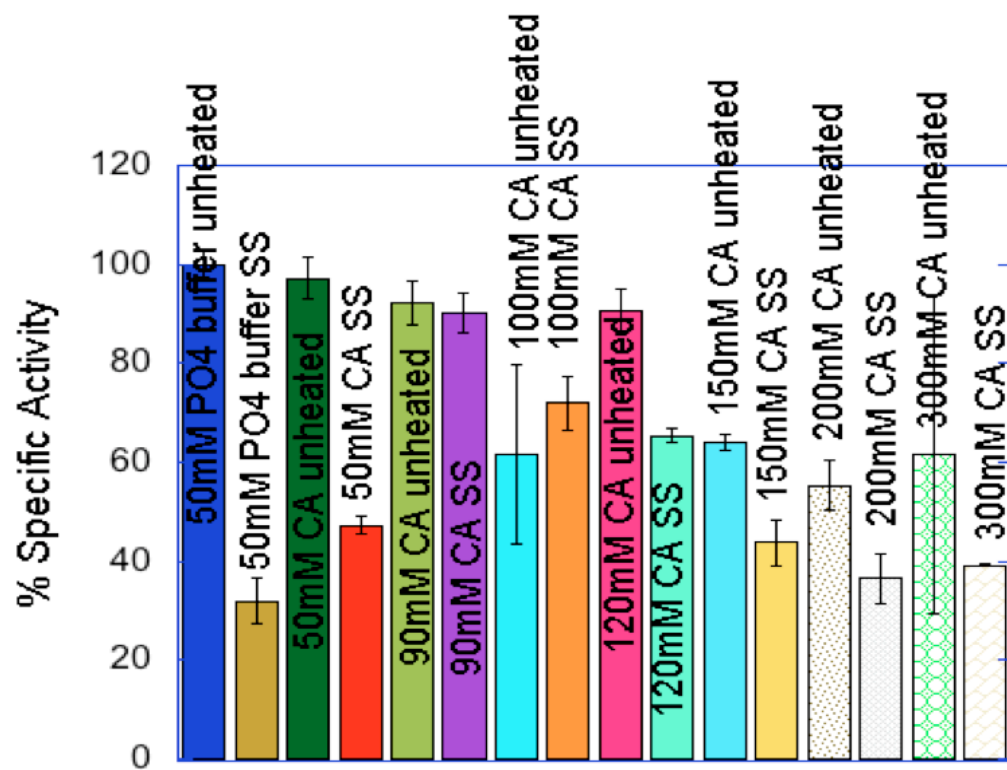
### 1.4.3. Activity study

Each protein exhibit different kind of activity. For example heme containing protein can dimerize guaiacol in the presence of hydrogen peroxide which can be measured by the product formation at 470nm. Non-metalloprotein like lysozyme can break the bacterial cell through their active site. In this study heme activity of hemoglobin showed an increase in the activity with an increasing concentration of CA for steam sterilized hemoglobin samples. Steam Sterilized myoglobin showed > 90% activity retention between range 90mM to 120mM CA. Interestingly catalase showed a 6-fold higher activity in the presence of CA sodium salt whereas the heated Catalase in the presence of CA didn't protect the activity of protein though structure retention was observed. Among the proteins studied lysozyme showed poor activity in the presence of CA. The increased activity of myoglobin at particular range 90-120mM CA shows that excessive CA results in interaction of CA with the active site whereas in case of hemoglobin higher concentration of CA helps in opening the activity site. When heme in the presence of CA was ran as a control it showed 90 % activity. If the activity was due to the removal of heme from protein at high temperature the activity should have been uniform at all concentration of CA. Also in case of the catalase and lysozyme steam sterilization resulted in complete loss of activity which could be due to the interaction of CA with the active sites upon steam sterilization. The strong interaction of CA with protein upon steam sterilization could explain the binding of CA to active site. It can be seen on the agarose gel that the steam sterilized protein became more negative and moved more towards the positive electrode compared to that of the unheated protein explaining that proteins form strong interaction with CA upon steam-sterilization. It is not clear the reason for metalloproteins to stay active after steam sterilization whereas no

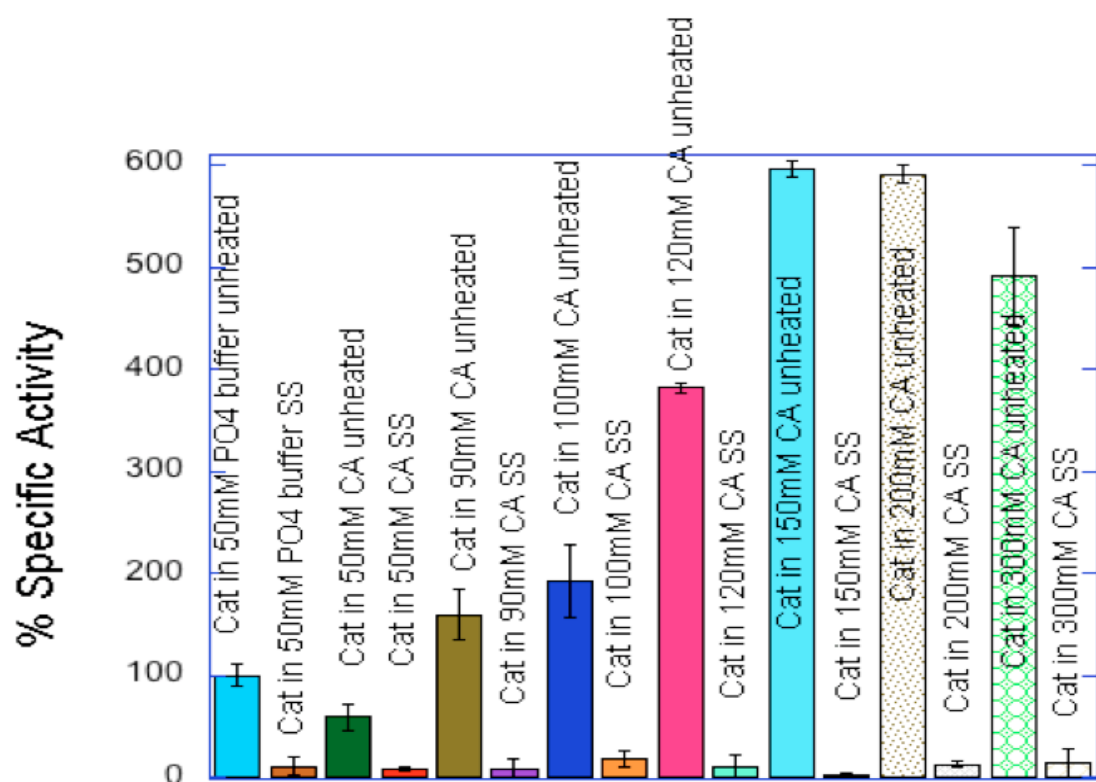
activity was observed in case of non-metalloproteins. A 6-fold increase in the activity of catalase was observed in the presence of higher concentration of CA. It is known in the literature that a low SDS concentration of 3mM can activate catalase up to 180% for decomposition of  $\text{H}_2\text{O}_2$ .<sup>11</sup> It was another observation made in the study that CA can activate catalase to greater percentage (~600% activity observed at 150-200mM CA) compared to that of SDS.



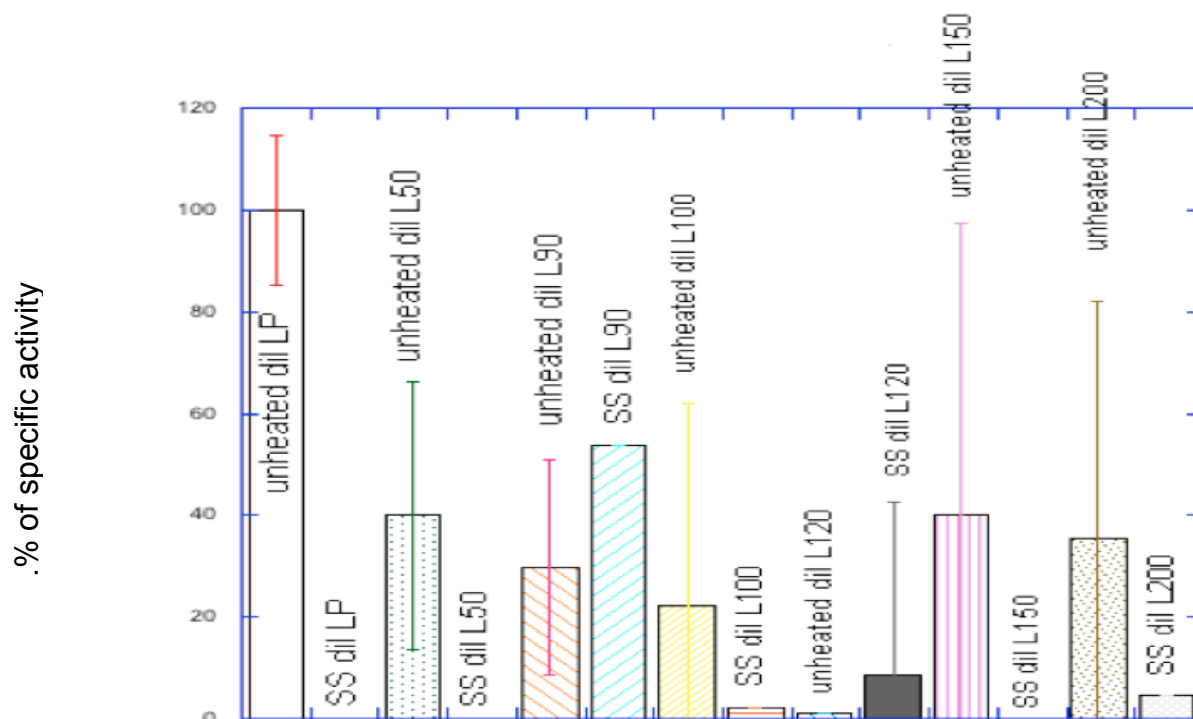
**Figure.1.21.% of specific activity of hemoglobin after steam sterilization in varying concentration of capric acid**



**Figure.1.22.% of specific activity of myoglobin after steam sterilization in varying concentration of capric acid**



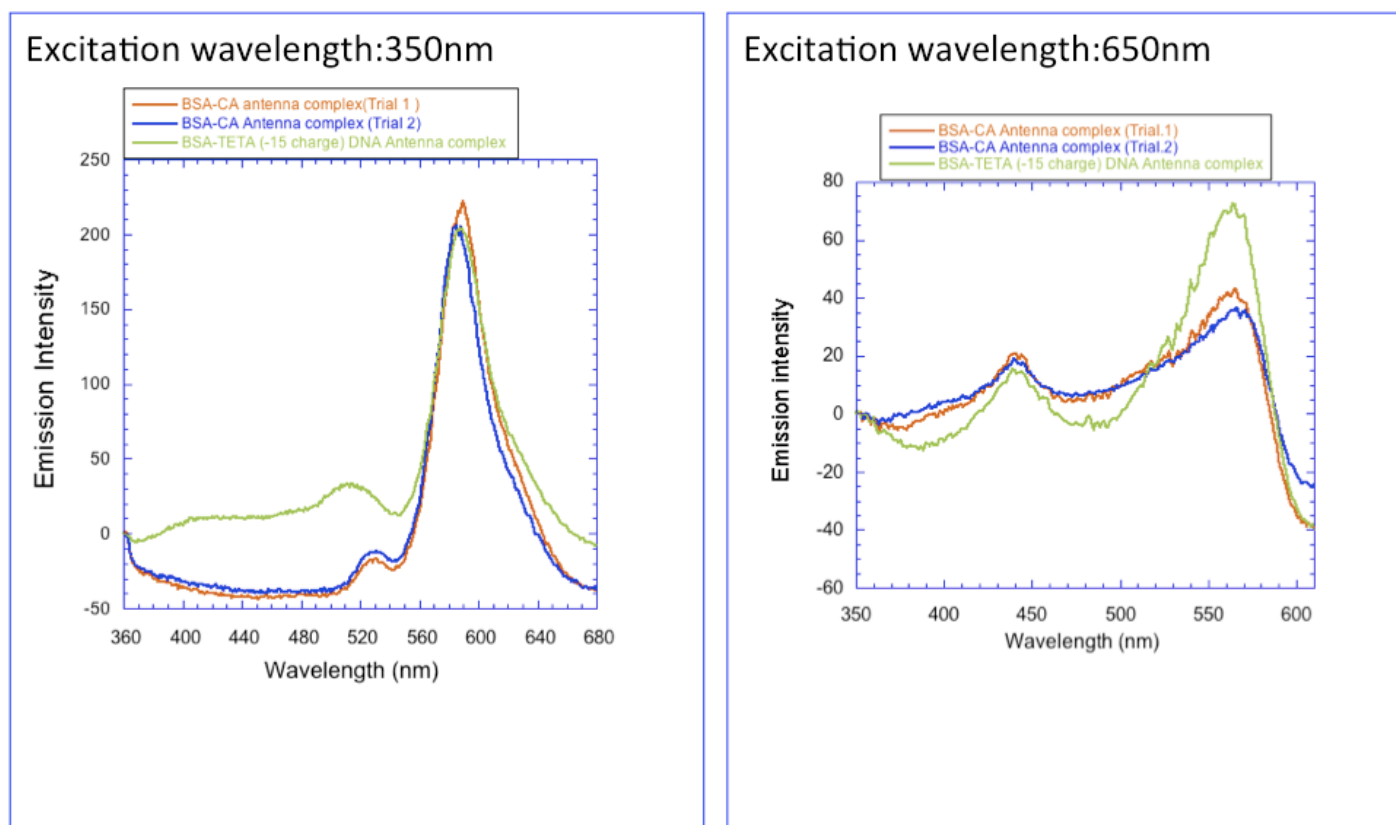
**Figure.1.23.% of specific activity of catalase after steam sterilization in varying concentration of capric acid**



**Figure.1.24.% of specific activity of Lysozyme after steam sterilization (SS)in varying concentration of capric acid**

#### 1.4.5 Energy Transfer complex

In our lab we have shown a self-assembly of four dyes in BSA/DNA complex to form artificial energy transfer cascade which has a potential application to harness solar energy. BSA was positively modified with Tetraethylene tetraamine (TETA) through edc chemistry and a physical complex of TETA modified BSA with negatively charged DNA was made. Four dyes starting with a highest energy donor was constructed in a cascade with other dyes as energy acceptors transferring energy. The dyes used as a highest energy donor was Hoeschst 33258 and the energy was transferred to coumarin 540A followed by that to the acceptor fluorescein and then from fluorescein it was transferred to the lowest energy acceptor Rhodamine B. In the present study the BSA/CA adduct was used to construct the energy transfer complex and was compared to the BSA-TETA/DNA system. Figure 1.25 A shows the emission spectra obtained after exciting the hoeschst dye. The energy was transferred from hoeschst to other dyes through the cascade and thus only the emission spectra of the Rhodamine B, the lowest energy acceptor, was observed. Similarly when the complex was excited at rhodamine B the dyes leading to energy transfer were observed showing the operational of dye cascade system in energy transfer (Figure 25.B). The BSA/CA adducts thus can bring the different dyes together to transfer energy like natural photosystem that occurs in plants and can find application in making solar cells.



**Figure.25.A) Emission spectra of BSA-CA adducts that measured at excitation wavelength of hoeschst (350nm).The energy was transferred in the dye cascade system and the lowest energy acceptor emission (rhodamine B) was observed as emission spectra. B) The emission spectra obtained after exciting at 650nm.**



#### **4. CONCLUSION:**

The present study thus demonstrates the novel role of CA in the structure retention and catalytic activity changes in different proteins. Though there are no definite correlation can be made based on the hydrophobic and ionic interactions or based on the thermodynamic parameters of the protein for interaction of protein –CA adducts, but it was observed that CA protected the aggregation of protein for steam sterilization. The SDS-PAGE gel electrophoresis shows a proof of covalent modification of proteins with CA at high temperature. A new material of protein-fatty acid adducts was thus synthesized by heating the proteins with fatty acids at high temperature. Also the potential application in constructing energy transfer complex makes a novel simple system for building artificial energy transfer complex. Since the protein-CA adducts are not immune responsive it could be used as a drug delivery machine. This protein fatty acid adducts need to be further explored for its potential application in medical and biomaterial field.

## **Chapter.2.**

### **Chemical modification of proteins with Octadecenyl succinic anhydride**

## **2.1ABSTRACT:**

Hydrophobicity of a protein is an important factor for its interaction with hydrophobic solid surfaces. This plays a significant role in making biomaterials that are not immune responsive *in vivo*. Since the protein contains fewer hydrophobic residues in the inner core the most common way of making biomaterials involve tedious way of changing the natural hydrophobic solid system to hydrophilic surface to enhance protein binding. In our study we use a simple and easy approach to make hydrophobic protein through covalent modification of amine group of protein with octadecenyl succinic anhydride (C18SA).<sup>12</sup> Agarose gel showed that hemoglobin and Bovine serum albumin were successfully modified with C18SA. Activity study of hemoglobin showed that the modification reaction was very fast and protected the denaturation of protein by THF, which was used as a solvent for dissolving C18SA. Solubility test of C18SA modified protein in organic solvents showed that the modified proteins were not hydrophobic enough to dissolve in organic solvents. Further studies are needed to use these hydrophobically modified proteins in making biomaterials or to be used in *in vivo* drug delivery.

**Keywords:** *Bovine serum albumin, Octadecenyl succinic anhydride, Agarose gel, biomaterial*

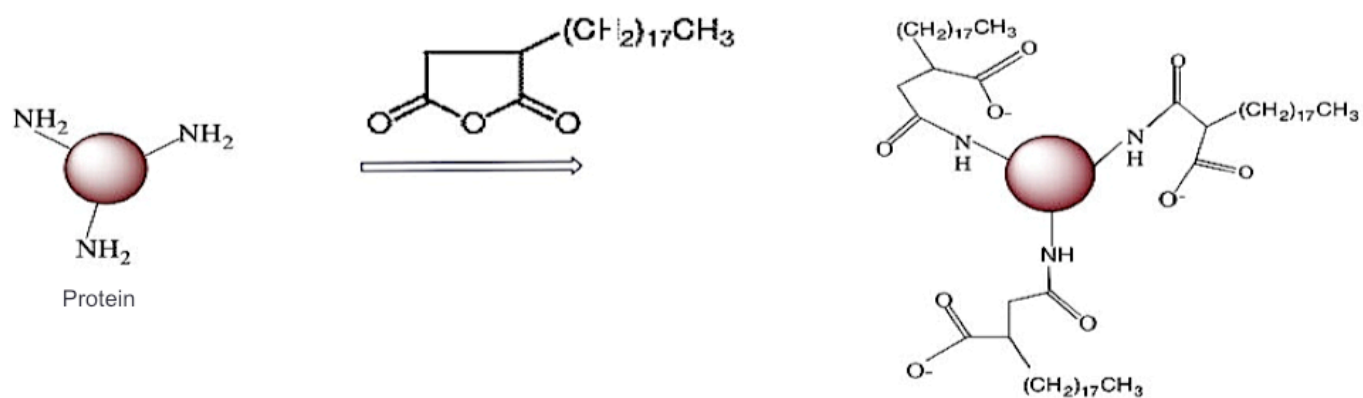
## 2.2INTRODUCTION:

Proteins are biological molecules made up of basic units of amino acids. The hydrophobicity of amino acid depends on the nature of the amino acid that constitutes the protein. The hydrophobic patches of most proteins are concentrated in the inner core of the proteins making them water-soluble. Making the protein hydrophobic will find an important application in surface modification of biomaterials using proteins. Biomaterials are generally defined as material that comes in contact with the biological system and doesn't induce any cellular response. The cell multiplication in tissue engineering depends completely on the adhesion of the cell to the biomaterials and amino acid sequence of arginine-glycine-aspartate in protein is known for helping in cell adhesion through cell membrane receptor such as integrins.<sup>13</sup>

Making the proteins hydrophobic would help in designing a biomaterial with protein surface by interacting the protein through hydrophobic interaction with hydrophobic solid materials. Another application of hydrophobically modified proteins that was hypothesized is in drug delivery. Hydrophobic proteins could be a biocompatible system to carry hydrophobic drugs and because of the size of the particle it would increase the blood residence time of the drug as the hydrophobic proposed micellar assembly of hydrophobic chain of C18SA around the protein would decrease the rate of renal filtration.<sup>14</sup> Also we hypothesized that modifying proteins hydrophobic will help in making biocatalyst that are functional in organic solvents.

In this present as discussed our aim was to modify proteins to make it hydrophobic protein for its useful applications. Different covalent chemistry reactions are identified in nature for modification of protein. Octadecenyl succinic anhydride (C18SA), which has an eighteen-

carbon chain, was used for the chemical modification of protein in this study to build hydrophobic layer around the protein. The protein modification was tried at different ratio of protein to C18SA and our results showed a gradual increase in the modification with increase in the number of C18SA per protein in the reaction mixture and also a reproducible complete modification was obtained. Solubility of protein in different organic solvents was also tested to analyze the application of hydrophobically modified protein in organic solvents.



**Scheme.2.1. Chemical modification of protein with octadecenyl succinic anhydride**

## 2.3 EXPERIMENTAL SECTION:

**2.3.1 Materials.** Hemoglobin and bovine serum albumin was purchased from MP biomedical and sigma-Aldrich respectively. Octadecenyl succinic anhydride (C18SA) was purchased from TCI America. A sodium phosphate buffer of 10mM Concentration was used with a pH 7.0. The Protein stock 100uM was made in 10 mM sodium phosphate buffer, pH7.0. Octadecenyl succinic anhydride stock of 5mM was made in 100% THF.

**2.3.2 Method.** The protein was reacted with C18SA in varying ratios (BSA: C18SA – 1:1, 1:20, 1:30, 1:40, and 1:50). The total reaction volume used was 2mL with the concentration of protein was kept constant as 50uM and the concentration of C18SA was varied according to the ratio. The sample was reacted for 3 hours and then characterized for chemical modification.

**2.3.3 Agarose Gel Electrophoresis.** Agarose (0.5% w/v) was dissolved in a hot Tris acetate buffer (40mM, pH.8) to prepare native agarose gels. Tris acetate (40 mM pH 8) was used as the running buffer to run the gel in a horizontal gel electrophoresis apparatus (Gibco model 200, Life Technologies Inc., MD) Samples were made with loading buffer (50% v/v glycerol and 0.01% m/m bromophenol blue), and loaded into the gel, and the gel electrophoresis was carried out at room temperature for 30 minutes at 100mV. The gel was then removed and stained overnight with 10% v/v acetic acid and 0.02% m/m Coomassie blue followed by destaining with 10% v/v acetic acid for 4 hours.

**2.3.4 Circular Dichroism (CD) studies.** Jasco J-710 CD spectrophotometer (Easton, MD) was used to measure the CD. A path length of 0.05cm quartz cuvettes was used in the wavelength range of 260- 190 nm. The spectra obtained were normalized per micro molar

bound enzyme per 1 cm path length and the structure retention was calculated for the steam sterilized protein samples.

**2.3.5 Dynamic light scattering experiment.** Sample were filtered with 0.2 micron filtered and particle size were determined by Dynamic light scattering instrument (Precision Detectors, Varian Inc) using a 1cm square plastic cuvette at 5658nm excitation laser source that was placed at 90° geometry for the purpose of excitation and monitoring.

**2.3.6 Activity Studies.** The peroxidase-like activity of Hb was used to study the % of activity retention after modification. Substrate O-methoxyphenol and oxidant H<sub>2</sub>O<sub>2</sub> were added to the protein solution. Oxidized product formation was monitored as a function of time in the kinetics mode reading the absorbance at 470nm using an HP 8453 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). From the plot of absorbance versus time, initial specific activities were calculated.

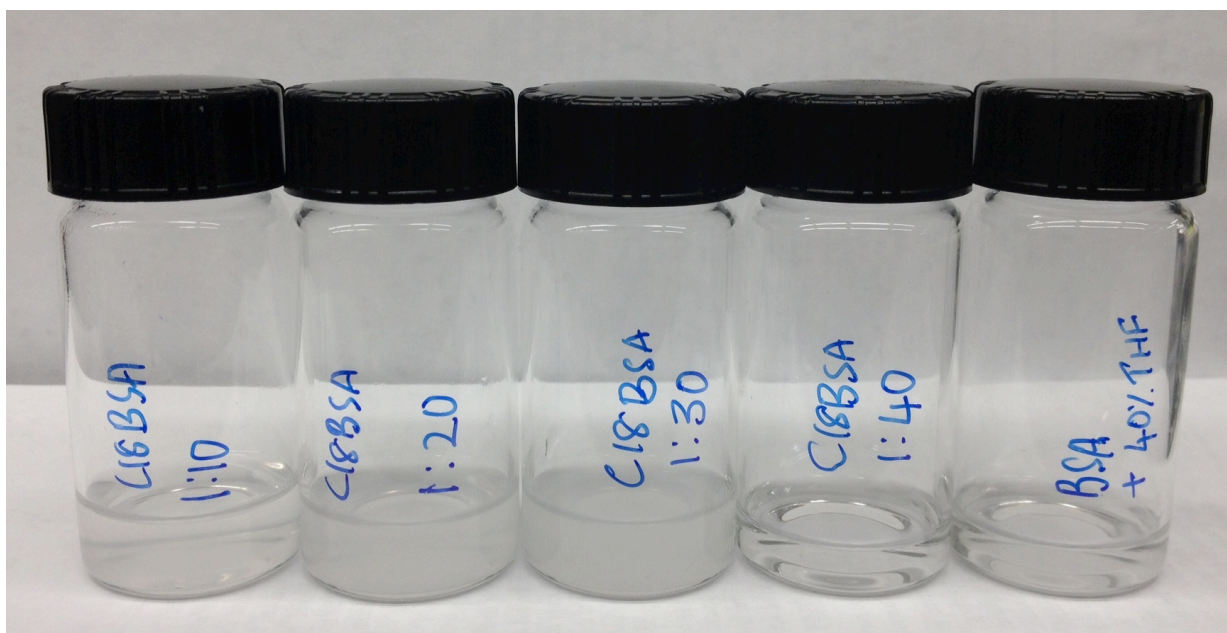
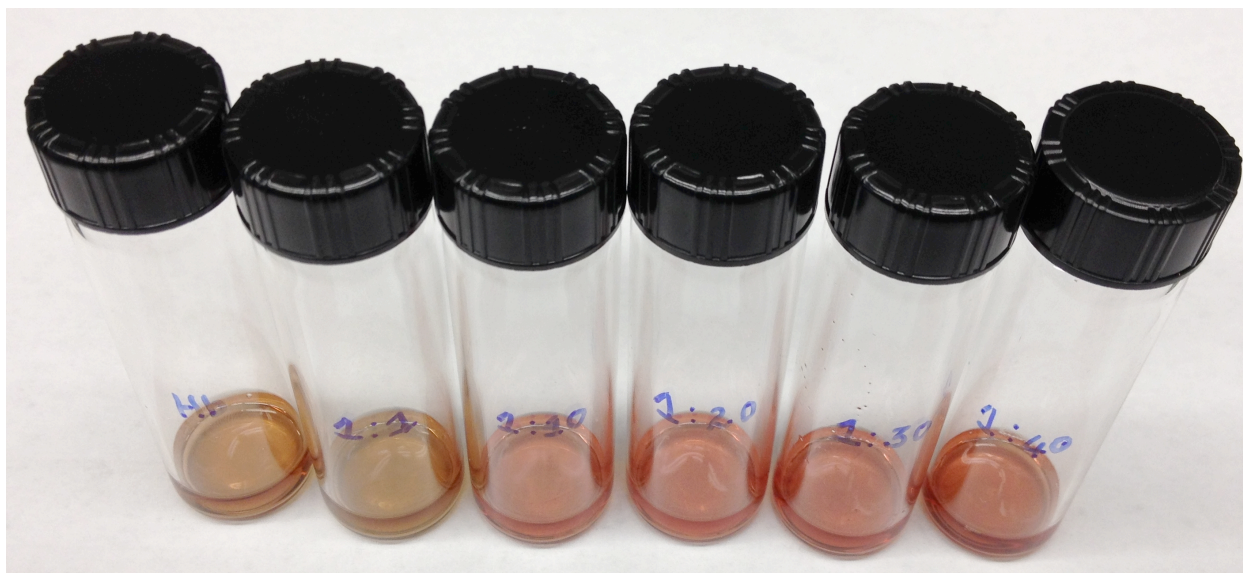
## **2.4 RESULTS AND DISCUSSION**

### **2.4.1 Agarose gel electrophoresis to study the chemical modification:**

The protein that was reacted with varying proportion to C18SA by dissolving the C18SA in THF as it is insoluble in water. The reacted sample was run on agarose gel to study the change in the charge after modification. The protein showed an increase in negative charge with increase in the number of C18SA per protein. This is due to the reaction of succinic anhydride of C18SA with amine group of protein and thus neutralizing a positive charge for every amine that is being modified. Modification of hemoglobin and bovine serum albumin showed similar trend and maximum charge change was observed for the highest

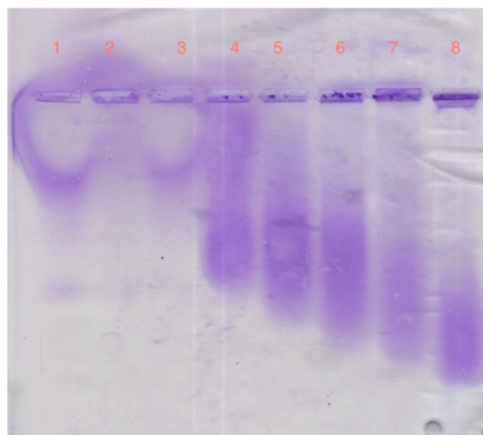


concentration of C18SA used (Figure 2.2A and Figure 2.3). The Hemoglobin sample was ran in corresponding % of THF as a control and no change in the charge was observed (Figure2.2B). This shows the change in the charge is due to the modification of protein with C18SA.



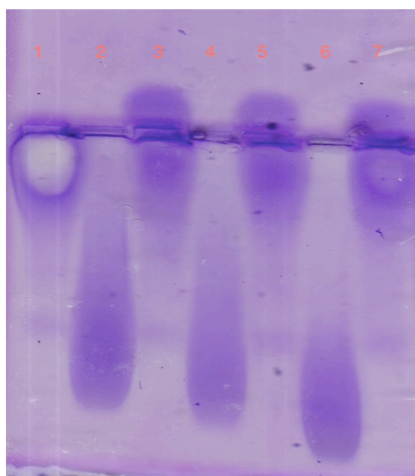
**Figure.2.1 A) C18SA modified Hemoglobin (Hb) with increasing ration of Hb:C18SA. Color change was observed with increase in the C18SA concentration.**

**B) C18SA modified BSA with increasing ratio of BSA: C18SA. The BSA: C18SA ratio of 1:20 and 1:30 became turbid after the reaction.**



- 1) Hb in 10mM PO<sub>4</sub>
- 2) Hb in 50% THF
- 3) Hb :C18 (1:1)
- 4) Hb:C18 (1:10)
- 5) Hb:C18 (1:20)
- 6) Hb :C18(1:30)
- 7) Hb :C18(1:40)
- 8) Hb:C18(1:50)

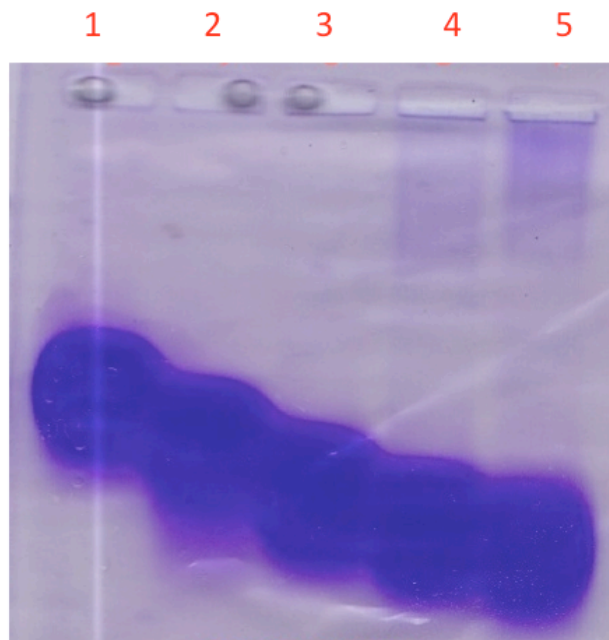
+ve electrode



- 1) Hb in 10mM PO<sub>4</sub>
- 2) Hb:C18 (1:30)-30%THF
- 3) Hb in 30%THF
- 4) Hb:C18(1:40) -40%THF
- 5) Hb in 40% THF
- 6) Hb:c18(1:50)-50% THF
- 7) Hb in 50% THF

+ve electrode

**Figure.2.2.A)Shows the Agarose gel ran for C18SA modified Hemoglobin (Hb) .As the Hb:C18SA ratio was increased the protein became more negative.Maximum change in charge was observed for 1:50. B) Shows the Agarose gel ran for C18SA modified hemoglobin (Hb) with a control sample of Hb in the presence of corresponding % of THF**



- 1) BSA in 10mM PO<sub>4</sub> buffer
- 2) 1:10
- 3) 1:20
- 4) 1:30
- 5) 1:40

+ve electrode

**Figure.2.3 Shows the Agarose gel ran for C18SA modified BSA. It was observed that as the ratio of BSA: C18SA was increased the protein became more negative. For BSA:C18SA ratio of 1:40 maximum change in the charge was observed.**

#### **2.4.2 Circular Dichroism study:**

The secondary structure retention of the protein was studied using the circular dichroism. The proteins showed 20-30% secondary structure loss after modification. The Soret CD was measured to understand the heme environment of hemoglobin after modification, showed that the heme environment was completely disturbed after modification (Figure).

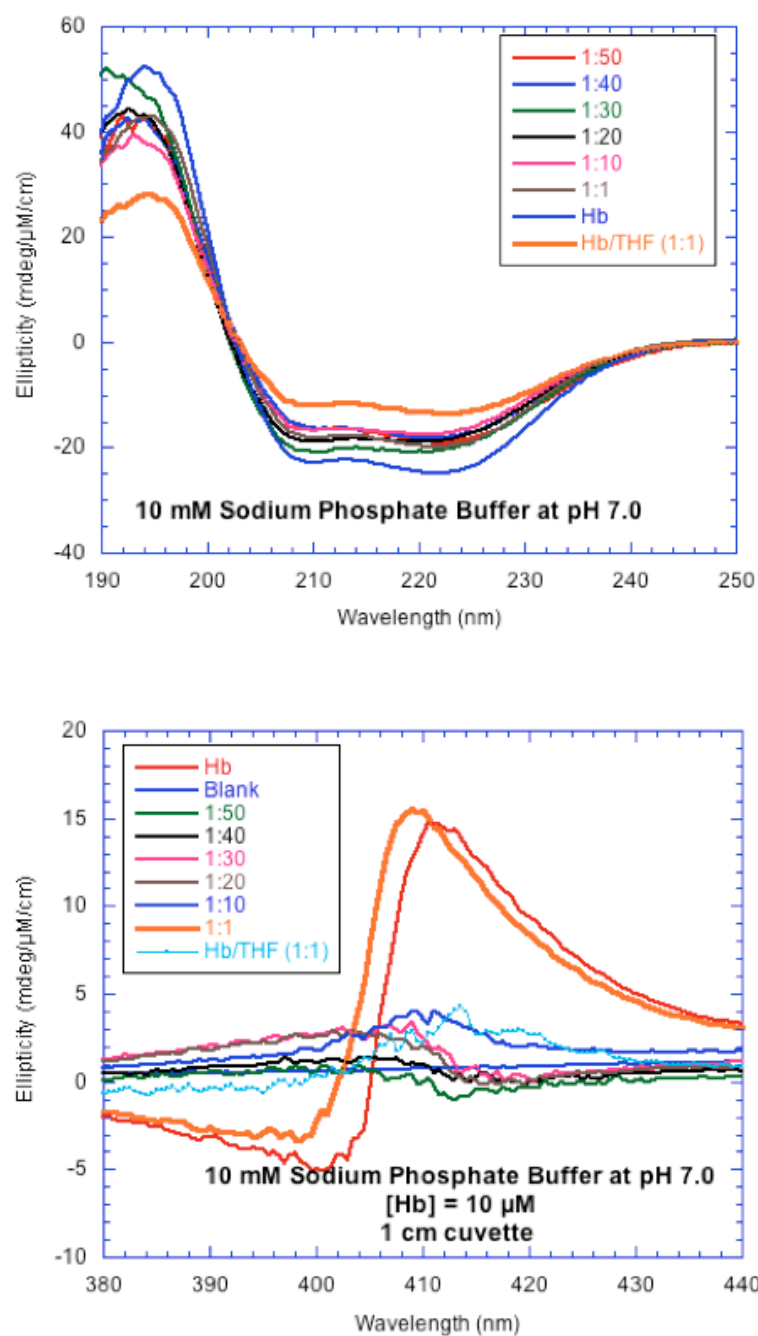
#### **2.4.3. Activity Study.**

Proteins were modified in the presence of THF, as the C18SA is insoluble in water. THF denatures the protein and results in loss of hemoglobin activity and the activity study showed that C18SA modification of the hemoglobin in THF showed protective effect on the activity to THF to a large extent at certain proportion of modification. The 1:1, 1:10 and 1:50 showed a greater extent of protection for THF (Figure .2.5). This shows that the succinic anhydride reaction of C18SA with amine group of protein is very fast and thus protects the denaturation of protein by THF.

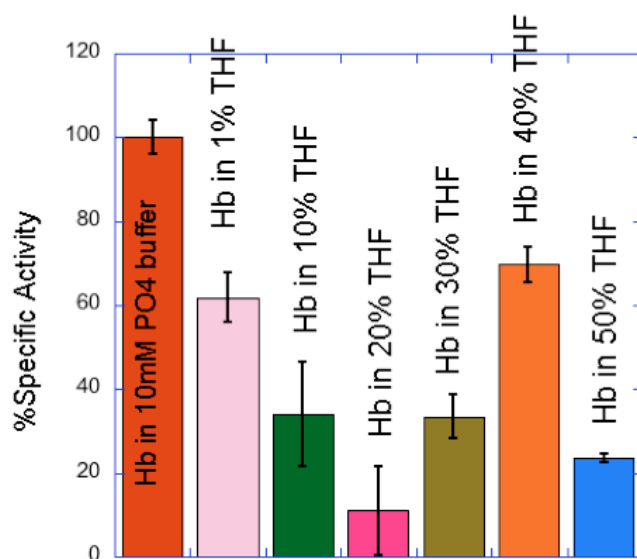
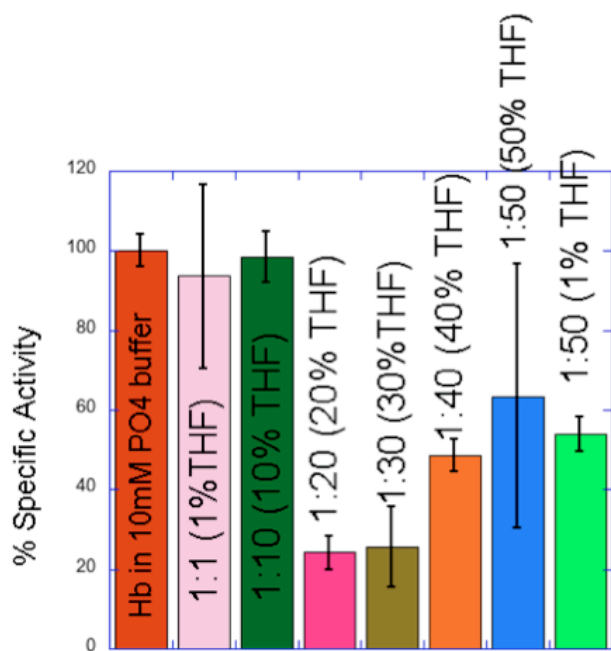
#### **2.4.4 Dynamic light scattering study.**

The dynamic light scattering study showed that 20-30% of modified hemoglobin showed an increasing trend (from ~35nm at 1:1 to ~70nm at 1:40 ratio of modification) in the size of the particle with increase in the number of lysine modified with C18SA. There was more than 50% of particle size that was found in the buffer (> 150nm) which was observed in all

the samples that interfered with the actual particle measurement (Table 2.2). Better experimental optimization could clearly show the increase in the protein size with increase in C18SA modification.



**Figure.2.4.A)** Shows the change in the UV-CD (secondary structure) of hemoglobin with increasing C18SA ratio. **B)** Shows the Soret CD (heme environment) of hemoglobin with increasing concentration of C18SA



**Figure.2.5.A) Shows the change in the activity of C18SA modified hemoglobin (Hb) B) Hb activity in different % of THF**



<b>Sample (concentration)</b>	<b>nm</b>	<b>nm</b>	<b>nm</b>
<b>10mM PO4 buffer</b>	2-2.8 nm(15-24%) 11-12 nm(7%)		148-169nm (57-76%)
<b>Hb in 10mM PO4 buffer (8uM)</b>	4.5-6 nm(9-10%) 1-2nm (3-4%)	35-40 nm(16-19%)	256-438nm (67-74%)
<b>Hb :C18SA 1:10 (8uM)</b>	2-5nm (5-6%)	45-59nm (33-39%)	224-454nm (56-58%) 1990nm (3%)
<b>Hb:C18SA 1:20 (1uM)</b>	2-4nm (3-6%)	43-58nm (22-29%)	220-416nm (69-74%)
<b>Hb:C18SA 1:30 (1uM)</b>	2-3nm (2-5%)	48-62nm(30-38%)	256-392nm (56-61%)
<b>Hb:C18SA 1:40 (1uM)</b>		62-69nm(16-25%)	264-322nm(75-83%)

**Table.2.2.Shows the diameter of the C18SA modified hemoglobin (Hb).It was observed that buffer showed a particle size >150nm which was consistently found in all the samples that interfered with the actual particle measurement.Column.3 shows an increasing trend in the size of the C18SA modified hemoglobin with increase in the ratio of C18SA.**

#### **2.4.5 Solubility test in organic solvents**

One of the applications hypothesized for making the proteins hydrophobic is to test the biocatalysis of proteins in organic solvents. It was hypothesized that if the proteins are modified hydrophobic enough it could dissolve in organic solvents and thus the catalytic activity can be tested in organic solvents. The C18SA modified hemoglobin was thus tested for solubility in different organic solvents (Table.2.3). The modified proteins still didn't dissolve in organic solvents. It could be concluded that the modification did not make the protein hydrophobic enough to dissolve in organic solvents.

Organic solvent	solubility
THF(50%)	soluble
DMF	Soluble (turned yellow)
Acetonitrile	Aggregated (insoluble)
Methylene chloride	Turned pale pink and cloudy (insoluble)
chloroform	Insoluble
Hexane	Insoluble
Toluene Butanol	Insoluble

**Table.2.3.Showing the solubility of C18SA modified hemoglobin in different organic solvents.200ul of 50uM C18SA modified BSA was mixed with 1mL of each organic solvent to test the solubility**

## **2.5.CONCLUSION:**

Agarose gel showed that hemoglobin and BSA were successfully modified with C18SA. The activity retention of hemoglobin after modification shows that the hydrophobic modification is very fast and protects the protein denaturation by THF that was added in the reaction mixture as a solvent for C18SA. Although the proteins were modified with C18SA the extent of modification was not high enough to dissolve the protein in organic solvents. The modified proteins can be further studied for the surface modification of biomaterials and can be tested for its biocompatibility. The proteins can also be tested for its potential application in invivo drug delivery.

## REFERENCES:

- (1)Cowan,D.A.Comp. *Biochem.Physiol.A.Physiol*,**1997**,118,429-438
- (2)Tischer.W,Kasche.V,*Trends Biotechnol*,**1999**,17,326-335
- (3)Surajit Chatterjee and Tushar Kanti Mukherjee, *J. Phys. Chem. B* ,**2013**, 117, 16110–16116
- (4)Yoshiko Moriyama, Yoshie Kawasaki, and Kunio Takeda,*Journal of colloidal and interface science* ,**2003**,257,41-46
- (5)José Wilson P. Carvalho, Fernanda Rosa Alves, Tatiana Batista,Francisco Adriano O. Carvalho, Patrícia S. Santiago, Marcel Tabaka, *Colloids and Surfaces B: Biointerfaces* ,**2013**,111, 561–570
- (6)Vindya Thilakarathne,Victoria A. Briand,Yuxiang Zhou,Rajeswari M Kasi and Challa V. Kumar,*Langmuir*,**2011**,27,7663-7671
- (7)Xiao-Ping Li, Adam M. Gilmore, Stefano Caffarri, Roberto Bassi, Talila Golan, David Kramer and Krishna K. Niyogi, *J. Biol. Chem.* **2004**, 279:22866-22874
- (8)Maehly,A.C.;Chance,B.*Methods Biochem.Anal.***1954**,1,357.
- (9)In *Worthington Enzyme Manual,Enzymes and Related Biochemicals*,Worthington,V.,Ed,Lakewood,NJ,**1993**,pp.77-80
- (10)Amano, K., Araki, Y., and Ito, E, *Eur J Biochem*, **1980**, 107, 547,
- (11)Mi A. Moosavi-Movahedi,Pure and applied chemistry,1994,66,71-75
- (12)Peng Xue, Jingnan Bao, Yon Jin Chuah, Nishanth V. Menon,Yilei Zhang, and Yuejun Kang, *Langmuir*, **2014**, 30, 3110–3117
- (13)Yingjun Wang,Ling Lu ,Yudong Zheng and Xiaofeng Chen, *J Biomed Mater Res A*. **2006** Mar 1;76(3):589-95
- (14)Ruchit Trivedi and Uday B Kompella, *Nanomedicine (Lond)*, **2010**; 5(3): 485–505.