INVESTIGATION ON THE EFFECT OF ZINC OXIDE NANOPARTICLES IN THE AGGREGATION OF HEN EGG LYSOZYME

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By

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This is to certify that the thesis entitled "**INVESTIGATION ON THE EFFECT OF ZINC OXIDE NANOPARTICLES IN THE AGGREGATION OF HEN EGG LYSOZYME**" by **Manoranjan Arakha (210bm2006)**, submitted to the National Institute of Technology, Rourkela for the Degree of Master of Technology is a record of bonafide research work, carried out by him in the Department of Biotechnology and Medical Engineering under my supervision. I believe that the thesis fulfils part of the requirements for the award of Master of Technology. The results embodied in the thesis have not been submitted for the award of any other degree

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Abbreviations

ZnO	Zinc oxide
SEM	Scanning electron microscope
DLS	Dynamic light scattering
XRD	X-ray diffraction
EDS	Energy dispersive spectroscopy
Pro	Protein
NP	Nanoparticles
CR	Congo red
Aβ	Amyloid fibrils
PSD	Particle size distribution

ABSTRACT

Protein misfolding and aggregation are responsible for several human pathologies commonly known as protein misfolding diseases. The various examples of protein misfolding diseases are: Alzheimer disease, spongiform encephalopathy, diabetes type 2, serpin-deficiency disorder, Huntington disease, Parkinson disease, amyloid polyneuropathy, and several others. The current research work was carried out to investigate the potential of ZnO nanoparticle to prevent lysozyme aggregation. ZnO nanoparticles were synthesized by chemical precipitation method from Zinc acetate dihydrate and urea. The optical, morphological and structural properties of synthesized ZnO nanoparticles have been studied using UV-Vis spectrophotometer, SEM, DLS, XRD and EDS. UV-Vis spectroscopic study shows that ZnO nanoparticles have surface plasmon resonance at 375nm. DLS analysis shows the average size of synthesized ZnO nanoparticle to be 68 nm. The EDS analysis shows the elemental composition of synthesized ZnO naoparticles and XRD study confirms the wurtzite structure of ZnO nanoparticles. The lysozyme aggregation was prepared by heating the native lysozyme at 100^0C. The effect of ZnO nanoparticles on the aggregation of lysozyme was studied. DLS analysis shows the mean size of the aggregates decreases with increasing concentration of ZnO nanoparticle (NP), which was further confirmed by SEM analysis. UV-Vis and Fluorescence spectroscopic studies were performed to analyze the structural changes of lysozyme upon binding with nanoparticles. The Congo red assay was performed to study the formation of amyloid fibrils.

Key words: Protein misfolding, Aggregation, Nanoparticles, Zinc oxide, Congo red assay

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

Proteins are the essential macromolecules for controlling most of the biological processes inside the cell. These proteins are synthesized on ribosome from the genetic informations that encoded in cellular DNA. After synthesis, these molecules fold into native structure to become more stable. There are some environmental factors which cause the misfolding of proteins. The inability of a protein molecule to adopt or to remain in its native conformation is termed as protein misfolding. Protein misfolding is the basis of some disorders like: cancer, degenerative diseases, metabolic pathologies that affect human most severely. It helps in causing diseases by altering the bio-availability of essential protein, like cystic fibrosis or by forming fibrillar aggregates with β-structure. Indeed protein misfolding and aggregation are responsible for about 40 human diseases. The fibrillar structures are accumulated in liver, heart, spleen and the central nervous system etc. In that case they are referred as amyloidal fibrils.

Now a day's nanotechnology has brought a great attention due to wide applications of nanoparticles in various fields of science and technology. Nanotechnology has emerged as the fore front of science and technology for last few decades. Generally it involves the production of devices or materials whose at least one dimension is in nanometre range i.e. 1-100 nm. Metal oxide nanoparticles which are inorganic in nature have attracted much attention for last few decades because they can withstand harse process conditions. Among metal oxides TiO_2, ZnO, MgO and CaO have drawn great attention for further studies as they are very safe to human beings and also for animals. The broad applications of nanoparticles are drug delivery, imaging and diagnosis. Due to its small size, nanoparticles possess high surface to volume ratio, which gives distinguishing features to nanoparticles.

Studies have shown that ZnO and silver nanoparticles have antibacterial and antifungal activity. The antimicrobial property of nanoparticles is determined by their size, shape, morphology, composition and crystallinity. Research are going on to synthesize nanoparticles ,designing nanodevices and application of these in different fields like medical, environment, energy, information and communication, heavy industry and customer goods etc.

Generally there are two parts of nanoparticles: the core material and a surface modifier. The surface modifier is responsible for change in physiochemical properties of core materials. The core material may be composed of biological materials like phospholipids, lipids, lactic acid, chitosan, and dextran or may be formed of carbon, chemical polymers, silica or metals. Various chemical compounds, drugs, probs and proteins are attached to nanoparticle surface by the help of covalent bonds or by adsorption.

When the chemical compounds attach to nanoparticle surface, the physiochemical properties changes. Thus the functionality of nanoparticles is enhanced or changed. The efficiency of nanoparticles for any applications depends upon the physiochemical characteristics of core material and surface modifiers. The composition of core materials along with surface modifiers makes the nanoparticles biodegradable and biocompatible. When nanoparticles are used as drug carriers, questions may arise about the toxicity of nanoparticles and this toxicity of nanoparticles is reduced by modifying their surface properties. Detail information about the interaction of nanoparticles with different molecules are not well known, but the interaction of nanoparticles with protein has drawn great attention because protein are the only one biological molecule which are fundamental to the proper functioning of cells and organisms.

In our present investigation we have synthesised ZnO nanoparticles and their interaction with hen egg lysozyme aggregates were studied. ZnO nanoparticles were synthesized by

chemical precipitation method and detail characterizations were done by UV-Vis spectrophotometer, SEM, DLS, XRD and EDS. Then these nanoparticles were interacted with aggregated hen egg lysozyme. The conformational change of lysozyme was studied by DLS, SEM ,UV-Vis spectrophotometer, fluorescence and spectrophotometer .Congo red assay was done to confirm that the formed aggregates are not amyloids.

1.1. Objectives

- Synthesis of ZnO nanoparticles by Chemical precipitation method
- Detail Characterization of synthesized ZnO nanoparticles
- Investigating the effect of ZnO nanoparticles on aggregated hen egg lysozyme. .

PLAN OF WORK

Plan of Work	**3rd Semester**		**4th Semester**	
	Mid Semester	End Semester	Mid Semester	End Semester
Literature Review				
ZnO-NP Synthesis and characterization				
Lysozyme Aggregation Analysis				
Manuscript Writing				

CHAPTER 2 : LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. The structure of proteins

Proteins are biomolecules in which amino acid chains are connected to each other by peptide bonds. Protein molecules are very diverse molecules and also vary in size from small peptides to large no of multimers. The common element which is present in every protein is the peptide backbone. This peptide backbone is formed by peptide bonds which link amino acid residues [1].According to the amino acids and their side chains, the variation of peptide backbone is observed. Generally 20 amino acids are there and they are grouped into different families basing on their side chains, such as acidic, basic, neutral and hydrophobic side chains. The primary structure of protein is determined by its amino acid sequences. The polypeptide chains fold into secondary elements like helix, sheet and turns which determine the three dimensional structure of protein [1].

2.2. Protein misfolding and aggregation

The Newly synthesized linear amino acid chain folds into its unique three dimensional structures to become functional. This unique structure is called native structure of protein. Protein misfolding is a very general tendency of protein molecules which occurs continuously. Certain mutation and amino acid composition are responsible for protein misfolding. Protein misfolding also occurred due to environmental changes [2].Certain environmental changes like increase in temperature, high or low pH, elevated glucose, agitation and oxidative agents also cause protein misfolding. Due to these agents the protein molecule loss their native conformation [3].The process by which protein losses their native conformation is called denaturation. Generally denaturation causes unfolding and partial misfolding of protein molecule. Due to lake of arrangement the partially misfolded protein

becomes non-functional and unfolded state is thermodynamically unfavourable and unstable. The unfolded protein has a propensity to form aggregates, which has lower energy and more stability [4]. Amyloid conformation is one specific aggregated form of protein molecules arises from protein misfolding. Amyloid contains linear, unbranched protein molecules or fibrils. Contrary to fibrillar proteins like collagen triple helix and keratin, amyloid contains linear unbranched protein or peptide fibrils [5].The special features of amyloid fibrils is that it binds fluorescent probs like Congo red and thioflavin derivatives. They commonly share a common secondary composition [6].

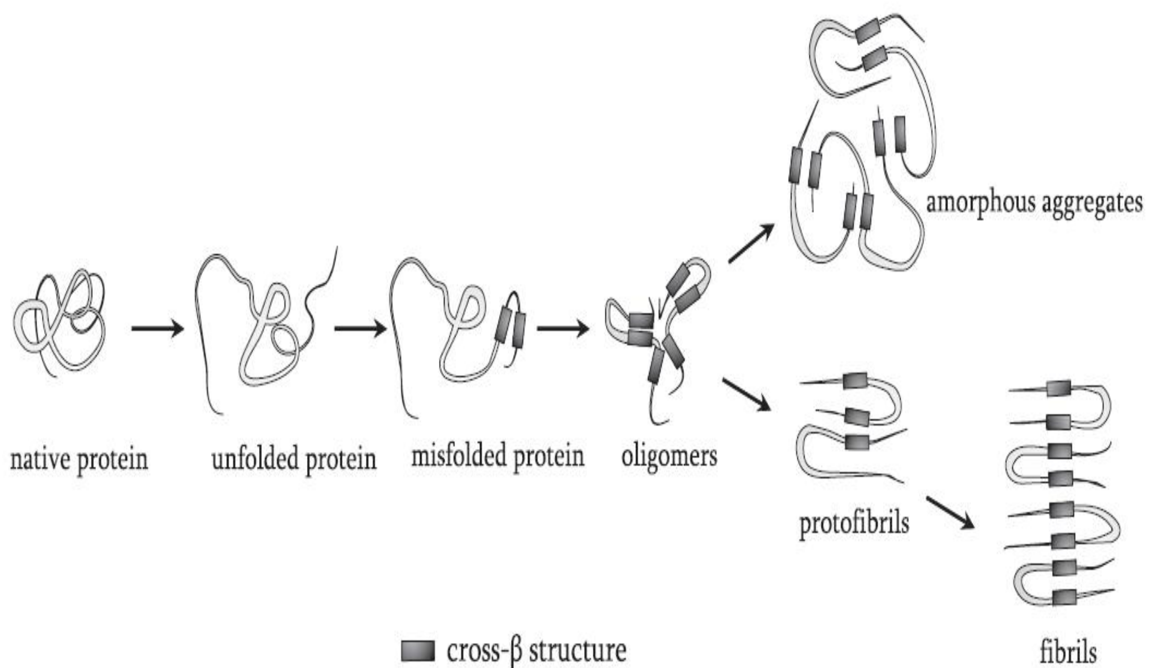

Figure 1.Protein misfolding and aggregation (Reproduced from [1])

2.3. Protein misfolding diseases

Protein misfolding diseases are group of disorders that contain aggregated protein. For last few years protein misfolding diseases have drawn great attention which are also classified as protein conformational disorder(PCD)[7].The various examples of protein misfolding

diseases are: Alzheimer disease, spongiform encephalopathy, diabetes type 2, Huntington disease, Parkinson disease, serpin-deficiency disorder, amyloid polyneuropathy, and several others. There are various similarities among these diseases at molecular levels [8].

2.4. Metal oxide nanoparticles

Nanotechnology has been considered as an immensely developing field due its wide applications in different fields of science and technology. The widely applicable word ʽNanoʾ is derived from a Greek word whose meaning is extremely small or dwarf [9].The nanotechnology concept was first given by Physist Richard Feynman in his talk "There is plenty of room at the bottom"[10].Nanotechnology can be considered as a multidisciplinary field which includes research and development of technologies in different field like nanotechnology, biotechnology, physics, chemistry and material science [9,11].The research on synthesis of nanoparticles has drawn great attention due to their distinguishing physical and chemical properties than the macroscopic particles[12].The rapid development of innovative synthesis protocols and various characterisation techniques are the clue points for rapid development of nanotechnology[13].Chemical and physical methods have been developed for synthesis of different types of nanoparticles, but the nanoparticles developed in these methods show poor morphology .Moreover these processes use toxic chemicals and elevated temperature for synthesis purpose which is toxic to environment[9,14].Biological synthesis methods are more favourable since these methods are eco-friendly.

2.5. Synthesis of nanoparticles

Basing on the different applications of nanoparticles, different protocols have been designed for synthesis of nanoparticles [15].The different methods used for synthesis of nanoparticles are:

- Physical method
- Chemical method
- Biological method

2.5.1. Physical method

The physical method often called top-down approach which includes methods like diffusion, irradiation, thermal decomposition; arc discharge etc .The thermal decomposition method which is one of top-down approaches, used for the creation of monodisperse nanoparticles. In this method fatty acids are allowed to dissolve in hot NaOH solution and then mixed with metal salt solution. Then this solution results the formation of metal precipitates. Crystals and short wires of cupper are allowed to be enclosed in glass ampoules in diffusion method. Then it is sealed at low pressure followed by annealing at 500^0c for 24 hours. Then the crystals are removed from ampoules and then cooled on a metallic plate at room temperature. In UV irradiation method, polycarbonate films are placed on glass microscope slide .Then this slide is exposed to UV radiation. Like above methods there are many methods used in top down approaches to synthesize metal nanoparticles [15].

2.5. 2.Chemical method

The chemical reduction method is an example of bottom up approach, used for synthesis of nanoparticles. In these methods different metal particles are reduced to form nanoparticles. Different metal particles used are sodium Borohydrate and sodium citrate etc [16].Other chemical reagents used are N,N-dimethylformamide(DMF)[9]poly(N-vinyl pyrrolidine(PVP),ethyl alcohol[10],tetra-n-tetra-fluroborate(TFATEB),CTAB[11]etc.

2.5.3. Biological method

Green synthesis method has advantages over other methods adopted for synthesis of nanoparticles.It is cost effective and eco-friendly. Moreover there is no need of using high

pressure, temperature and toxic chemicals. Now a days nanoparticles are synthesized from various sources like bacteria and fungi, plant extract etc [9, 14].

2.6. Zinc oxide nanoparticles

Zinc oxide is an inorganic compound having formula ZnO. This is a white powder generally insoluble in water. The structure of ZnO nanoparticles has been investigated computationally using new atomistic potentials. Mechanical properties like internal stress and adhesion characteristics are required to maintain patterning accuracy and durability for various applications of nanopartcles. The structure of ZnO is generally hexagonal wurtzite.Generally ZnO crystallizes into hexagonal wurzite and Zinc blend.

2.7. Application of ZnO nanoparticles

In recent years Zinc oxide has been considered as an important semiconductor having wide band gap (3.37 eV), and large exciton-binding energy (60 meV) [20]. ZnO has been preferred as a highly multitasking metal oxide due to its unique optical and electrical properties[21].In many industrial areas such as solar cells, UV light-emitting devices, photocatalysts, gas sensors, pharmaceutical and cosmetic industries ZnO nanoparticles have been widely used [22-26] .Moreover metal nanoparticles have surface Plasmon resonance properties in UV-visible region ,nontoxic, self-cleansing[27].ZnO nanoparticles are also biocompatible to skin and having antimicrobial, dermatological properties. ZnO nanoparticles are used in sunscreen because it act as a UV blocker[28].ZnO nanoparticles have strong resistance to microorganisms and this property is due to the generation of reactive oxygen species on the surface of nanoparticles.

2.8. Synthesis of Zinc Oxide nanaoparticles

There are several physical, chemical procedures for synthesis of ZnO nanoparticle in large quantities in a short period of time. Among them simple-solution based methods, chemical precipitation, sol gel, solvothermal/hydrothermal, electrochemical and photochemical reduction method are preferable methods.ZnO nanoparticles can be synthesized form plant leaf extract, bacteria, fungi and enzymes by using green synthesis methods. Green synthesis methods are eco-friendly approach and compatible for pharmaceutical and other biomedical applications because no toxic chemicals are used in these methods [29].

2.9. Lysozyme

Lysozyme is a protein of single chain polypeptide having 129 amino acids and having molecular mass of 14.307 kda and isoelectric point at 11.35.These amino acids are cross linked with four disulfide bridges. It has the property to catalyse β (1-4) linkages between N-acetylmuraminic acid and N-acetyl-D-glucosamine residues which are present peptidoglycan and catalyse N-acetyl-D-glucosamine residues in chitodextrin [30].Lysozyme often used to lyse bacterial cells by hydrolysing peptidoglycan present in cell walls. Gram positive cells are more susceptible than gram negative bacteria for lysozyme hydrolysis since gram positive bacteria have high proportion of peptidoglycal in their cell wall. Gram negative bacteria have less susceptible due lack of high proportion of peptidoglycan in their cell wall. However the hydrolysis of these bacteria cells become more easy in the presence of EDTA that chelates metal ions present in outer bacterial membrane. This lysozyme is purified from chicken egg white then it is crystallized three times followed by dialysis and then supplied as lyophilized powder. It has been estimated that protein content by UV absorbance is 90% and rest of the content is buffer salts like sodium acetate and sodium chloride. Lysozyme has been used as lysing agent for the purification of plasmid DNA. The activity of lysozyme depends both on

P^H and ionic strength. The activity of lysozyme is stable over a broad P^H , ranging from 6.0 to 9.0.The activity of this enzyme observed maximal at P^H 6.2 over a wide range of ionic strengths(0.02-0.100M) than at P^H 9.2(0.01-0.06M). The inhibitor of lysozyme is indolderivatives; it binds and distorts the active site. Another inhibitor is imidazole which helps in the formation of a charge-transfer complex. Various surface active agents like sodium dodecyl sulphate, sodium dodecanate and dodecyl alcohol can also inhibit lysozyme.

2.10. Nanoparticle protein interaction

When nanoparticles enter into the body they interact with biological fluids like plasma .Nanoparticles in these physiological environment bind with proteins to form a coat which is known as ʽprotein coronaʼ. It is important to understand how and why proteins are absorbed to nanoparticle surface because corona is the key factors which determine the fact of nanomaterials in vivo [31].The surface properties of nanoparticles determine the binding of nanoparticles with protein. The surface properties of nanoparticles are determined by characteristics of nanoparticles such as the shape, chemical composition, surface functionalization, angle of curvature, porosity and crystalinity, roughness and hydrophilisity or hydrophobicity etc. The important forces that are responsible for nanoparticle –protein interaction are Vander Walls forces, London dispersion forces, polarization and lone-pair electrons and hydrogen bond. The mathematical model of nanoparticle-protein interaction can be predicted by taking the assumption that all inter-molecular interaction has equal contribution [32].

Nanodescriptors can be created using the adsorption coefficients of probe compounds. These nanodescriptors represent the contribution and relative strengths of each molecular interaction. As an example, this model helped to predict, for example the adsorption of various small molecules onto carbon nanotubes [32]. In summary, the affinity of

nanoparticles to biomolecules is determined by the contribution of multiple site of adsorption on the NPs surfaces (that are close to the amino-acids residues of the proteins), and the contribution of various forces that depend on the proteins and the type of nanoparticles. Predicting this kind of interaction could be very helpful in nanomedicine to eliminate certain type of nanoparticles based on their relative nanodescriptors [33].

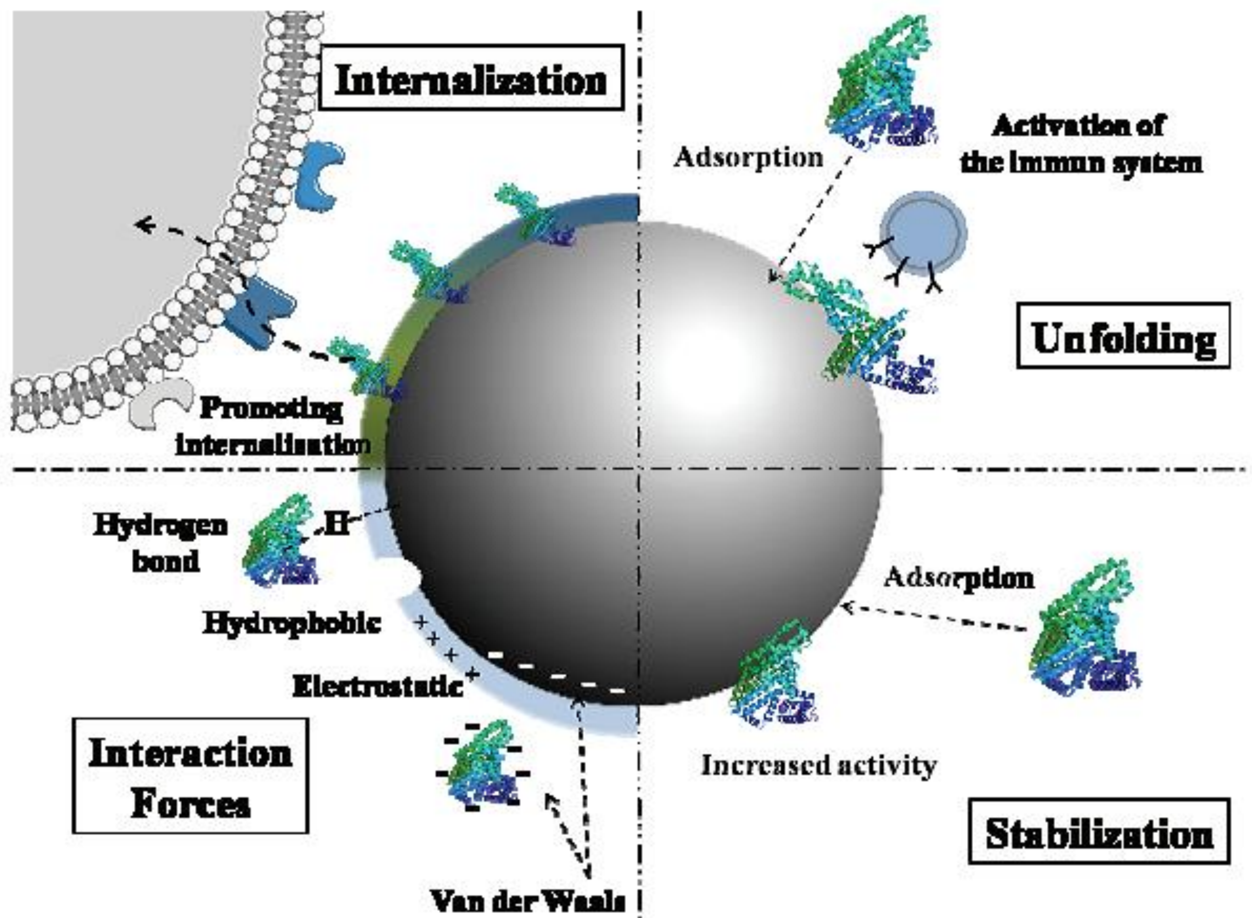

Figure2. The Effects of NPs on proteins, and the forces of interactions. (Reproduced from [34])

The nanoparticle-protein interaction study gives a brief and help in understanding the potential biological injuries like changes in protein fibrillation, loss of function like enzymatic activity impairment and exposure of new epitopes [34].

2.10.1. Conformational changes of protein upon binding with nanoparticles

The secondary structure of proteins changes when they bind to planar surface, but when they bind to nanoparticles there is no change in secondary structure. The proteins retain their original structure due to high curvature of nanoparticles. However a wide study in various surfaces of nanoprticles and proteins suggested that a disturbance of protein structure still happens. The unfolding kinetic studies of β-lactoglobin and lysozyme were done to study the conformational studies. It has been studied that when above proteins were absorbed onto silica nanoparicles, there was changes in secondary and tertiary structural level[35,36].From many studies it has been confirmed that when proteins are absorbed onto nanoparticles, there are loss of α-helical content, with or without increase in β-sheets[35,37].

It has been studied that Bovine serum albumin (BSA) is a good model protein for protein conformational studies. At different pH BSA undergoes conformational changes. From the circular dicroism studies it has been found that when gold nanoparticles bind BSA ,there is decrease in α-helical structure and increase in β-sheets and turns and it was also studied by Fourier transform infrared(FTIR) spectroscopy[35].In few cases the thermodynamic stability of the protein is decreased when they are absorbed onto nanoparticle surfaces, but in most cases the native structure of protein does not change.[38].When the thermodynamic stability decreases the proteins become sensitive to denaturating agents like urea. If such problems occur then the structural changes of protein may lead to loss of biological function when they introduce inside the body.

Therefore it is very essential to study the protein nanoparticle interaction, to know which properties of nanoparticles are responsible for perturbation of protein conformation. In general it has been studied that structural changes of protein occur due to intrinsic properties of protein combined with various features of nanoparticles. Recent studies have suggested

that how the concentration of nanoparticles on the surface of protein influence the structure and function of protein [35, 36].

2.10.2. The size of the nanoparticles influence the structure and function of proteins

The surface curvature which is responsible for protein nanoparticle interaction is determined by size of the nanoparticles.The nanoparticles having larger surface area possess low surface curvature where as that of small size have high surface curvature. An experimental study of nanoparticle size influencing the structure and function of protein has been performed taking lysozyme silica nanoparticle conjugates [37].From this study it was concluded that smaller nanoparticles strongly favour native like protein structure due to which lysozyme has intrinsic enzyme activity. When lysozyme adsorbed onto nanoparticle surface there is reduction in both α-helical content and enzymatic activity of lysozyme.When RNase adsorbed on silica nanoparticles similar matter occurred. The thermodynamic stability of RNase decreases upon binding to silica nanoparticles [38].

A simple model shown below explains the mechanism for the effect of different size of nanoparticles on protein surface. The greater perturbation of native protein occurred for larger nanoparticles, because larger nanoparticles provide larger surface area for contact of protein on surface of nanoparticles [38].

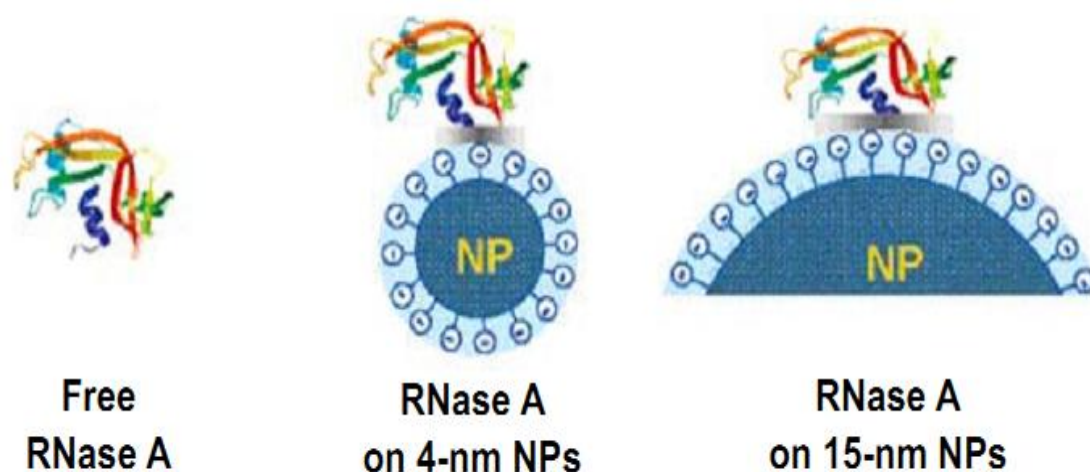

Figure 3.Interaction of RNase with different diameters of silica nanoparticles (Reproduced from 38)

2.10.3. Conformational changes of protein affected by surface concentration

When the proteins concentration are kept constant and the concentration of nanoparticles vary then different surface concentrations can be obtained. When there is high surface concentration of nanoparticles then more proteins are absorbed onto nanoparticles surface so that a crowed surface environment created which facilitate protein-protein interaction. During the nanoparticle-protein interaction different surface concentration of nanoparticles can be obtained by keeping the concentration of protein constant and varying the quantity of nanoparticles. More proteins are absorbed onto the nanoparticle surface when the surface concentration of nanoparticles will be more. Wu and colleagues suggested that both lysozyme and β-lactoglobulin unfold to a greater extent when the surface concentration of the silica nanoparticles was lower than when the concentration of protein was higher [34, 39].It has been suggested that the interaction between protein and hydrophobic surface of silica nanoparticles occurred at lower surface concentration. Concentration results in the reduction of net energy barrier to protein unfolding. From the above study we can conclude that

immobilizing enzymes at high packing density onto nanoparticles will lead to less loss of activity of enzyme than lower surface densities.

2.10. 4.Chaperon activity of nanoparticles

It was widely accepted that when proteins adsorbed onto nanoparticle surface, the structure of protein is perturbed to a varying extents. To improve the protein folding it is interested to know whether the nanoparticles act as a chaperon. The protein surface in native protein is generally rich in hydrophilic residues. The hydrophobic core of the protein is generally buried when the proteins found in native state. The outer surface or hydrophilic surface generally interacts with the aqueous environment. When protein unfolds the hydrophobic core is exposed which causes protein aggregation. In order to proper folding the protein need chaperons like GroEL and GroES, which bind to the unfolded protein and make them proper folding to native state [40, 41].Chaperons are generally used in biotechnology to create biologically active recombinant proteins [42].Two types of nanoparticles have been found to be having refolding capacity such like a nanogel and another is hydrogel.

CHAPTER 3: MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Chemicals

For all experimental studies pure and analytical grade chemicals were used. For synthesis of ZnO nanoparticles Zinc acetate, urea were purchased from sigma Aldrich Pvt Ltd.The chemicals required for protein aggregation studies such as hen egg lysozyme, Congo red and other chemicals required for buffer preparation were also purchased from sigma Aldrich Pvt Ltd.

3.2. Glassware and Apparatus

All glass wares (Measuring cylinders, Beakers, Conical flasks, and Test tubes etc.) were purchased from Borosil, India.

3.3. Synthesis of ZnO nanoprticles

The Zinc oxide nanoparticles were synthesized by using chemical precipitation methods from Zinc acetate and urea as described by C R Bhattacharjee et al. (2011).Zinc acetate dehydrates and urea solutions were prepared in deionised water maintaining molar concentration of 0.1 M each. The Zinc oxide and urea solutions were mixed in a beaker with volumetrically ratio of 1:4 respectively. The solution was magnetically stirred at room temperature for homogeneous solution. Then the solution was transferred into a round bottom flask. After sealing the flask it was heated at 115^0c for 1.5 hour in a muffle furnace. A white precipitate was formed after the completion of reaction. The precipitate was centrifuged at 6000 rpm for 10 min and washed in distil water to remove any absorbed chemicals or ions, so that there will be less possibility of agglomeration. Then the precipitate was dried at 100^0c for 1 hour and then calcined in a muffle furnace at 350^0 C for 1 hour. Then the resulting powered obtained was ZnO nanoparticles.

3.4. Characterization of ZnO nanoparticles

3.4.1. DLS particle size analyser

Dynamic light scattering (DLS) which is based on the laser diffraction method with multiple scattering techniques was employed to study the average particle size of ZnO nanoparticles. The prepared sample was dispersed in deionised water followed by ultra-sonication. Then the particle distribution in liquid was studied in a computer controlled particle size analyser (ZETA sizer Nanoseries,Malvern instrument Nano Zs).

3.4.2. Scanning electron microscopy

In this research work JSM-6480 LV SEM machine was employed to study the morphology of synthesized nanoparticles. The experiment was performed at an accelerating voltage of 20 kV.

3.4.3. UV-Vis spectroscopy

The synthesized ZnO nanoparticles were characterised by Perkin-Elmer UV-Vis spectrophotometer, Lamda.35, to know the Plasmon resonance property of ZnO nanoparticles.First the ZnO nanoparticles sample was sonicated in an ultrasonicator for uniform dispersion and then the aqueous component was taken and analysed for Plasmon resonance property at room temperature.

3.4.4. XRD and EDS methods

In this research work Philips PAN analytical machine was employed for X-ray diffraction studies. The phase variety and grain size was determined in X-ray diffraction studies. The synthesized ZnO nanoparticles sample was studies with CUKα radiation at voltage of 30 kV and current of 20 MA with scan rate of 0.04^0/sec. Different phases present in the prepared samples were identified with search and match facility available in X′ pert high score

software. The particle size of the prepared samples were determined by using Scherrer′s equation as follows

$$D \approx \frac{0.9\lambda}{\beta \cos\theta}$$

Where D is the crystal size, λ is the wavelength of X-ray, Θ is the Braggs angle in radians and B is the full width at half maximum of the peak in radians.

The study of elemental composition of sample was carried out by energy dispersive X-ray spectroscopy (EDS) with the machine SEM (JEOL JSM_5800).

3.5. Preparation of Lysozyme Aggregation

Hen egg lysozyme aggregates were prepared by heating the native protein lysozyme. In this study100µM of lysozyme solution was taken. Then it was heated in a water bath maintaining the temperature at 100^0C. After heating, the native protein was unfolded as high temperature causes unfolding of protein. Then due to lake of proper environment the protein misfolded and formed aggregates.

3.6. Interaction of ZnO nanoparticles with aggregated lysozyme

During the cooling process of lysozyme aggregation , ZnO nanoparticles of 100 µM were added to the aggregated protein with various protein: nanoparticle ratios such as 5:1, 2:1, 1:1, 1:2, 1:5, and a sample without nanoparticle was kept as control. The above prepared samples were incubated overnight at 37^0C. The nanopartcle protein interaction was studied by particle size analyzer (DLS), scanning electron microscope, UV-Vis spectrophotometer, and fluorescence spectroscopy and zeta potential measurement.

3.7. Characterization of protein nanoparticles conjugates

3.7.1. DLS analysis

The formation of protein nanoparticles conjugates were first studied by DLS analysis. Five samples were prepared with protein nanoprticles ratio of 5:1,2:1,1:1,1:2,1:5 etc and a control sample of aggregated lysozyme without nanoparticles was prepared and all are incubated overnight at 37^0 C. The samples were characterized by DLS particle size analyser to determine the average size of aggregated protein samples

3.7.2. SEM analysis for protein nanoparticles conjugates

For SEM analysis two samples were prepared; one is aggregated lysozyme and other is protein nanoparticle conjugate with 1:5 molar ratio and these are incubated overnight at 37^0C .These samples were analysed by Jeol JSM-6480 LV SEM at an accelerating voltage of 20 KeV.

3.7.3. UV-Vis spectroscopic analysis

Native lysozyme was dissolved in 0.01mM PBS buffer then the absorbance spectra were taken. Protein nanoparticle conjugates were prepared with ratio of 5:1,2:1,1:1,1:2,1:5 and a control of only aggregated lysozyme was also prepared and all were incubated overnight at 37^0 C. Then UV-Vis spectra were taken for all samples with Perkin Elmer, Lamda-35.

3.7.4. Fluorescence spectroscopic study

Three samples of protein nanoparticles conjugates with ratio of 5:1, 1:1 and 1:5 were prepared and a control sample of aggregated protein was prepared and incubated overnight at 37^0 C. Then fluorescence spectra of native lysozyme and above prepared samples were taken with perkin-Elmer LS-55- Luminescence spectrometer. Fluorescence spectra were measured by excitation at 295 nm and

emission at 310-430 nm. The fluorescence spectra of ZnO nanoparticles was taken in this range and found that ZnO nanoparticles do not exhibit fluorescence at 310-430 nm.

3.7.5. Zeta potential measurements

The protein nanoparticles conjugate samples were prepared with different ratio of protein nanoparticles such as 5:1, 2:1, 1:1, 1:2, 1:5 etc. Zeta potential measurements were taken for native lysozyme , aggregated lysozyme and for all protein nanoparticle conjugate samples.

3.7.6. Congo red assay

The Congo red assay was performed to detect whether any amyloid fibrils were formed during the aggregation study of protein. A stock solution of Congo red having concentration of 100 μM was prepared in 90% filtered 0.01 mM Phosphate buffer solution. The constituents of above buffer solution was (0.0027MKCl and 0.137M NaCl; pH 7.4) and 10 %(v/v) ethanol. The above solution was filtered three times using filter paper to remove any Congo red micelles. The protein nanoparticle conjugate samples were mixed with the solution of Congo red in phosphate buffer to yield a final concentration of 10 μM .The Congo red-protein nanoparticle conjugate samples were incubated at room temperature for 15 minutes before spectral analysis. The control sample of Congo red without protein nanoparticle conjugates was also prepared. The concentration of Congo red was same as concentration used in protein nanoparticle conjugate samples. Then a preaggreegated protein samples without Congo red was prepared. The absorbance of all test samples and control was taken at 541 nm and 403 nm. The following formula was applied to determine the concentration of CR band (i.e. Congo red-amyloid)

$$CR\text{-}A\beta = A_t^{541}/47{,}800 - A_t^{403}/68{,}300 - A_{cr}^{403}/86200$$

Where CR stands for Congo red, Aβ stands for amyloid fibrils, A_t is the absorbance of test sample with CR, A_{cr} is the absorbance of Congo red. The results were expressed as μg/ml

CHAPTER 4: RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Synthesis and characterization of ZnO nanoparticles

4.1.1. Synthesis of ZnO nanoparticles prepared by chemical precipitation

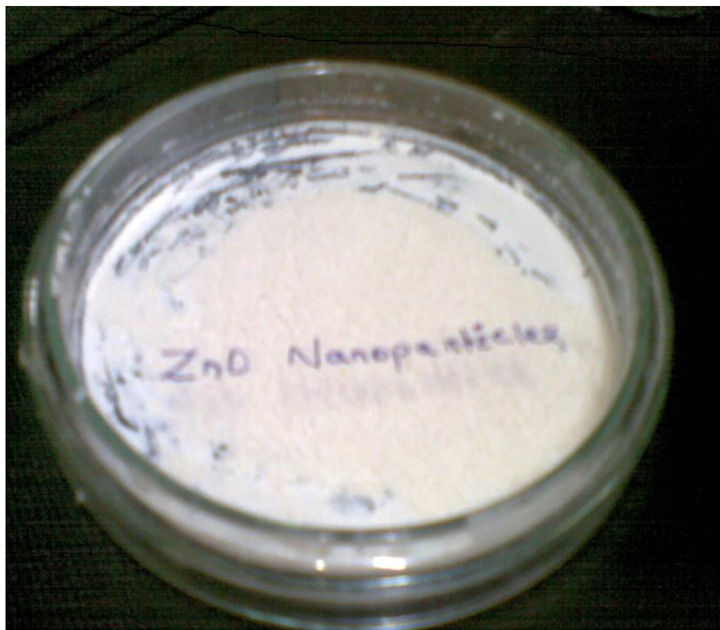

Figure 4: Synthesized ZnO nanoparticles by chemical precipitation method

The figure 4 shows the ZnO nanoparticles synthesized by chemical precipitation method according to the procedure discussed in ' material and method .The powder form of synthesized ZnO nanoparticles looks white in colour.

4.2. Characterization of ZnO nanoparticles

4.2.1. DLS particle size analyser

The particle size distribution (PSD) of synthesized ZnO nanoparticles by chemical precipitation method is shown in figure 5 and 6.According to the graph the particle size distribution of ZnO nanoparticles ranges from10 nm100 nm. Form the DLS figure it is clear that the solution contains nanoparticles having various sizes which is according to SEM data.

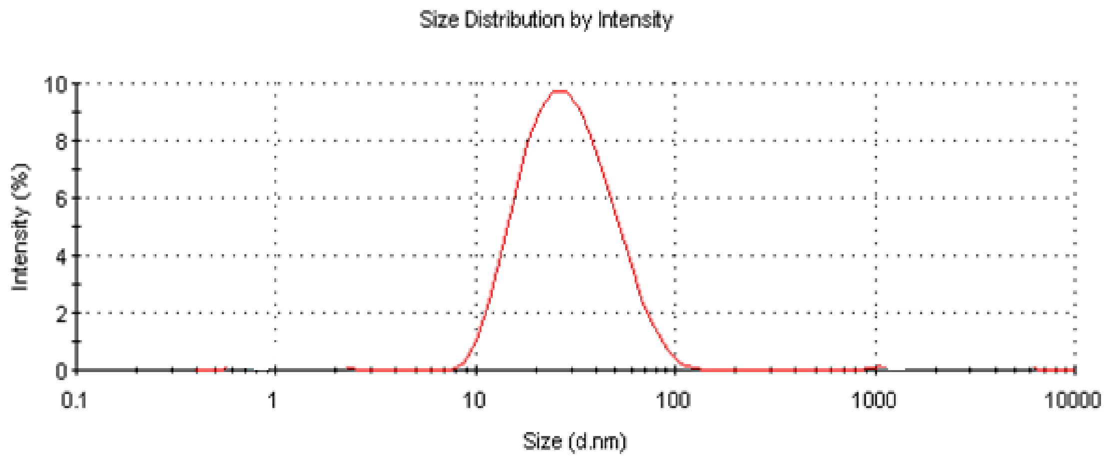

Figure: 5: Particle size distribution of ZnO nanoparticles

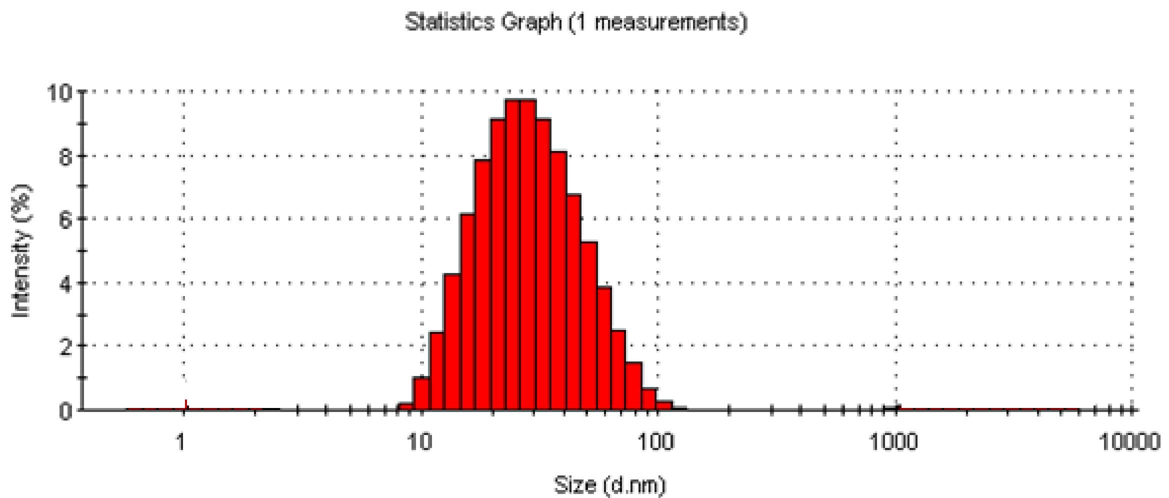

Figure 6: Statistics of particle size distribution of ZnO nanoparticles

4.2.2. SEM analysis of ZnO nanoparticles

Figure 7 shows the SEM image of ZnO nanoparticles synthesized by chemical precipitation method. Most of the particles in the powder are within 100 nm in size, and showing low agglomeration rate. Most of the particles are spherical in shape.

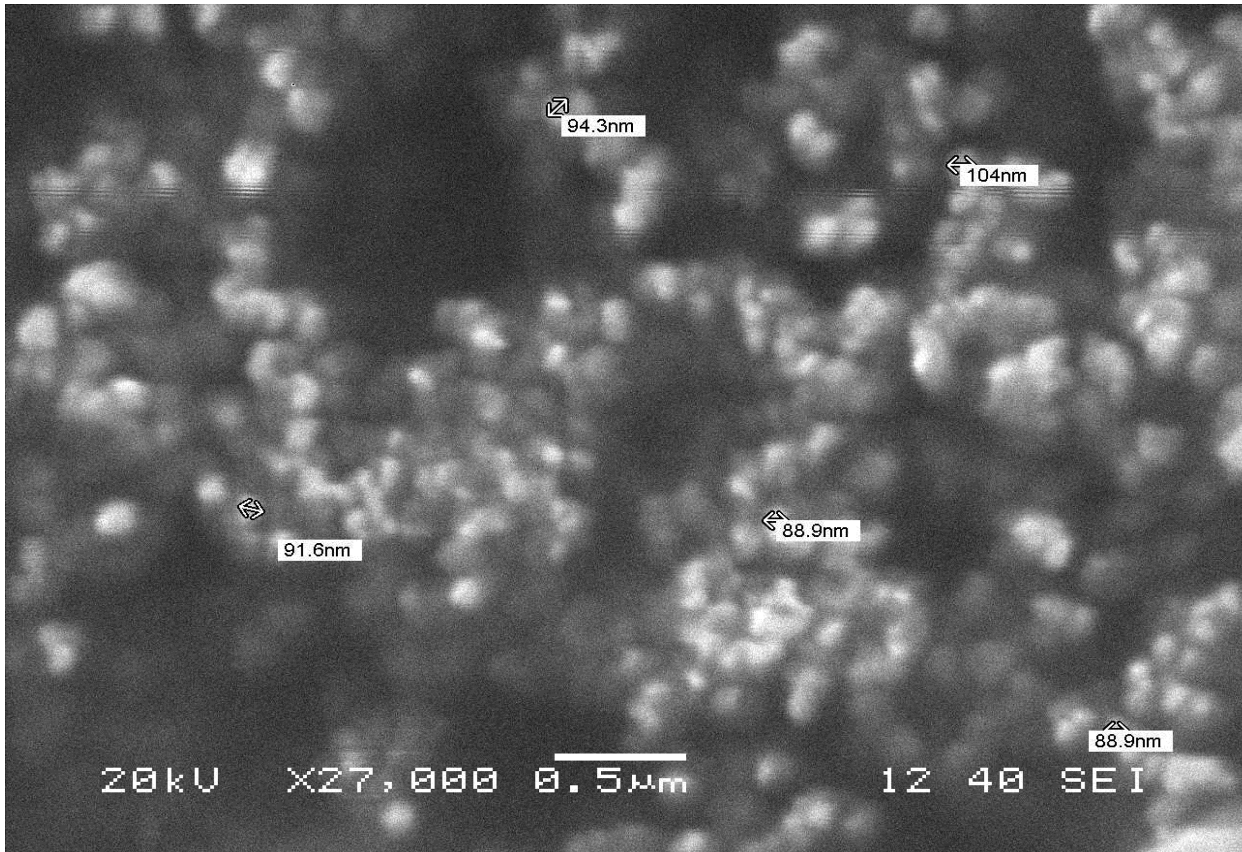

Figure 7: SEM micrograph of ZnO nanoparticles at X 27,000

4.2.3. UV-vis absorption studies

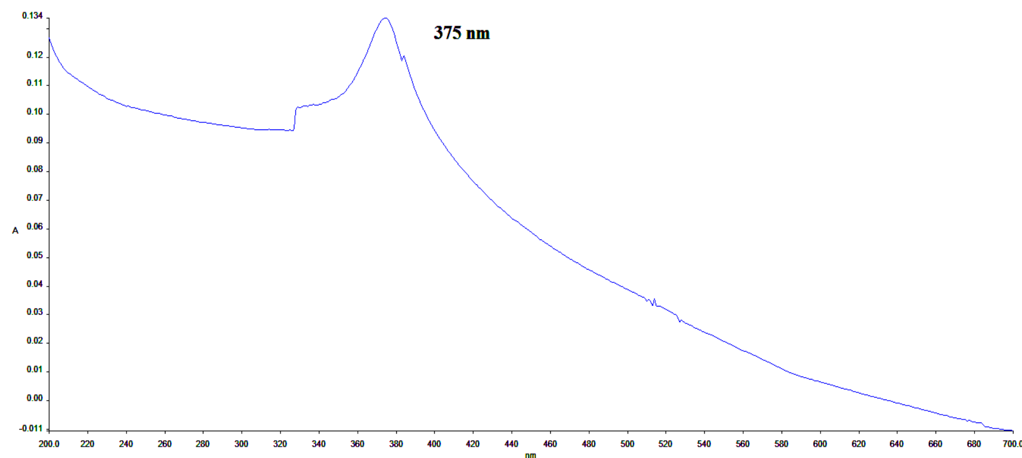

Figure 8: UV-Vis spectra of the ZnO nanoparticles

Figure 8 shows the UV-Vis absorption spectra of ZnO nanoparticles. The absorption spectra of ZnO nanoparticles shows peaks at 375 nm and this is due to the plasmon resonance of Zinc

oxide nanoparticles. The ZnO nanoparticles have distinctive colour in colloidal solution due to its tiny dimension. The electron cloud on the surface of nanoparticles oscillates and absorbs electromagnetic radiation at particular energy. The particular type of resonance arises is called surface Plasmon resonance. The shifting of absorption spectra towards red end or blue end depends upon the particle size, shape, surrounding medium and state of aggregation.

4.2.4. XRD and EDS analysis

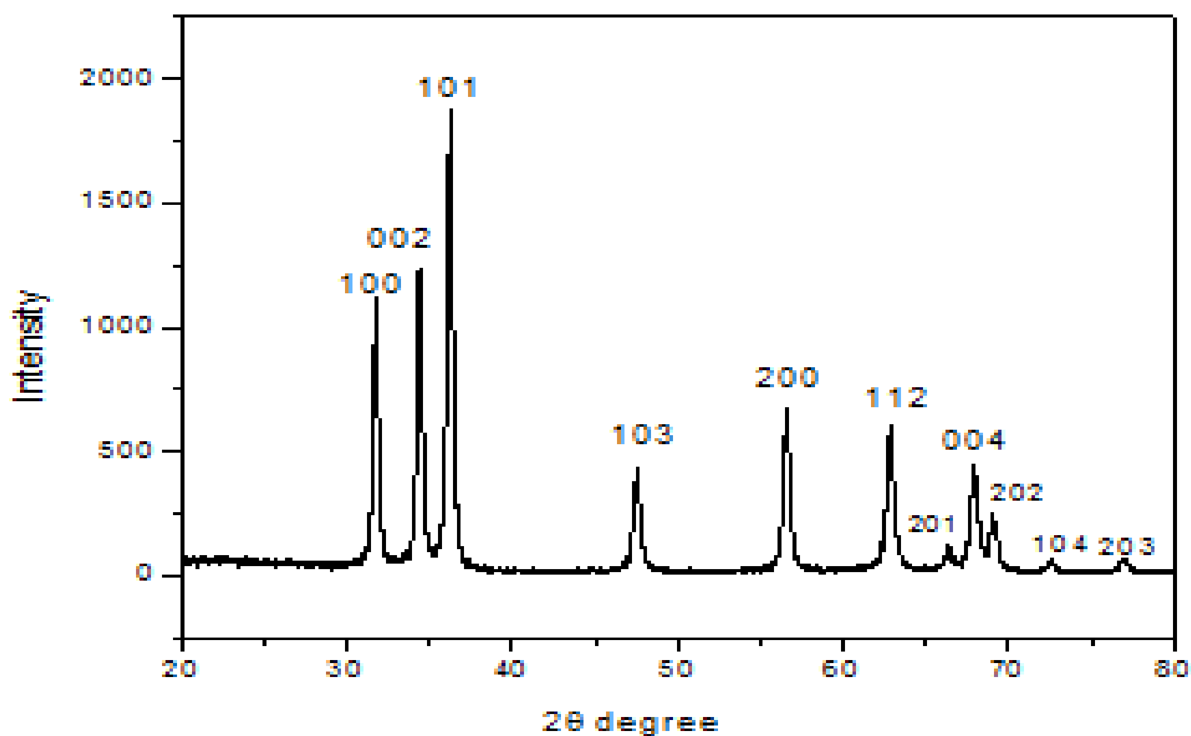

Figure 9: XRD patterns of ZnO nanoparticles

Figure 9 shows the XRD pattern of ZnO nanoparticles. The XRD pattern confirms the crystalline structure of ZnO nanoparticles. The picks of the diffractogram are well indexed to hexagonal phase of Zinc oxide powder. The synthesized ZnO powders are having wurtzite structure. Any peaks related to impurities are absent in diffractogram.

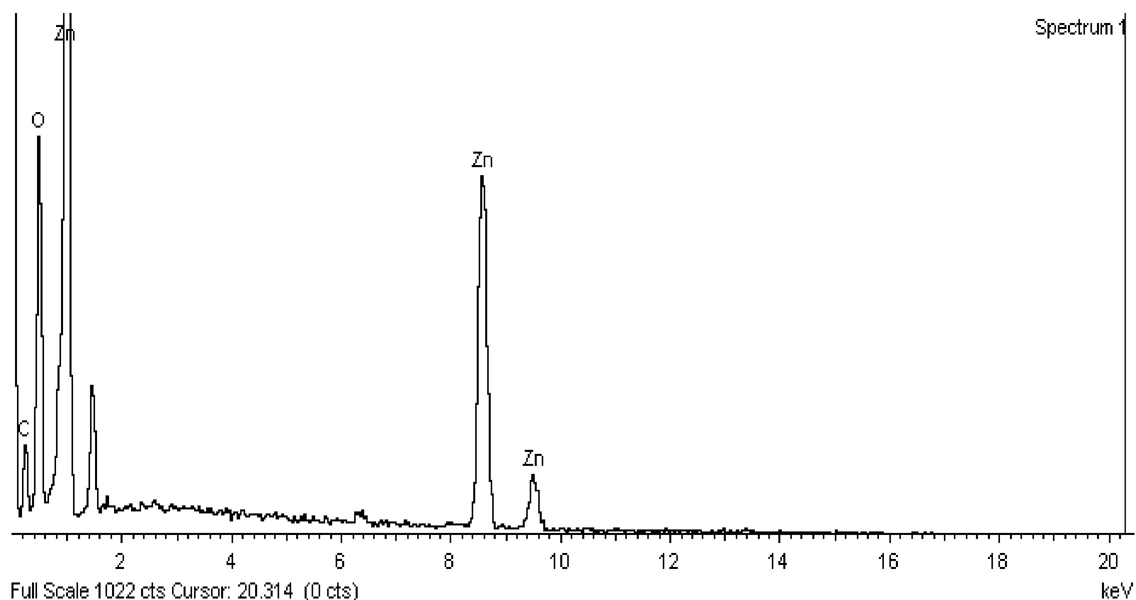

Figure-10: EDS micrograph of ZnO nanoparticles

The figure 10 shows the EDS analysis of ZnO nanoparticles which confirms the elemental composition of ZnO nanoparticles. The EDS analysis display the optical absorption peaks of ZnO nanoparticles and these absorption peaks are due the surface Plasmon resonance of Zinc oxide nanoparticles.

4.3. Characterization of nanoparticle protein conjugates

4.3.1. DLS analysis

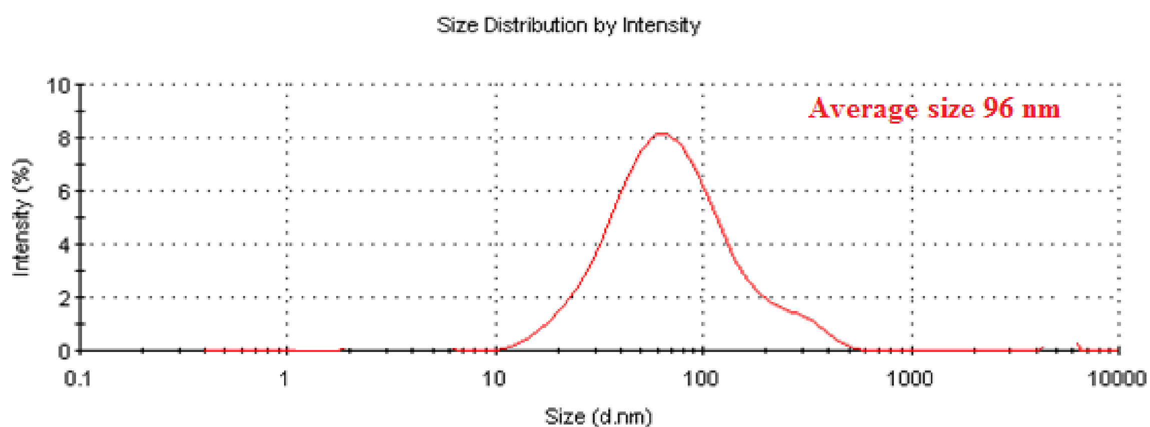

Figure 11: Particle size distribution of native lysozyme

Figure 12: particle size distribution of aggregated lysozyme

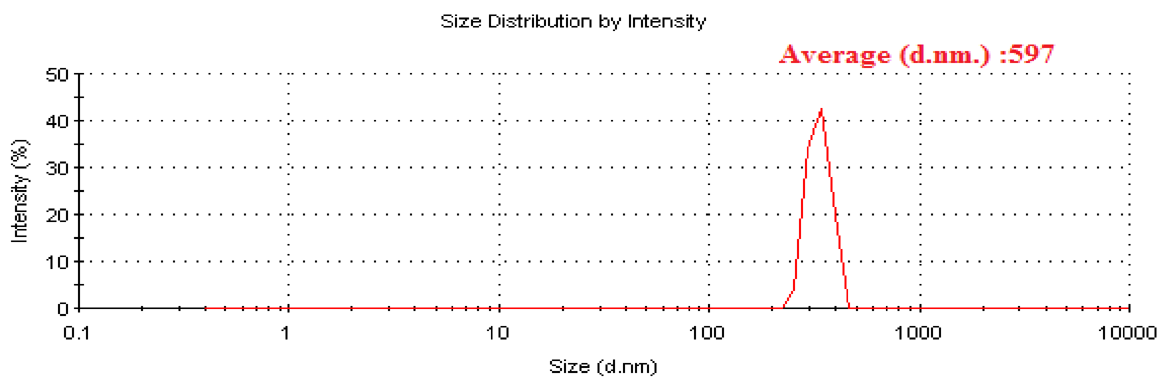

Figure 13: particle size distribution of protein nanoparticle sample (5:1)

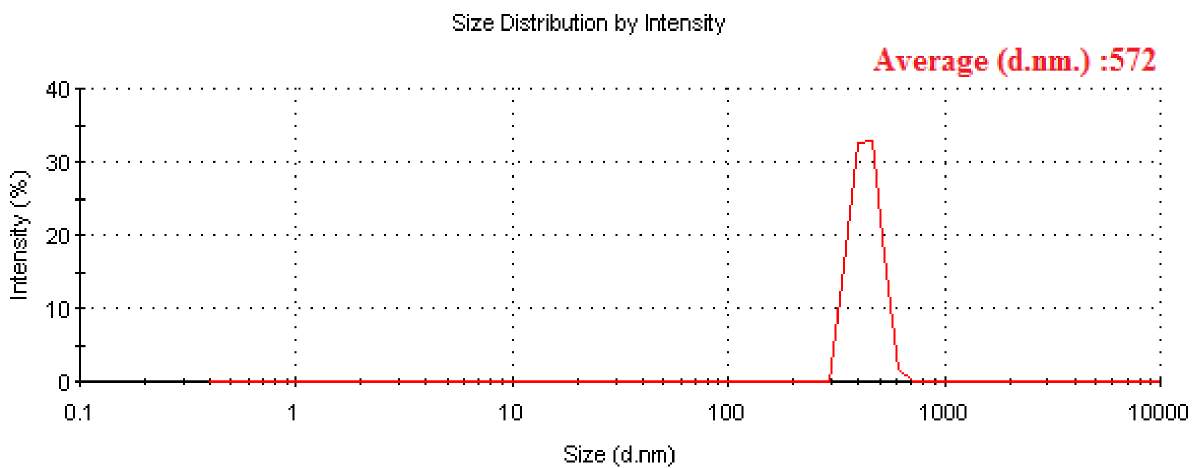

Figure 14: particle size distribution of protein nanoparticle sample (2:1)

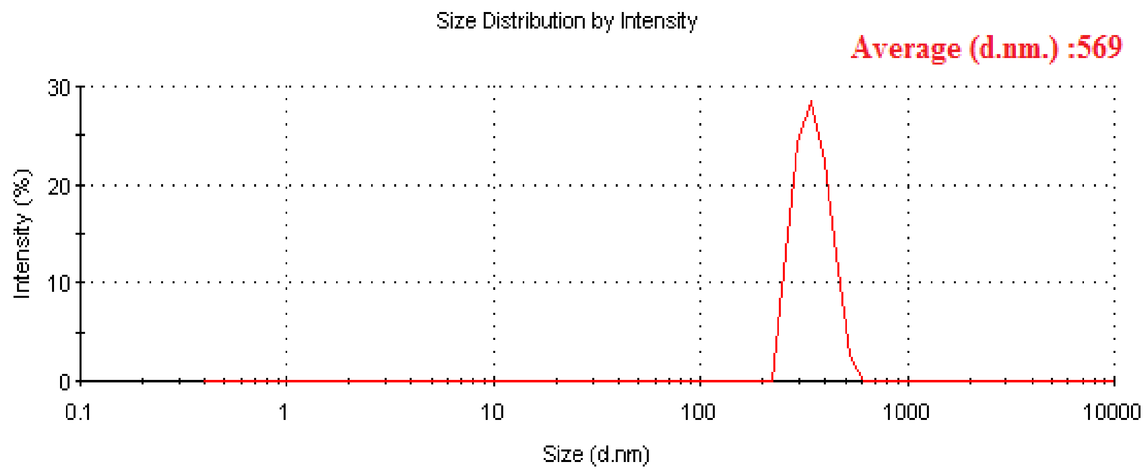

Figure 15: particle size distribution of protein nanoparticle sample (1:1)

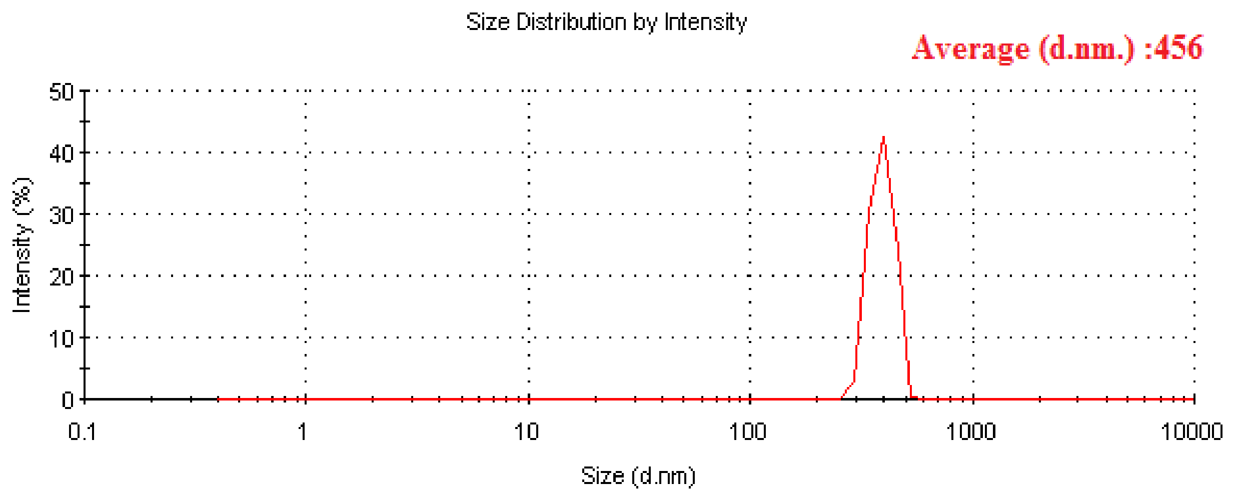

Figure 16: particle size distribution of protein nanoparticle sample (1:2)

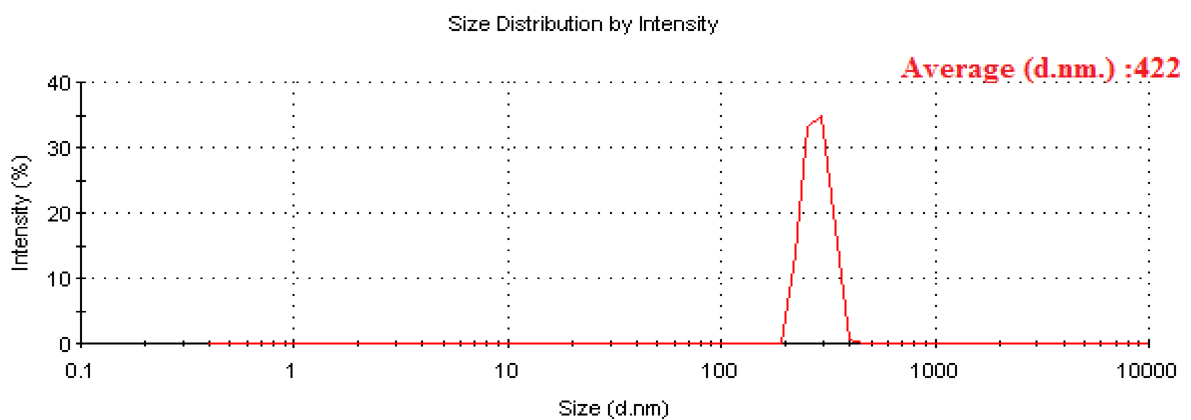

Figure 17: particle size distribution of protein nanoparticle sample (1:5)

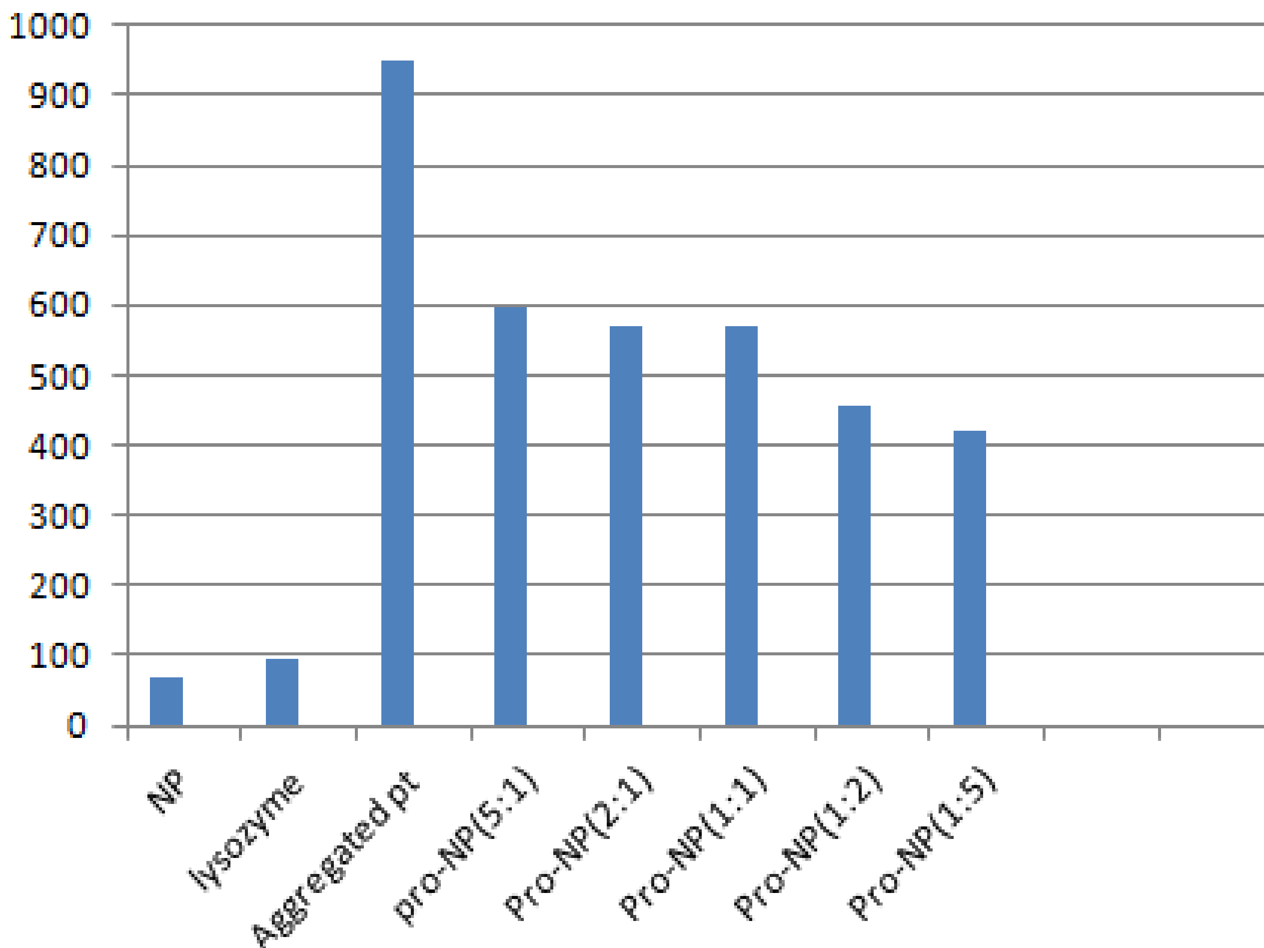

Figure 18: Average particle size of different samples of nanoparticle protein conjugates

The figures above show the DLS analysis of protein nanoparticle conjugates. The symbols NP and pro suggest for nanoparticles and protein respectively. Analysis suggests that solution containing only lysozyme shows average size of particles at 96 nm but aggregated protein shows high rate of agglomeration since the average size of particles in this solution has greater than others (figure 12) .But as the concentration of nanoparticles increase in solution the rate of aggregation decreases(Figure 13-17). This might indicates that nanoparticles have the potential to prevent aggregation of protein. The fig-15 shows the bar diagram which indicates the decrease in rate of aggregation with increasing the concentration of nanoparticles.

4.3.2. SEM analysis

The lysozyme ZnO conjugates were also studied by SEM analysis. Figure-19 shows the SEM micrograph of aggregated lysozyme. It can be conclude that after the native structure was lost the protein misfolds and then aggregates. When we introduce nanoparticles (with protein nanoparticle ratio of (1:5), the nanoparticles bound to the aggregated protein and then prevented the rate of aggregation. This result has been shown clearly in figure 20. So it can be concluded that ZnO nanoparticles prevent the rate of aggregation of lysozyme.

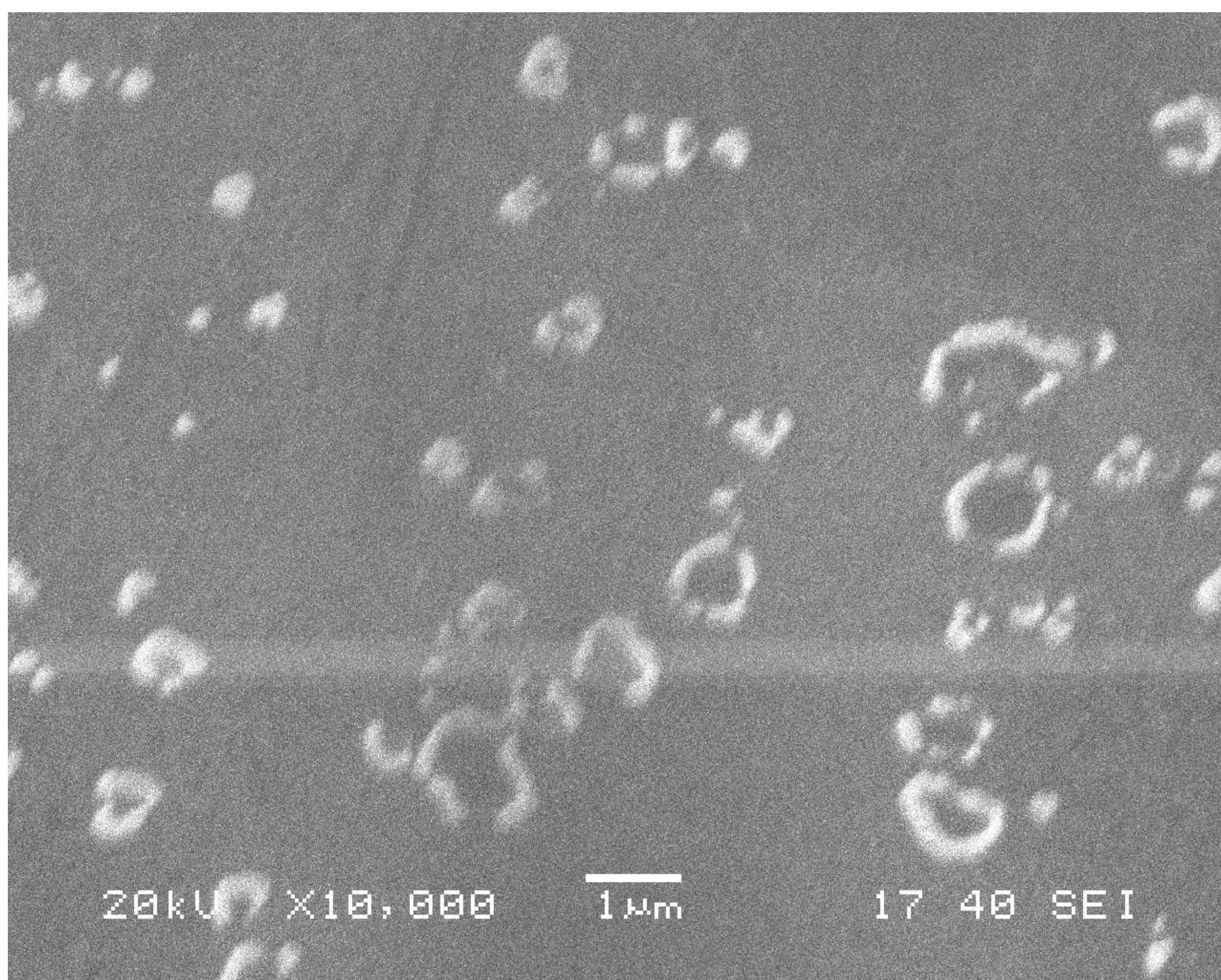

Figure: 19 SEM image showing the aggregated lysozyme

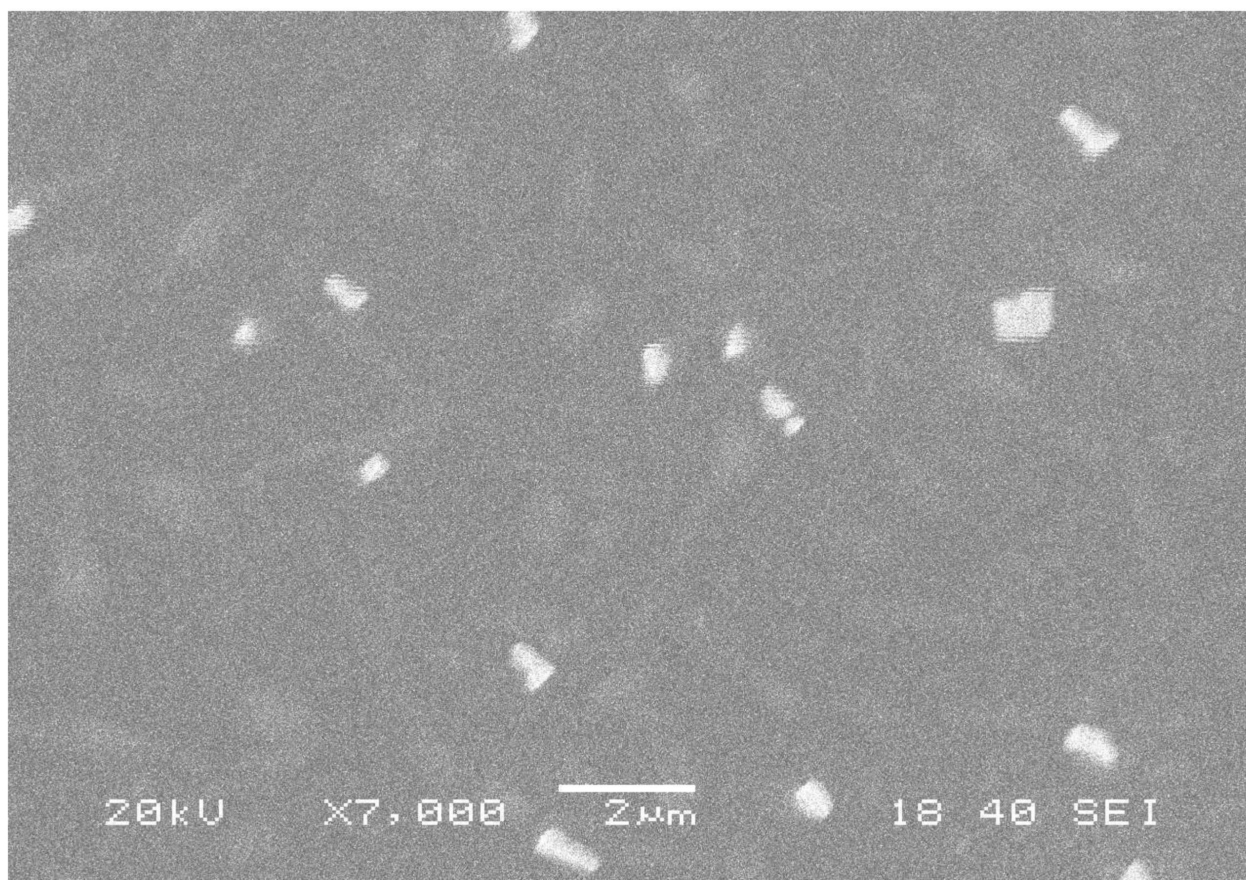

Figure: 20 SEM image showing the prevention of aggregation by ZnO nanoparticles.

4.3.3. UV-Vis spectroscopy analysis

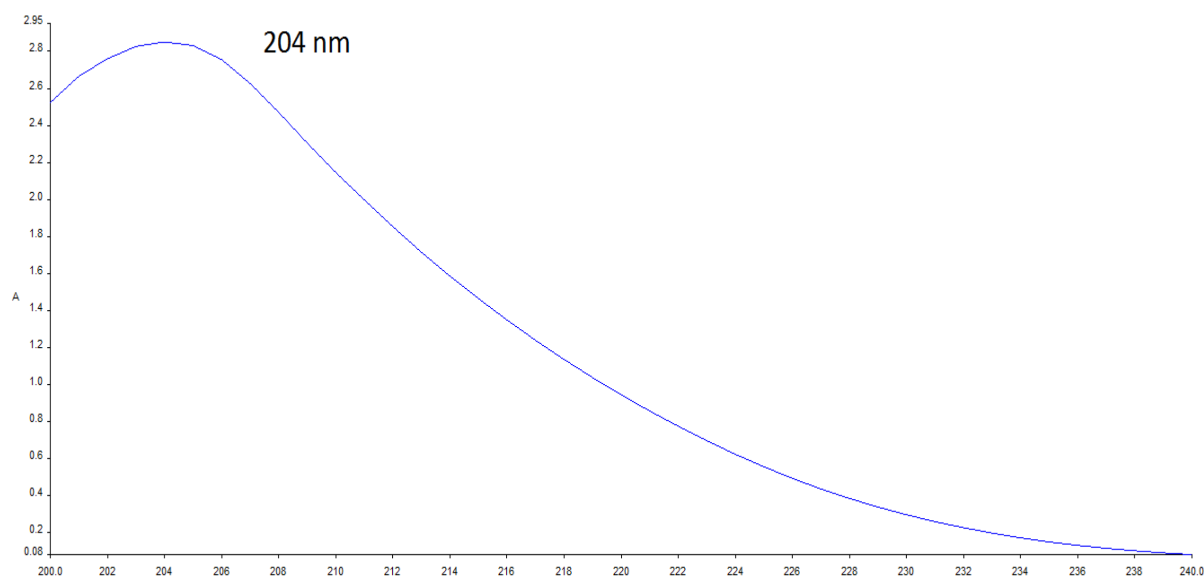

Figure 21: UV-Vis spectra of lysozyme dissolved in 0.01 mM PBS buffer

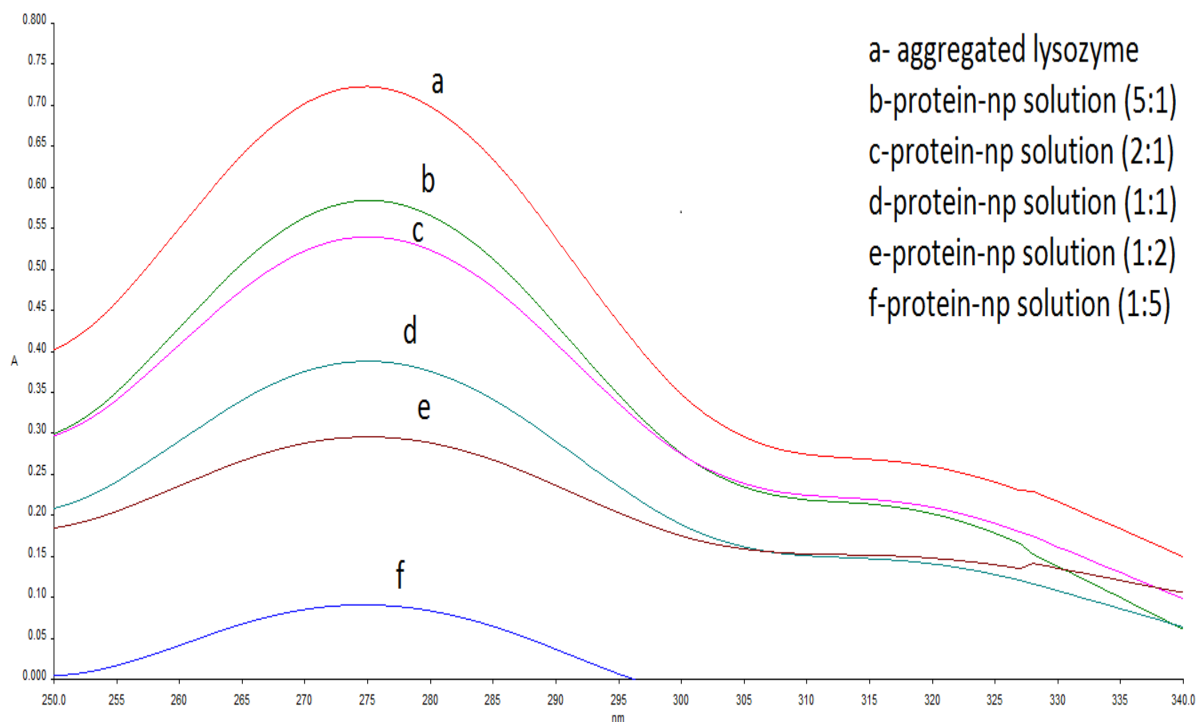

Figure 22: UV-Vis spectra of ptotein nanoparticle solutions

The figure 21 shows the absorbance spectra of lysozyme in 0.1mM PBS buffer. The peak of the curve found to be at 204 nm .The ZnO nanoparticles induced conformational changes of lysozyme is represented in figure 22. From the figure 22, curve a represents the curve for aggregated lysozyme. From the figure it can be conclude that after heating there is loss of native structure of lysozyme, which may indicate that lysozyme losses activity after heating. The absorption spectra for aggregated protein shows peak about at 275 nm. The curve suggests that after unfolding of lysozyme, the tryptophan comes out which gives absorbance spectra at 275 nm. The curves b to f represents the reduction in absorbance but no shifting in peaks. It may be suggested that nanoparticles prevent the aggregation of lysozyme.

4.3.4. Fluorescence spectroscopy analysis

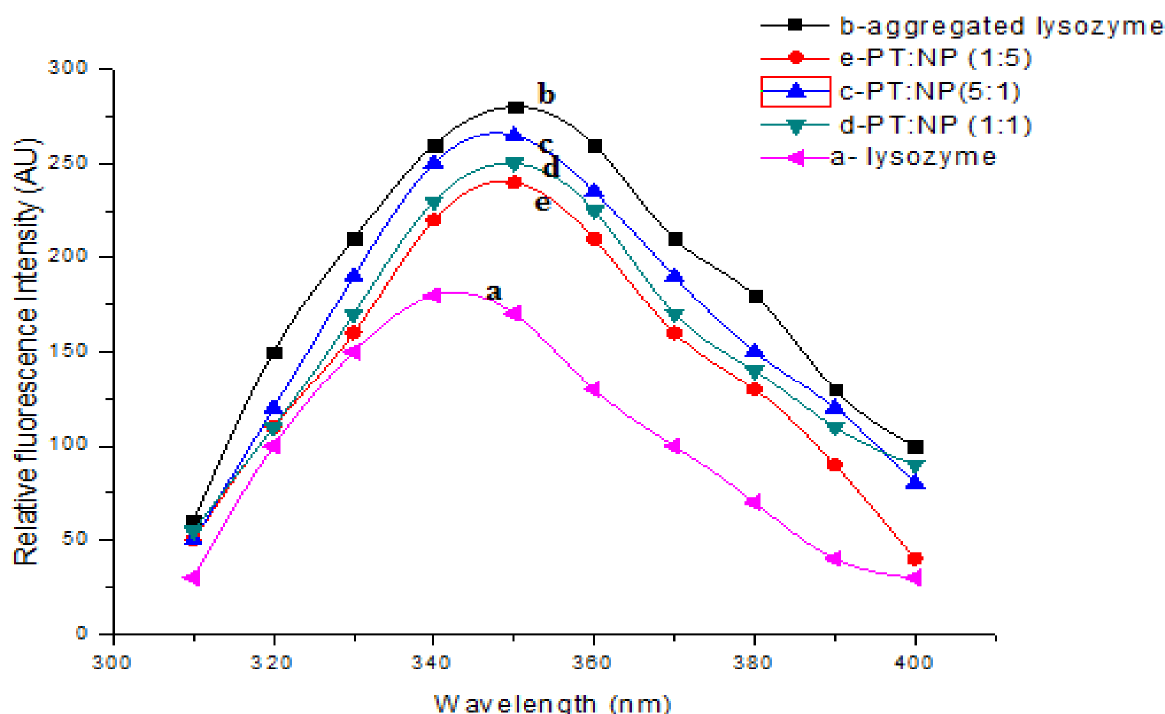

Figure 23: Fluorescence emission spectra of Lysozyme and lysozyme nanoparticle conjugates at different concentration of lysozyme and nanoparticles

The effect of ZnO nanoparticles on aggregated lysozyme was studied by using Tryptophan fluorescence. In the figure 23 curve a shows the fluorescence emission spectra for native lysozyme dissolved in 0.01mM PBS buffer. It reveals that the protein in native stage has λ_{max} at approximately 340 nm. In the presence of ZnO nanoparticles, the λ_{max} shifted to about 350 nm. This reveals the change in tertiary structure of lysozyme upon misfolding. When nanoparticles are introduced there is reduction in tryptophan florescence intensity .When nanoparticles bind with protein molecule, brings down the fluorescence intensity of tryptophan which indicate a havoc conformational change of protein molecule. This may be concluded that upon binding with nanoparticles the tryptophan molecules are transferred to more polar environment. So in presence of nanoparticles the tryptophan fluorescence was quenched drastically.

4.3.5. Zeta potential measurements

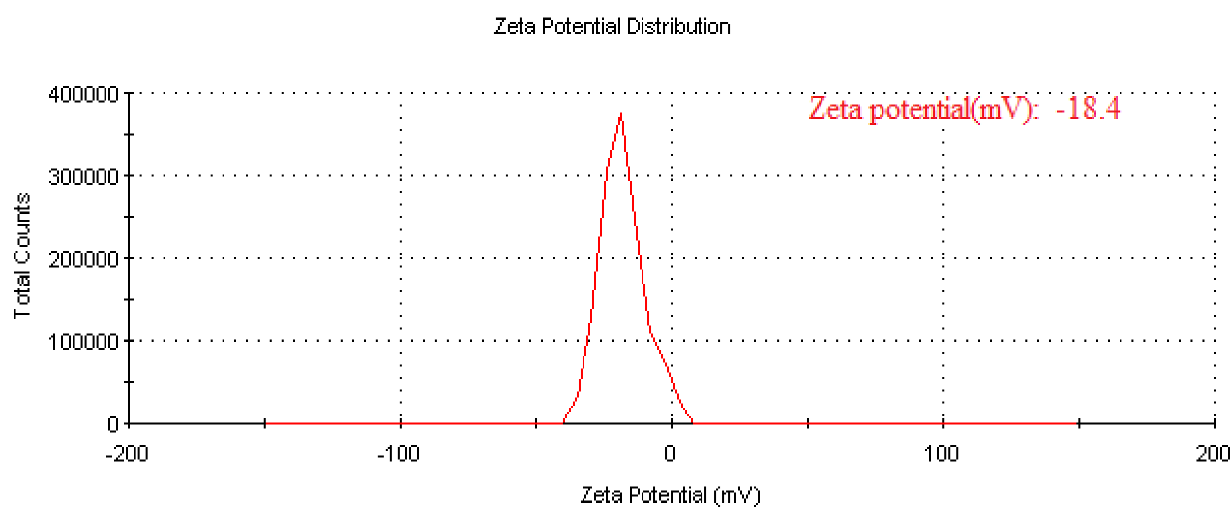

Figure 24: Zeta potential distribution of native lysozyme

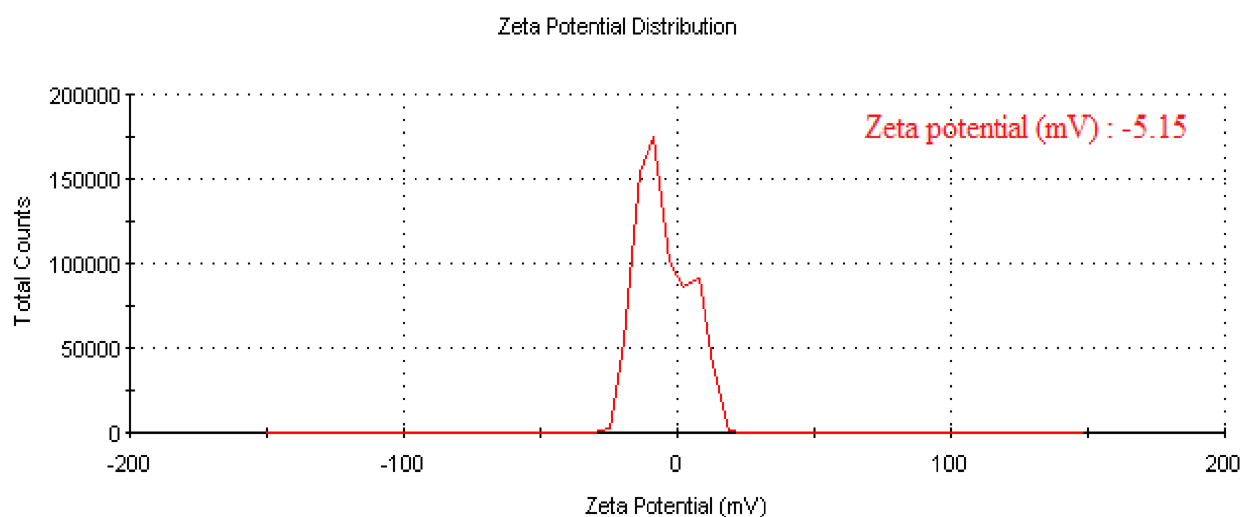

Figure 25: Zeta potential distribution of aggregated lysozyme

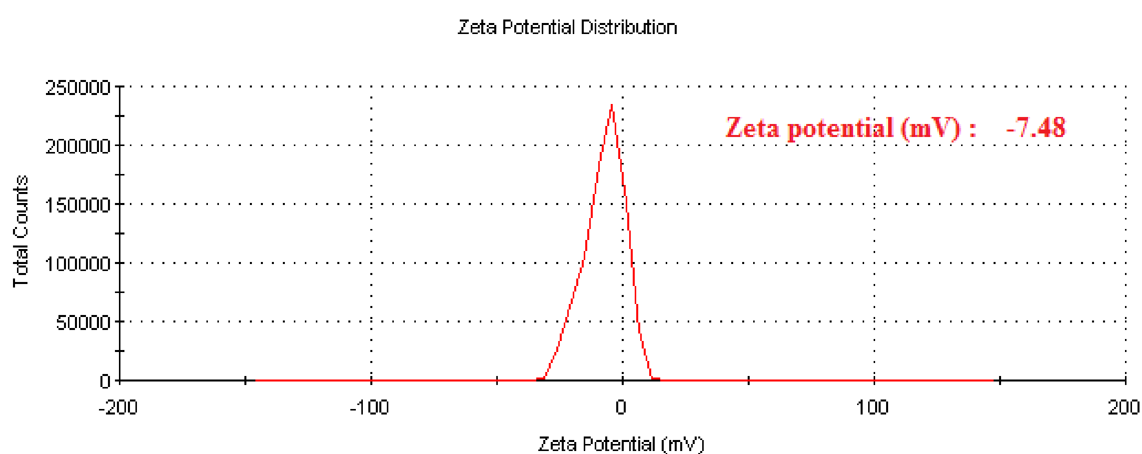

Figure 26: Zeta potential distribution of protein-nanoparticle solution (5:1)

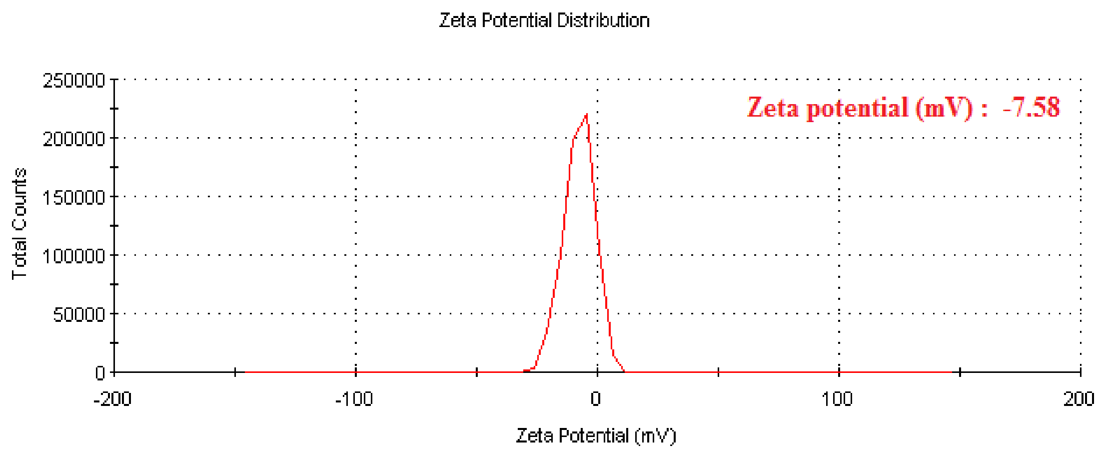

Figure 27: Zeta potential distribution of protein-nanoparticle solution (2:1)

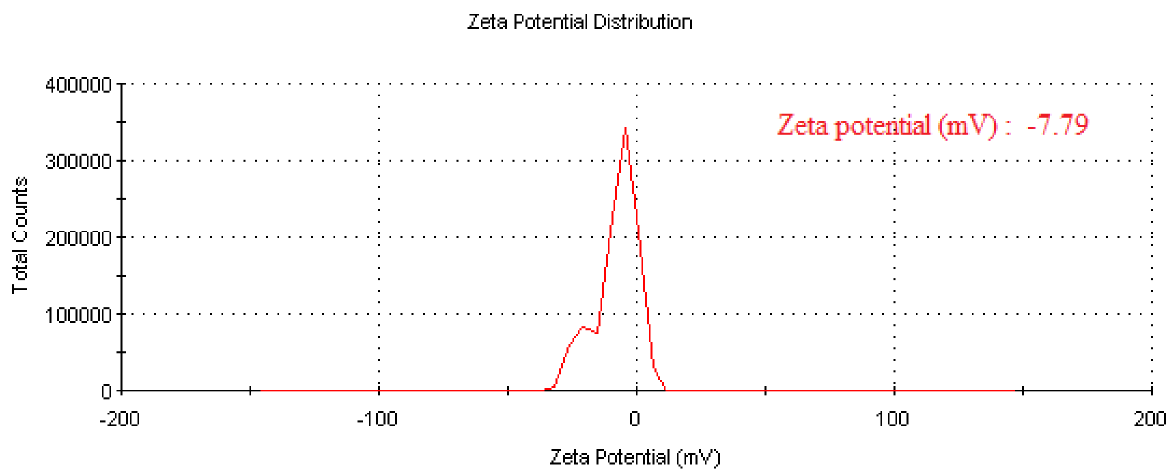

Figure 28: Zeta potential distribution of protein-nanoparticle solution (1:1)

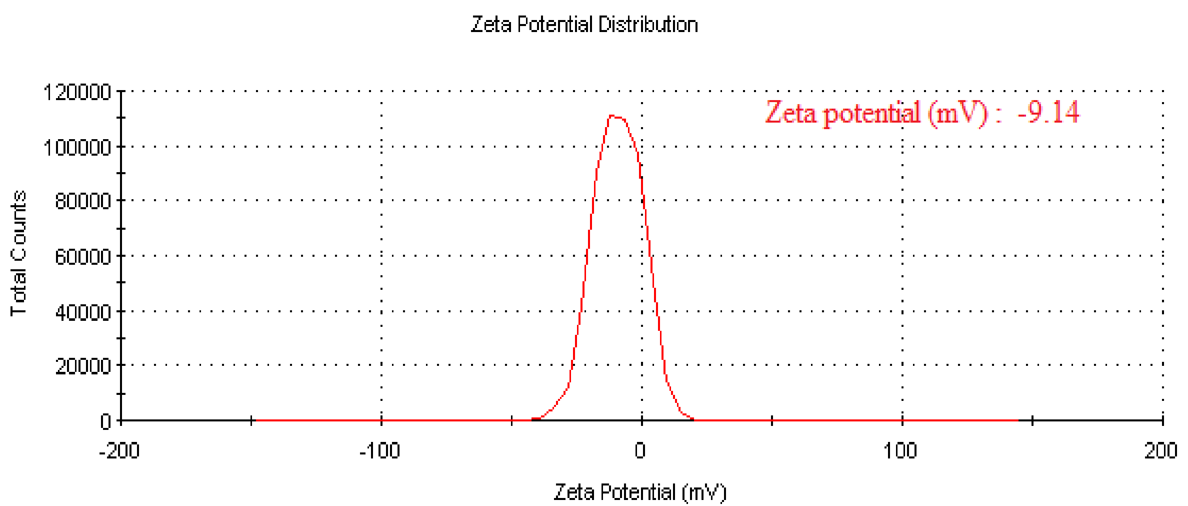

Figure 29: Zeta potential distribution of protein- nanoparticle solution (1:2)

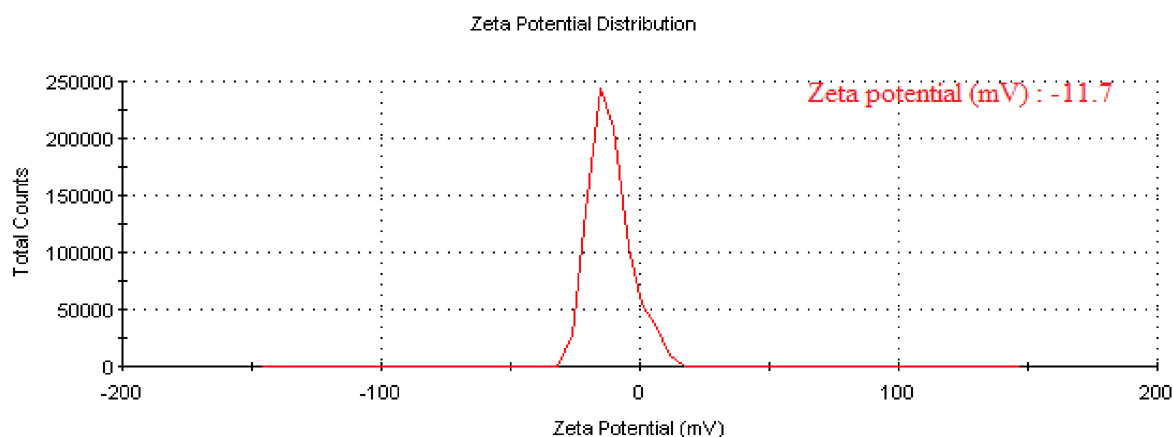

Figure 30: Zeta potential distribution of protein-nanoparticle solution (1:5)

The Zeta potential analysis was done for native lysozyme and also for aggregated lysozyme with and without different concentration of nanoparticles.The analysis shows that the native lysozyme solution is more stable having high value of zeta potential compared to others(figure 24).When the protein misfolds it leads to agglomeration causing protein to be unstable which have low value of zeta potential(figure 25).When the nanoparticles are introduced the value of zeta potential increases corresponding to the concentration of nanoparticles added. From the above figures(26-30) it is clear that the value of zeta potential increases for samples 5:1,2:1,1:1,1:2,1:5 respectively. Since the value of Zeta potential is increasing, it may be concluded that the nanoparticles are preventing aggregation of protein molecules, increasing the stability of protein molecule.

4.3.6. Congo red assay

The Congo red assay was done to measure the presence of any amyloid fibrils in the aggregated lysozyme protein solution, according to the protocol given in the materials and methods section. In the figures below, curve shows the absorbance spectra for Congo red. Then curve b shows the absorbance spectra of aggregated lysozyme with Congo red and

curves c,d,e,f and g show the absorbance spectra of protein nanoparticles solutions having ratio of 5:1,2:1,1:1,1:2,1:5 respectively with Congo red. The absorbance of Congo red, aggregated lysozyme and nanoparticle- aggregated lysozyme conjugate with Congo red were taken at 541 nm and 403 nm. Then the amount of amyloid fibrils was calculated according to the following formula given in materials and methods as follows

$$CR\text{-}A\beta = A_t^{541}/47{,}800 - A_t^{403}/68{,}300 - A_{cr}^{403}/86200$$

From the figure it has been confirmed that the peaks obtained are same as without congo red (as figure 22).So it has been concluded that Congo red did not bind aggregated lysozyme. Since the congo red has the property to bind only amyloid fibrils, so there is no amyloid fibrils formation in the above solution (table 1).

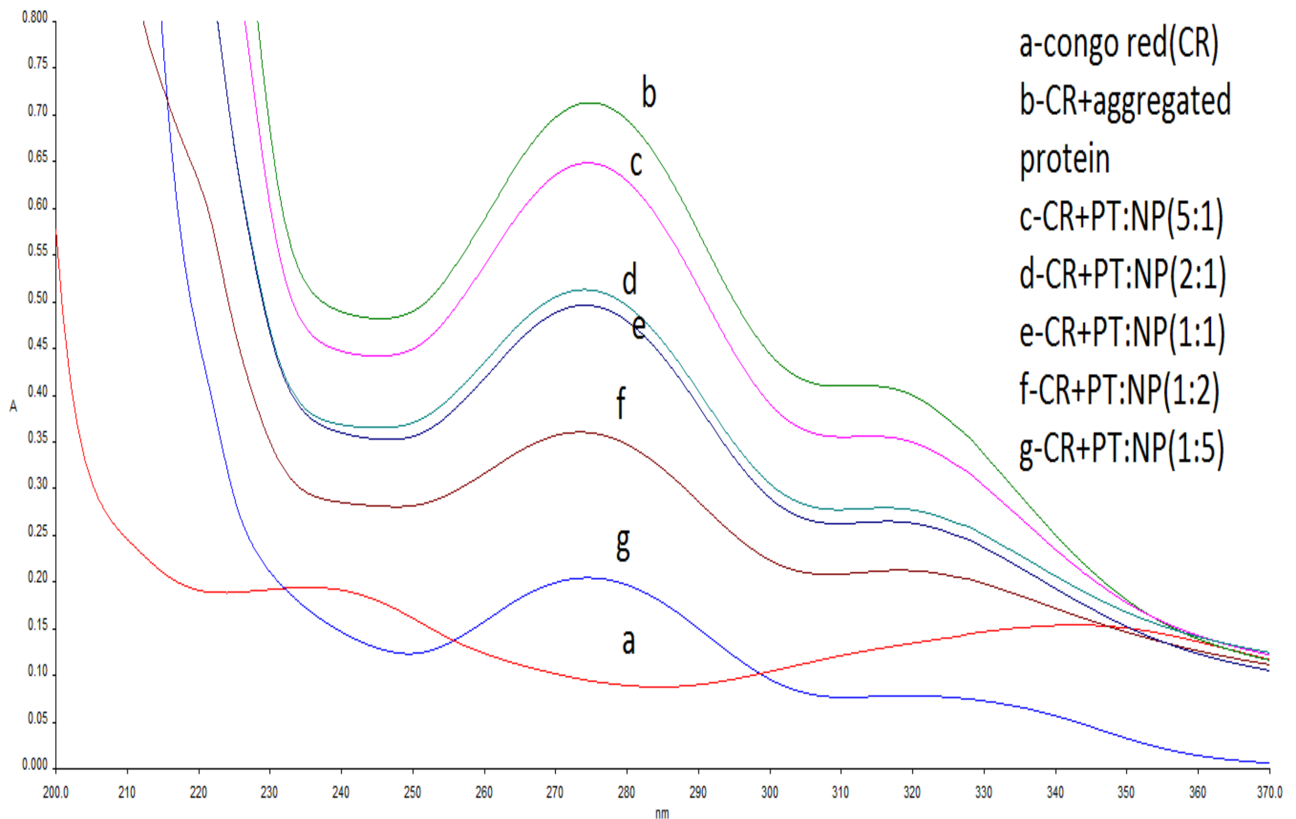

Figure 31: Absorbance spectra of Congo assay

Table-1: Amyloid fibrils in protein nanoparticle solution (The results were expressed as µg/ml)

Name of samples	ABS at 541 nm	ABS at 403 nm	Aβ fibrils (µg/mL)
Congo red	0.083642	0.069936	-----
Aggregated protein	0.084510	0.070141	0
Protein: NP (5:1)	0.084829	0.070137	0
Protein: NP (2:1)	0.089769	0.075464	0
Protein: NP (1:1)	0.99970	0.081472	0
Protein: NP (1:2)	0.11221	0.084731	0
Protein: NP (1:5)	0.095781	0.077694	0

CHAPTER 5: CONCLUSION

5. CONCLUSION:

Zinc oxide nanoparticles were successfully synthesized by using chemical precipitation method. The DLS analysis shows that the size of nanoparticles ranges from 10-100 nm. The UV-Vis spectroscopic study shows the Plasmon resonance property at 375 nm. The XRD study confirms the wurtzite structure of ZnO nanoparticles. The EDS analysis shows the elemental composition of ZnO nanoparticles. The effect of ZnO nanoparticles on aggregation of lysozyme was studied by various characterization techniques. The DLS and SEM analysis confirms the potential of ZnO nanoparticles to prevent the aggregation of hen egg lysozyme. The UV-Vis and fluorescence spectroscopy analysis suggest the conformational changes of lysozyme upon binding with ZnO nanoparticles. The Zeta potential measurement suggests that stability of aggregated lysozyme increases with increase in concentration of ZnO nanoparticles. From Congo red assay we confirm that there is no amyloid formation in our nanoparticle protein conjugate samples. Therefore it can be concluded that ZnO nanoparticles have the potential to prevent lysozyme aggregates.

CHAPTER 6
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6. REFERENCES

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