Interaction Of Metal Nano Particles With Protein: A Spectroscopic Study To Monitor Protein Conformational Changes

By

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CERTIFICATE OF ORIGINALITY

This is to certify that the work titled "**Interaction of Metal Nanoparticles with proteins: A Spectroscopic Study to Monitor Protein Conformational Changes"**submitted by **Apoorva Bangroo** in partial fulfillment of the requirements for the award ofthe degree of Bachelors of Technology in Biotechnology, of Jaypee University of InformationTechnology, Solan has been carried out under the supervision of **Dr. Abhishek Chaudhary**. This work hasnot been submitted partially or wholly to any other University or Institute for the awardof this or any other degree or diploma**.**

Signature of Supervisor

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Signature and name of the student

Apoorva Bangroo (131568)

Date:

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Summary

Research and advancements in Nanobiotechnology are well known reasons for admitting this technology in day-to-day life because it tends to provide answers and substitutes to technological, environmental and health challenges. Nanostructures are the entity of interest for all applications of Nanobiotechnology where size and shape of nanoparticles determine their essential properties.Our study is based on the conformational changes that occur on the proteins when interacted with different nanoparticles. As we know changes in the structure of the proteins can cause various diseases like neurodegenerative for whichno treatment till date has been found because of the obstacle that is Blood-Brain which can be overcome by nanoparticles. As there is increasing demand for different nanoparticles, so it becomes necessary to evolve synthesis procedures which are cost-effective and environment-friendly as well. The associated research is majorly focusing on the synthesis and on the physico-chemical nature which dominates the formation of nanoparticle-protein complex, its impact on the structure of adsorbed proteins and overall implication these interactions have on cellular functions.Chemical synthesis of nanoparticles is known to be more toxic as it involves the use of chemicals whereas green synthesis has proven to be less toxic and more environmental friendly because of the use of biological routes such as microorganisms, plants and viruses or their byproducts which can be such as proteins and lipids with the help of various biotechnological tools. To examine the effects of nanoparticles on proteins, the interaction between Bovine Serum Albumin (BSA) with Zinc and Gold nanoparticles at diverseconcentrations were tested. The interaction, BSA conformations, kinetics and adsorption were estimated by UV-Visible spectrophotometer, Dynamic light scattering (DLS), Circular Dichorism, TEM and Fluorescence Quenching.DLS, UV-visible, Circular dichroismspectrometric and analysis confirms the interaction with minor changes in size of the protein. Fluorescence quenching analysis verifies the side-on or end-on interaction of BSA to NPs whereas TEM determines the size and morphology of the nanoparticles. The data of the present study determines the detailed assessment of BSA adsorption on nanoparticles progressing with mechanism, kinetics and isotherm of the adsorption.

CHAPTER 1

INTRODUCTION

Nanoparticles (NPs) are those objects ranging in size from 1-100nm so because of their varying sizes they differ from there bulk materials in properties. There properties are unique because of which they may be suitable in distinct range of applications and wherefore they have attached significant notice specifically in the field of biomedical. The application of nanodrugs, nanovaccines and nanoparticles are being entirely examined. In spite, our understanding about the biocompatibility and hazards of exposure to nanomaterials is precise, so exposure to these nanomaterials for humans can be coincidental, for example occupational exposure or voluntarily like the use of nano-enabled consumer commodities.There are an rising count of investigations that exhibit unfavorable effects of nanomaterials in in-vitro cellular systems, but it is uncertain whether the accessible data can be reliably anticipated to conclude the adverse effects of nanobiotechnology for humans. Hence, there is an urgent need to understand the molecular mechanisms of nanoparticles to biological system interaction.

In biological medium, nanoparticles may perhaps mesh with biomolecules like proteins, nucleic acids, lipids and even biological metabolites due to their nano-size and big surface area to mass ratio is of meticulousmeaning in the adsorption of proteins on the shell of nanoparticles. The configuration of nanoparticle-protein complexes is known as nanoparticle-protein corona. A number of consequences of protein adsorption on nanoparticle surface can be observed. On the whole, the nanoparticle-protein complex can manipulate the biological reactivity of nanoparticles [1, 2].

Proteins are polypeptides with definite pH of the immediate medium. Adsorption of proteins at nano-bio interface is aided by numerous forces such as hydrogen bonds, salvation forces, van-der-waal interaction, etc. The complete nanoparticle-protein complex development is multifactorial procedure and it depends not only on the uniqueness of nanoparticle but also on the interacting capability of protein and the surrounding medium. A definite association and dissociation rate for the protein decides the durability of their interaction with the nanoparticle surface. Irretrievable or at least long term binding of proteins on the nanoparticle leads to the formation of a “hard-corona” whereas instant reversible binding of proteins which have more rapidly switch over rates defines “soft-corona” [3].

Serum, plasma or cellular protein represents complex biological systems and it is well thought-out that nanoparticles can form nano-bio complexes when open to the elements in several different systems in-vivo. An inhaled nanoparticle passes through mucosal layer, lung epithelial cells and finally enters into the blood stream. Likewise, at cellular level followed by phagocytosis by a monocyte, the nanoparticle is taken into endosomes which eventually combine with lysosomes. Each of these represents distinctive environment and has precise properties with reverence to their protein composition, enzymatic activities, pH, ion composition, etc.

Hence, to study the conformational changes of the structure of macromolecule i.e. proteins, nanoparticles were synthesized having different physical parameters so that it can be used to study various effect on the protein structure with these nanoparticles. There are various types of nanoparticles like Silver, Gold, Zinc, etc. having different structureswhich can be observed in the figure 1. New nanotools are repeatedly made by refining the applications of the nanobiotechnology which are already being used. The imaging of native biomolecules, biological membranes and tissues is another major topic for the nanobiology researchers. There are additional topics which include the use of cantilever array sensors and the application of nanophotonics for manipulating molecular processes in living cells [2].

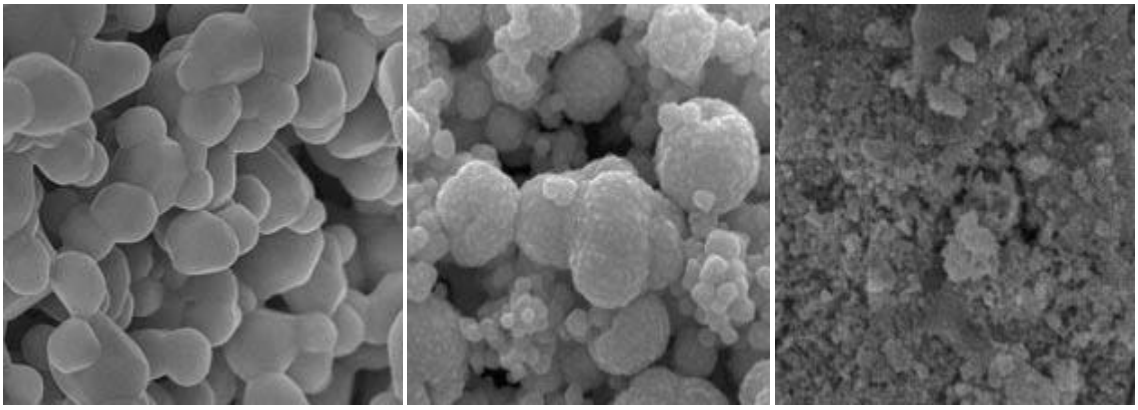

Fig1. Electron Microscope of Zinc, Gold and Silver nanoparticles [1]

There are two ways to synthesize nanoparticles which are as follows: (1) Top-down (2) Bottom-up

Top-down approach is defined as break down of bulk material to nano-sized particles [4, 5]. **Bottom-up approach** is defined as miniaturization of materials, components with further self-assembly process which leads to the formation of nanostructures [5, 6, 7]. Along with these there are three methods for preparing nanomaterials and they are: (1) Physical methods (2) Chemical methods (3) Biological

method. The most commonly used is **chemical synthesis** of nanoparticles which involves number of steps and it is considered to be more toxic and hazardous for living organisms as well as for environment so, to overcome the disadvantages of chemical synthesis method the other method which is called as green synthesis is preferred. **Green synthesis** method involves the use of microorganisms, plants, viruses or their byproducts such as proteins and lipids because of involvement of biological routes this method has been proven to be least toxic to living organisms and to environment also. In case of plants different parts can be used for the synthesis of nanoparticles like roots, leaves, stem, flower, etc. There are many plants which have medicinal properties but there availability is a matter of concern hence, *Catharanthus roseus* an important medicinal plant is easily available so, it can be used for synthesizing nanoparticles via biological method. This plant belongs to the family Apocynaceae; the other universal names are periwinkle, Madagascar periwinkle, sadabhar. Traditionally, *Catharanthus roseus* has been used in folk drug to take care of diabetes, high blood pressure and diarrhea. Though, inmodern medicine alkaloids and chemotherapeutic agents from C. *roseus* known for pain relieving property in cancer treatment. The plant is known to control foremost diseases such as leukemia and diabetes. It is cultivated primarily for its alkaloids, which are having anticancer activities [8]. Nanoparticles are of major interest because of the following reasons:

1. Better higher surface-to-volume ratio.
2. Biocompatibility.
3. Controlled delivery of drugs.
4. Site specific targeting can be achieved by attaching targeting ligands to surface of particles.
5. Subsequent clearance of drugs so as to achieve in drug therapeutic efficacy and reduction in side effects.

Nanoparticles can be characterized by various techniques in order to identify whether particles have formed, their size morphology and also to know which functional group is present on them and also to detect the nanoparticles- protein interaction. The techniques involved are:

1. UV-Vis spectroscopy: refers to absorption spectroscopy or reflectance spectroscopy in the UV-Vis spectral region. The range of UV region is 190 nm- 380 nm and that of visible region is 380 nm- 750 nm so, the range of UV-Vis is 190 nm- 380 nm. It is used to determine whether the

particles have formed or not and also size of particles. It is based on Beer- Lambert's law which says absorbance is directly proportional to concentration of the sample and the path length:

$$\mathbf{A \propto CL}$$

2. Dynamic Light Scattering (DLS): It is based on determination of particle size by measuring the random changes in the intensity of light scattered from a suspension or solution. It is used to determine the size distribution profile of small particles in suspension solutions [9].

3. Fourier Transform Infrared Spectroscopy (FTIR): It is a technique that is used to attain infrared spectrum of absorption or emission of solid, liquid or gas. It is based on the principle that most molecules absorb light in the infra-red region of the electromagnetic spectrum. This absorption corresponds specifically to the bonds present in the molecule. The frequency range is measured as wave numbers typically over the range 4000 – 600 cm^{-1}. It is used to determine the functional group present on the nanoparticles [10].

4. Transmission Electron Microscopy (TEM): It is a system in which a beam of electron is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through it. It is based on Illumination - Source is a beam of high velocity electrons accelerated under vacuum, focused by condenser lens (electromagnetic bending of electron beam) onto specimen. It measures the size of nanoparticles, and their morphology [10].

5. Circular Dichorism: It is a system which involves circularly polarized light, i.e., the differential absorption of left- and right-handed light. It is used to measure changes in secondary structure of proteins which depends on chiral properties of proteins [10].

6. Fluorescence Spectroscopy: It is a type of electromagnetic spectroscopy that analyzes fluorescence from a sample. It comprises ofusing a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and makes them to emit light; typically, but not necessarily, visible light. It used is to measure the change in spectra due to the interaction of protein with the nanoparticles [11].

7. Zeta Potential: The potential difference existing between the surfaces of a solid particle immersed in a conducting liquid (e.g. water) and the bulk of the liquid. It is an important parameter that is related to nanoparticles stability or aggregation in dispersion, and can have significant implications on product performance.

8. X-ray powder Diffraction: It is a fast analytical technique primarily used for phase identification of a crystalline material and can give information on unit cell dimensions. The analyzed material is finely ground, homogenized, and average bulk composition is determined.It is based on the interaction of the incident rays with the sample produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law ($n\lambda=2d \sin \theta$). It gives crystal structure [11].

Hence in our study we have used the following characterization techniques to conclude our work which are as follows:

i. Dynamic Light Scattering
ii. Zeta Potential
iii. Fluorescence Spectroscopy
iv. Circular Dichorism
v. Transmission Electron Microscopy

CHAPTER 2

Literature Survey

It is an assumption that adopting nanomaterials specifically the nanoparticles will have an important place in the future of science, technology and also the medicine. Introducing nanoparticles is completely based on their properties, functionality and effect on the system. During the industrial revolution in twentieth century resulted in the aggregation of large amount of toxic waste which was responsible for health hazards. In this work we are focusing on the different methods which are involved in the green synthesis of nanoparticles using specific biomolecules which are present in the plant extracts as precursors with emphasizing on the changes that occurs on the surface of protein after interaction with the nanoparticles [10].

Protein structure is three-dimensional arrangement of atoms in protein structures consisting of one or more long chains of amino acid residues. They form a vast array of functions within organisms, including catalysis of metabolic reactions, replication of DNA, and response to stimuli along with the transportation of molecules from one location to another. They differ from each other on the basis of their sequence of amino acids, which is dictated by the nucleotide sequence of their genes and results in protein folding into a specific three dimensional structure that will determine its activity [12]. Most proteins fold into unique 3-dimensional structures. The shape into which a protein naturally folds is called as its native conformation. On the basis of their folding, the protein structure can be classified as: primary structure (linear), secondary (α-helix or β-pleated), tertiary structure refers to the three-dimensional structure in which α-helixes and β-pleated sheets are folded into a globular structure and the last is quaternary structure which is also a three-dimensional structure which is stabilized by the non-covalent interactions and disulfide bonds as shown in figure 2.

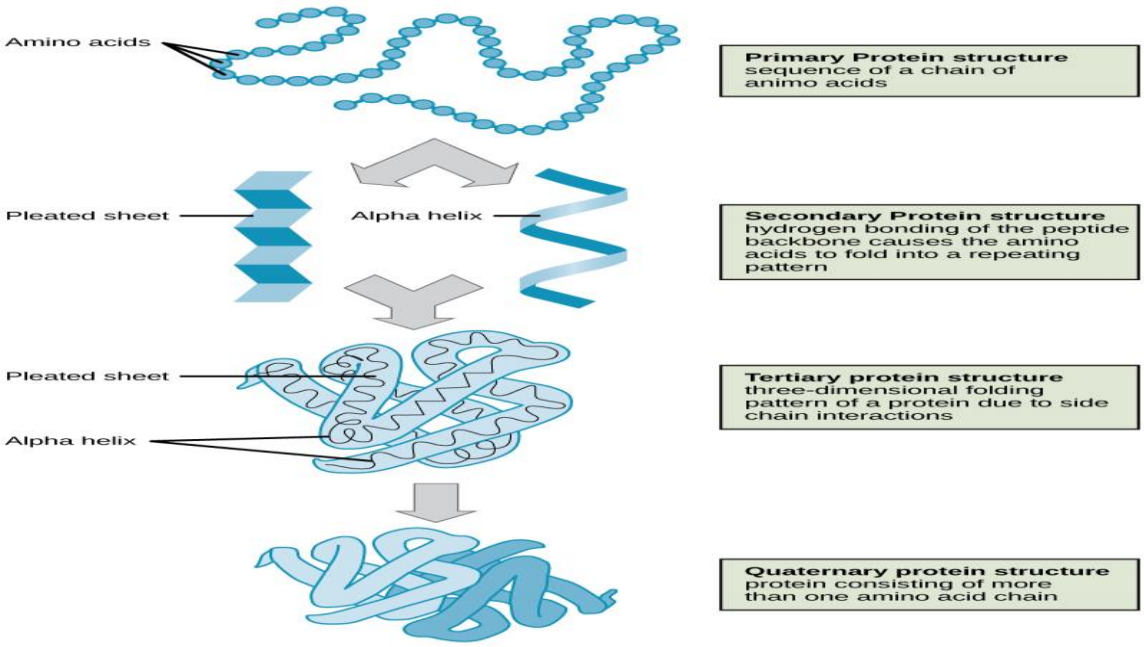

Fig2. Different structure of protein

Hence changes in these structures after interacting with different nanoparticles is studied through circular dichroism. In order to monitor the conformational changes on the protein, the studies were done on BSA (Bovine Serum Albumin) because it is a serum albumin protein which is derived from *Bos taurus* (domestic cow).The total length BSA precursor protein is 607 amino acids but an N-terminal 18-residue signal peptide is cut from the precursor protein upon secretion, due to this the initial protein product contains 589 amino acid residues whereas an additional four amino acids when cleaved yields the 583 amino acid residues that is the mature BSA. In a study it has been shown that the conformational changes that occurred in Bovine Serum Albumin (BSA) when they were made to interact with gold nanoparticles [10]. Adsorption and binding modes of BSA on gold nanoparticles was monitored by UV-Vis and fluorescence. It was found that gold nanoparticlesremoved the fluorescence emission of tryptophan residues of BSA and also conformational changes that occurred because of temperature. Hence it was concluded that protein conformational transition temperature depends on pH along with monitoring of linking between BSA and gold nanoparticles. Researchers have done studies on BSA protein because it has its structural resemblance with Human Serum

Albumin (HSA). It has also been noted that BSA adsorbs on aluminum oxide surface as a monolayer by using 30-36% of its total negative charge and that too additional BSA molecules from the medium, bind onto this monolayer as dimmers [13]. Titanium oxide nanoparticle was shown to cause conformational changes and shrink the polymerization of tubulin, which is an fundamental cytoskeletal protein [14]. Spectroscopic investigation of zinc oxide (ZnO) nanoparticle interaction with BSA showed no structural perturbation to the complete structure but still negligible conformational changes were observed hence an irreparable conformational change in the secondary structure of protein called as transferring was observed on the interaction with SPIONS. Detailed studies along these lines can be functional in designing protein-nanoparticle complex surface for the upcoming applications [13, 14]. It was noted that nanoparticles which have a diameter range of 5-100 nm strongly binds to the human blood proteins which are albumin, fibrinogen, γ-globulin, histone and insulin. The binding of nanoparticles with protein depends on the size of the nanoparticle and also the native structure of the protein because of the binding the thickness of the adsorbed protein layer gradually increases with that of the size of the nanoparticle [2, 15].

There are different types of nanoparticles which are responsible for showing different activities like anti-bacterial, anti- viral, and anti- cancer, etc also there are many factors which are responsible for causing cytotoxicity and genotoxicity which can be concentration of NPs, dispersion, size and surface functionalization [16]. As silver nanoparticles are known for their anti-microbial activity was capped with starch and it was readily able to interact with the small protein which is known as Bovine α-lactalbumin (BLA) via the formation of protein corona and the result was there was a drastic decrease in the bactericidal potential of AgNPs along with the structural changes. In order to maximize their bactericidal activity and to reduce the conformational changes in the protein there was a strategyin which polyethylene glycol (PEG)-capped AgNP was developed and it showed hemocompatibility. The same procedure was done on larger proteins called Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA). Hence it was proved that PEG-capped AgNP achieves the improved biocompatibility in actual physiological conditions because of which it can become a better therapeutic avenue in many disorders that are caused by bacteria [17]. Pseudo-first order kinetics can be determined with equilibrium contact-time of 30 minutes.

The pseudo first order reaction can be expressed via following mathematical equation

$$[\log(q_e - q_t) = \log(q_e) - k_1 t] \qquad \text{eq.(1)}$$

where q_e *and* q_t are the concentration of protein BSA molecules that are adsorbed on AgNP surface at equilibrium and at different time t whereas k_1 the adsorption is the steady rate of pseudo first- order model for the adsorption (min^{-1}) [18]. The basis of nanoparticle bio-reactivity is basically the interaction of the nanoparticles with the proteins which give mount to the formation of nanoparticle-protein corona i.e., the nanoparticles are enshrouded by a layer of biomolecules, predominantly proteins, mediating its interactions with cells because of this nanoparticle-protein corona cellular uptake, accumulation, degradation and clearance of these particles are influenced. Surface of nanoparticle can be the source for conformational changes in adsorbed protein molecules which can influence the bio-reactivity of the nanoparticles but when understanding these interactions they can be helpful in generating bio-compatible nanoparticles which can have controlled surface characteristics in the biological environment [19]. As discussed above about the functions of silver nanoparticles now because of those functions there were many changes made in diagnosis and treatment of diseases which were ultimately able to prevent various types of diseases in humans. As cancer is most common disease so, developing a therapeutic approach for its cure is most important as silver nanoparticles has the ability to destroy cancer cells with respect to non-cancerous cells at low irradiation power and low toxic for the body as well. The nanoparticles surface can also induce thermodynamic instability to the proteins which are absorbed by making it susceptible to the chemical denaturation. The fate of proteins after binding with the nanoparticles is somewhat governed by their own chemical properties [20].

Synthesis of nanoparticles can be done either via chemical method or green method. When using green method further it can be carried out by many different macro and microscopic organisms like plants, bacteria, fungi, seaweeds and also microalgae. Green synthesis method has proven to effectively control many endemic diseases while showing less adverse effects [10]. Mainly plants are used for biological route because plants contain compounds such as alkaloids, flavonoids, saponins, steroids, tannins and many other nutritional compounds. These natural compounds are extracted from different parts of plants like stem, leaves, roots, etc., and there are

also secondary metabolites present in them which act as reducing and stabilizing agent for the bio-reduction of metallic nanoparticles. This approach is considered as inexpensive, single-step and environmental friendly as well [9].

Till now maximum work is done on either silver or gold but there is another nanoparticle known as gadolinium which has been found to target specific peptides for β- amyloid plaques which are known to cause a neurodegenerative disease known as Alzheimer's disease. Alzheimer's disease is a progressive mental disorder which is known to cause as many as 26.6 million people all over the world. There are many drugs available today for the treatment but still there is no cure and also early diagnosis is not found. Hence nanoparticles will stimulate conformational changes in the protein which will lead to fibril formation and can be used for the treatment of the disease because given the small size of nanoparticles, it is quite likely that they will encounter various types of cells and also translocate across membrane barriers in an organism. Nanoparticles which are less than 100nm in diameter will enter the cells; less than 40nm will enter the nucleus while below 35nm will cross the blood brain barrier. There are basic two proteins which are responsible for causing this disease and they are amyloid and tau protein. Amyloid protein is mainly characterized by extracellular deposition of non-soluble fibrillar proteinaceous aggregates which are extremely structured into cross-β structure and they are known as amyloid fibrils. Early accurate protein typing is necessary for patient's treatment those who are suffering from neurodegenerative diseases. There are many neuroimaging techniques which provide structural details and permits to assess the amyloid burden but the technique cannot distinguish between different amyloid deposits so, for this adequate multimodal imaging nanoparticles which will target specific amyloid fibrils that will provide a useful tool for amyloidoses typing and its early diagnosis are very necessary. Functionalized gadolinium-based MRI nanoparticles along with the peptides will be extremely specific for Aβ fibrils. There is scope of these nanoparticles which when grafted with the peptides will help in distinguishing the dissimilar amyloid proteins which they tested with Aβ (1-42) and also with mutated-(V30M) transthyretin (TTR) fibrils. Tau proteins are those proteins which are responsible for stabilizing microtubules and they are abundantly in the neurons of the central nervous system. When this protein is abnormally phosphorylated it results into Paired Helical Filament (PHF) and Neurofibrillary Tangles (NFTs). There are six isoforms of this protein which are in the range from 352-441 amino acids and all

these six isoforms are present in hyperphosphorylated state in paired helical filaments from Alzheimer's disease. Hyperphosphorylated tau protein disassembles microtubules and sequesters normal tau, MAP1, MAP2 and ubiquitin into the tangles of PHFs and this insoluble structure is responsible for damaging cytoplasmic functions along with interference with the axonal transport which leads to cell death. Gold nanoparticles are coated with monoclonal ani-tau antibody which is based on two-photon scattering assay and it can be used for the detection of Alzheimer's tau protein in 1pg/ml level which is noted to be about two orders of magnitude lower than the cut-off values which was noted as 195pg/ml for tau protein in the cerebrospinal fluid. When gold nanoparticles which are coated with ani-tau antibody are mixed with tau protein it has been reported that intensity of two-photon Rayleigh scattering is increased by about 16 times. This assay is very sensitive for tau protein and it helps in distinguishing tau protein from BSA which is known to be the most abundant protein in cerebrospinal fluid [11, 21].

The array of plasma protein binding to single walled carbon nanotubes (SWCNT) was fibrinogen succeded by immunoglobulin, transferring and albumin. Dislodgment of albumin by other cell, lysate proteins was established for nanoparticles was investigated [22]. In contrast to this plasma protein binding to very minute super paramagnetic iron oxide (SPION) nanoparticle surface did not follow the theory of being exposed to plasma proteins [23]. Therefore dislocation of proteins with time is not the universal rule which can be used for all types of nanoparticles.Interaction of single wall carbon nanotube (SWCNT) and multi wall carbon nanotube (MWCNT) having different surface tension with tau protein resulted in more structural changes in case of interaction with SWCNT. SWCNT and MWCNT are also responsible for damaging the viability and affecting the complexity of PC12 cells in different modes of cytotoxicity. MWCNT is responsible for causing cell death via necrosis whereas apoptosis is induced by SWCNT and these suggests that surface tension can be used to determine the conformational changes in the protein and also the how structure of nanoparticle affects neurotoxicity [22]. SWCNTs were able to differentially persuade the loss of structure and catalytic action for the investigated enzymes. The resulted observed were like that RNAse and lysozyme retained their indigenous structures on silica nanoparticles whereas albumin and lactoperoxidase underwent an irretrievable conformational change [24].

Adsorption of protein on the surface of nanoparticle depends on the affinity of the protein towards the nanoparticle surface but also on its ability to entirely occupy the surface. The approach in which protein molecules assemble themselves on the surface of the nanoparticle may distress the biological reactivity of the latter at the cellular level [23]. Plasma proteins such as Human Serum Albumin (HSA) transferring were revealed to adsorb in a monolayer manner on iron-platinum nanoparticle surface [25].

A recent study has showed the occurrence of structural modification of protein with gold nanoparticles. In comparison to positively charged and neutral nanoparticles, the negatively charged nanoparticles of gold were exposed to bind more efficiently to fibrinogen in an point of reference which led to the cytokine release in human monocytic T-helper cells in in-vitro [24]. Chemical fabrication of the nanoparticles surface to avoid proteins to adsorb and it can be carried out by using polyethylene glycol (PEG), thus it is also known as “PEGylation”. This imparts a “sheath character” to nanoparticle surface which will shield it from being recognized by immune cells [26].

The experimental approach which is employed by most of the current studies which involves thorough study of single protein with the nanoparticle surface in comparison to the entire nanoparticle-protein complex which consists of many diverse types of proteins. Intentional use and mixture of the accessible analytical techniques are needed to analyze different aspects of nanoparticle-protein interactions all together [20].

CHAPTER 3

METHODOLOGY

Proposed strategy for the project:

- Synthesizing the nanoparticles via chemical and green synthesis method
- Characterizing the synthesized nanoparticles via different methods like UV-Vis, DLS, etc.
- Studying the interaction of nanoparticles with proteins

PROTOCOL 1: Chemical synthesis of nanoparticles

Materials

Zinc Chloride ($ZnCl_2$)and sodium thiosulfate ($Na_2S_2O_3.5H_2O$) are used. Reagent grade potassium hydroxide (KOH), are used to adjust the pH values. Distilled water with a conductivity of 18.2MΩ was used throughout the experiment.

Principle: Chemical synthesis is based on redox reaction i.e. one compound would be oxidized while other one will be reduced.An oxidation-reduction (redox) reaction is a type of chemical reactionthat involves a transfer of electrons between two species. An oxidation-reduction reaction is any chemical reaction in which the oxidation number of a molecule, atom, or ion changes by gaining or losing an electron.

Procedure 1:

1) Stock solution of $ZnCl_2$ was prepared.
2) 10ml of $ZnCl_2$ was taken from the stock and transferred to conical flask which then was kept on magnetic stirrer.
3) Freshly prepared $Na_2S_2O_3.5H_2O$ was added to the flask under mild stirring conditions for 9 minutes.
4) After 9 minutes second addition of $Na_2S_2O_3.5H_2O$ was done with the volume ranging from 20-50ml.
5) The reaction took about 20-30 minutes to complete.
6) After the reaction was completed the solution was transferred in the falcon and kept at room temperature for overnight incubation.
7) The solution was then observed after 24 hours.

Procedure 2:

1) Stock solution of $ZnCl_2$ was prepared.
2) $Na_2S_2O_3.5H_2O$ and KOH solution was freshly prepared.
3) 10ml of $ZnCl_2$ was taken from the stock and transferred to conical flask which then was kept on magnetic stirrer.
4) Freshly prepared $Na_2S_2O_3.5H_2O$ was added to the flask under mild stirring conditions after 9 minutes.
5) KOH was then added to the reaction.
6) After adding KOH second addition of $Na_2S_2O_3.5H_2O$ was done with the volume ranging from 20-50ml.
7) The reaction took about 20-30 minutes to complete.
8) After the reaction was completed the solution was transferred in the falcon and kept at room temperature for overnight incubation.
9) The solution was then observed after 24 hours.

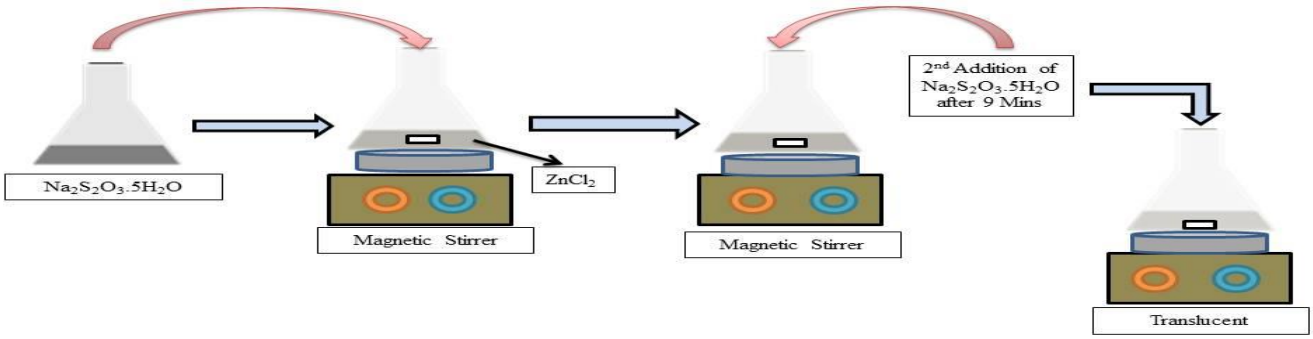

Fig3. Reaction of Zinc Chloride with Sodium Thiosulfate

Procedure 3:

1) Stock solution of 2mM Chloroauric acid ($HAuCl_4$) was prepared.
2) $Na_2S_2O_3.5H_2O$ was freshly prepared.
3) 12ml of $HAuCl_4$was taken from the stock and was transferred to the conical flask which was then kept at magnetic stirrer.
4) Freshly prepared $Na_2S_2O_3.5H_2O$ (10ml) was added to the flask under mild stirring condition immediately after the conical flask with $HAuCl_4$ was kept on the stirrer.
5) After 9 minutes again $Na_2S_2O_3.5H_2O$ (2ml) was added which act as the stabilizing agent.
6) The reaction took about 90 minutes to complete.
7) After the reaction was completed it was transferred to the falcon and kept in the refrigerator for overnight incubation.

PROTOCOL 2: Green Synthesis of nanoparticles

Leaf extract of *Catharanthus roseus*, Zinc Chloride ($ZnCl_2$), $AgNO_3$ and ethanol are used.$AgNO_3$ is used as the shaping agent. Distilled water with a conductivity of 18.2MΩ was used throughout the experiment [10].

Procedure 1:

1. Leaves were boiled in the distilled water for about 30 minutes.
2. Leaves were transferred in the beaker containing alcohol and heated mildly for about 15-20 minutes so that its pigment was absorbed.
3. Extract was filtered through the Wattmann filter paper.
4. $ZnCl_2$ solution was prepared and it was kept on magnetic stirrer.
5. Then leaf extract was added.
6. The reaction took about 120 minutes to complete.
7. After completion the sample was kept at room temperature for overnight incubation.
8. The solution was then characterized.

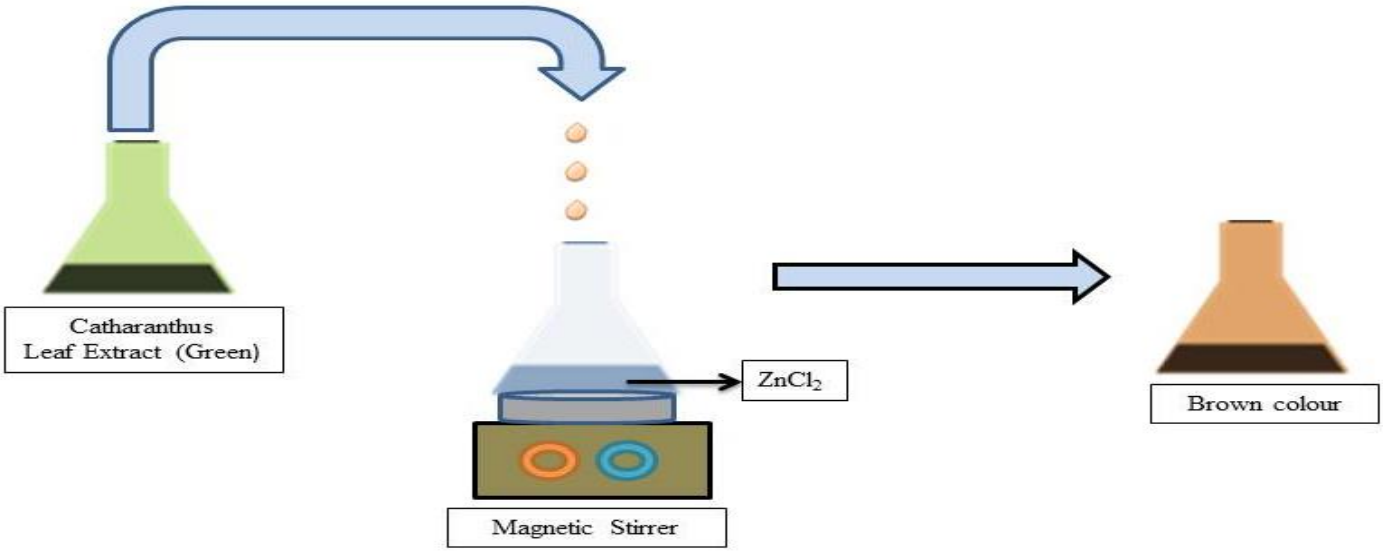

Fig4. Reaction of Catharanthus Leaf Extract with Zinc Chloride

Procedure 2:

1. Leaves were boiled in the distilled water for about 30 minutes.
2. Leaves were transferred in the beaker containing alcohol and heated mildly for about 15-20 minutes so that its pigment was absorbed.
3. Extract was filtered through the Wattmann filter paper.
4. $ZnCl_2$ solution was prepared and it is kept on magnetic stirrer.
5. Then leaf extract was added.
6. KI solution was prepared and then added.
7. The reaction took about 120 minutes to complete.
8. After completion the sample was kept at room temperature for overnight incubation.
9. The solution was then characterized.

Procedure 3:

1. Leaves were boiled in the distilled water for about 30 minutes.
2. Leaves were transferred in the beaker containing alcohol and heated mildly for about 15-20 minutes so that its pigment was absorbed.
3. Extract was filtered through the Wattmann filter paper.
4. $ZnCl_2$ solution was prepared and it is kept on magnetic stirrer.
5. Then leaf extract was added.
6. KI solution was prepared and then added.
7. $AgNO_3$ was added at the last.
8. The reaction took about 120 minutes to complete.
9. After completion the sample was kept at room temperature for overnight incubation.
10. The solution was then characterized.

CHAPTER 3

Results and Discussions

Results

In this research we explored the kinetics of nanoparticles i.e., the time taken by the particles to get synthesized. We observed the colour difference when compared the individual reactant as shown in figure 4 and 5 with the combination as shown in figure 6. The reaction took almost 120 minutes to get complete in which we observed the color changes at different time intervals. The intensity of the color increased as the reaction proceeded as shown in figure 7 and 8 and then it was observed for 24 hours in which it was seen that the reaction was stable. To confirm the size of as synthesized particles, DLS measurement was performed which predicted the size and distribution of the nanoparticles as observed in figure 9, average hydrodynamic size was found 18 nm. To confirm the stability of nanoparticles, zeta potential was measured, which came out to be +28mV it revealed the stability in colloidal solution which can have significant implications on its performance. The TEM image (Fig. 15 (a)&(b)) of the *Catharanthus roseus* leaf extract-ZnO nanoparticles showed spherical particles in the nano-size range. Then we explored the effect of nanoparticles on the BSA protein through which we concluded that some conformational changes occur in the native structure of BSA when compared with the standard BSA and nanoparticles via fluorescence spectroscopy and Circular Dichorism as shown in figure 10 and 11. The interaction of BSA with different concentrations of nanoparticles was used to perform the fluorescence and CD spectroscopy measurement. The result revealed that the emission of BSA in nanoparticles conjugate was quenched with increasing concentration of nanoparticles which indeed indicated that nanoparticles acted as a quencher. Fluorescence quenching usually occurs by two mechanisms,dynamic and static, bank on the nature of interaction between quencher and BSA [27]. Dynamic quenching usuallyoccurs when quencher molecules have adequate energy to collidewith the fluorophore of BSA at excited state, to carry it to theground state. On the other hand, static quenching outcomes fromthe formation of non-fluorescent ground state intricate betweenquencher and fluorophore. The fluorescence quenching datawere firm by using the Stern–Volmer equation eq^{n} (2).

$$\mathbf{F_0/F = K_{sv}\,[Q] + 1 = 1 + K_q\tau_0[Q]} \qquad \text{eq(2)}$$

where F_0 and F are the fluorescence intensities of BSA in the lack and existence of quencher, Ksv the Stern–Volmer constant, Kq the biomolecular quenching rate constant, [Q] the concentration of quencher, and τ_0 the average lifetime of BSA, 10^{-8}s [28]. A linear Stern–Volmer plot between F_0/F against the concentration of *Catharanthus roseus*–ZnO NPs was obtained according to eq^n (2), and the result was presented in Fig. 13(a). For static quenching, the binding constant (K_b) and thenumber of binding sites (n) between *Catharanthus roseus*–ZnO NPs and BSA weredetermined with the following equation eq^n (3) [10].

$$\mathbf{Log[F_0 - F] = logK_b + nlog[Q]} \qquad \text{eq(3)}$$

where K_b is the binding constant of *Catharanthus roseus*–ZnO NPs with BSA, [Q] the concentration of *Catharanthus roseus*–ZnO NPs, and 'n' isthe number of binding sites. Fig. 13(b) shows the linear plot of log[(F_0-F)/F] against log[*Catharanthus roseus*–ZnO NPs] according to eqn (3). Fig. 11 illustrates the CD spectra of BSA both in the absence and in the presence of *Catharanthus roseus*–ZnO NPs as recorded in the wavelength range of 200–280 nm. The mean residue ellipticity (MRE) came out to be 21269 by equation(4) and %α helicity before and after interaction came out to be 62.9% and 53.4% (eq5) by the following equation:

$$\mathbf{MRE = \theta/C_p nl \times 10} \qquad \text{eq(4)}$$

$$\mathbf{\%\alpha\ helicity = -(MRE - 2340)/30300 \times 100} \qquad \text{eq(5)}$$

Where θ is ellipticity observed, C_p is concentration of protein, n is number of amino acids and l is the path length of the cuvette [33]. By analyzing these results it is proved that change in secondary structure of BSA was caused due to its interaction with the nanoparticles and it was observed that the vital features of BSA remained undamaged.

1. Synthesis of Nanoparticles

Fig5. *Catharanthus roseus* Leaf Extract

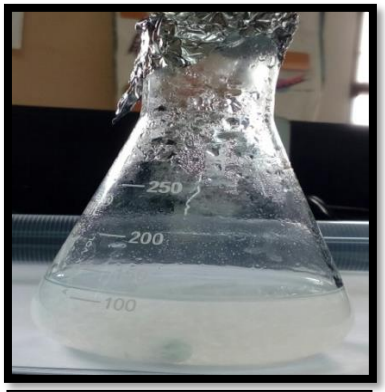

Fig6. $ZnCl_2$ Solution

Fig7.$ZnCl_2$+Leaf Extract+KI+ $AgNO_3$

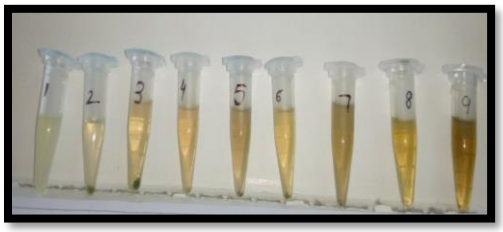

Fig8.$ZnCl_2$+Leaf Extract+KI (at different time intervals)

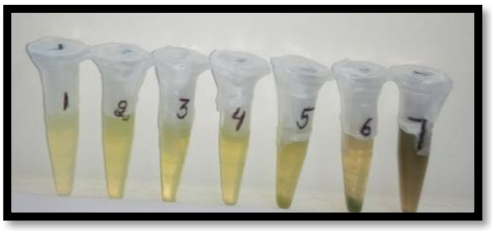

Fig9. $ZnCl_2$ + Leaf Extract (at different time intervals)

2. Characterization of nanoparticles

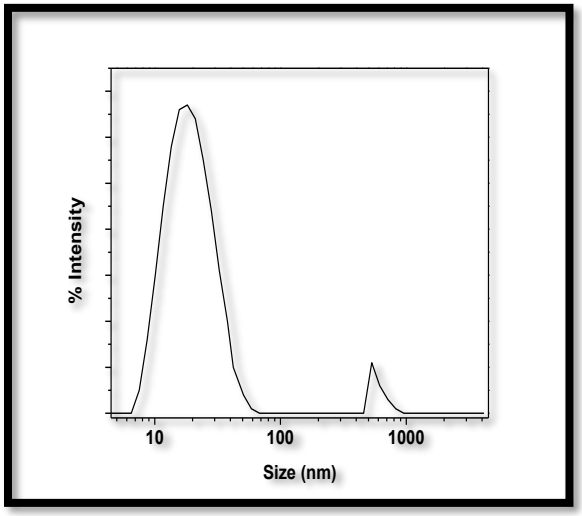

Fig10. Size distribution of nanoparticles using DLS technique

Zeta Potential= +28mV

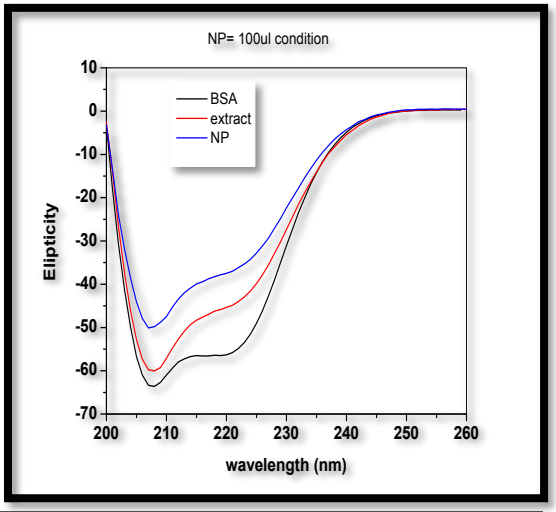

Fig11. CD analysis of nanoparticles with BSA

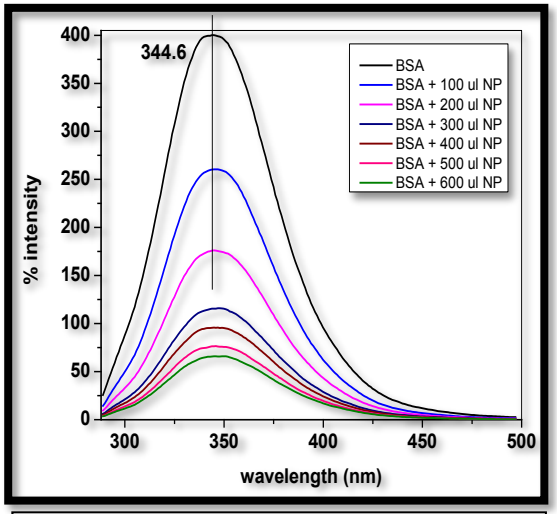

Fig12.Trp emission of BSA with different concentration of nanoparticles

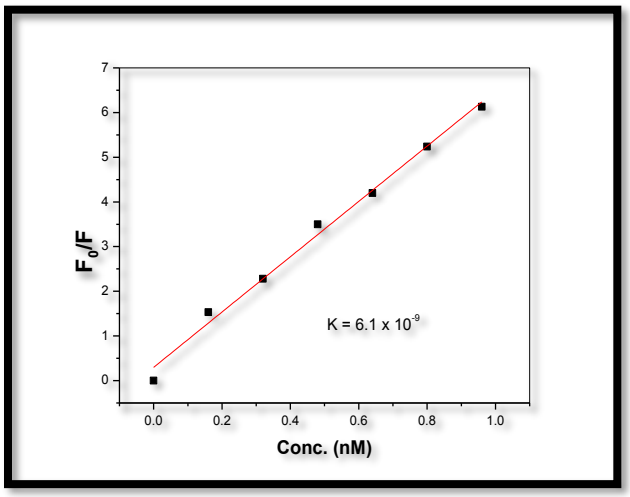

Fig13(a)

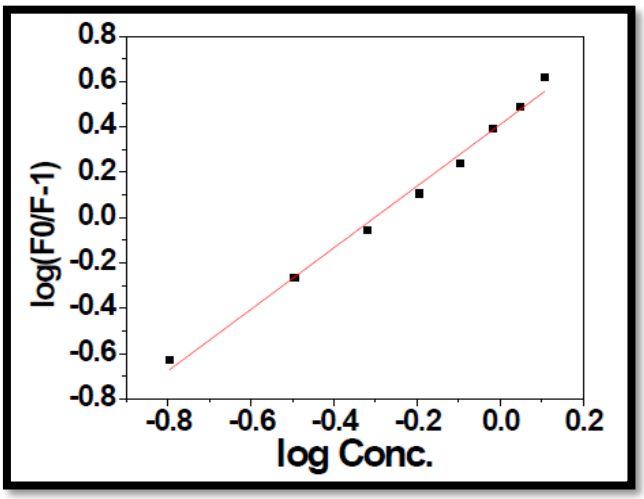

Fig13(b)

Fig13(a)&(b).Stern–Volmer plotting of BSA with *Catharanthus roseus*–ZnONPs at different concentrations. (B) The plot of log[(F_0 - F)/F] versus log [*Catharanthus roseus*]–ZnO NPs].

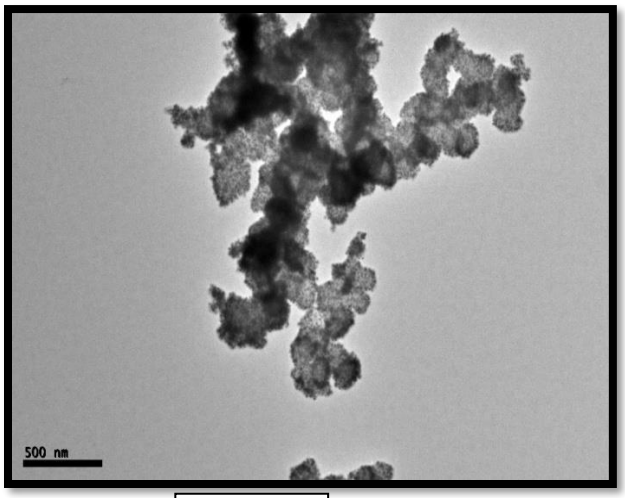

Fig 14(a)

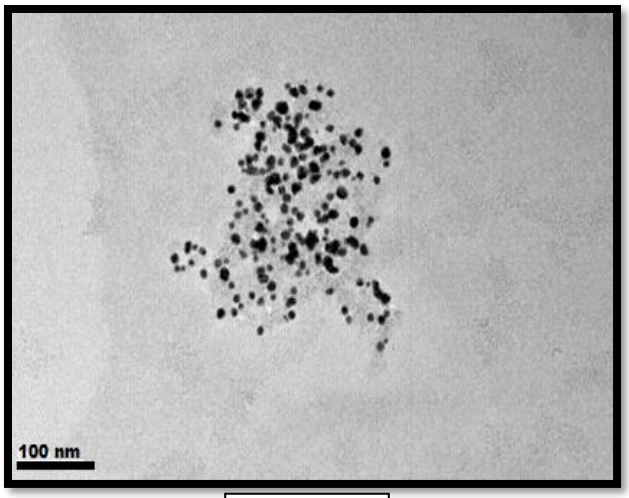

Fig 14(b)

Fig14 (a) & (b). Analysis of morphology of NPs via TEM at different wavelengths

Discussion

BSA showed its typical fluorescence spectra about 345nm. The fluorescence intensity was seen to decrease gradually without any peak shift at 345nm with the increasing concentration of nanoparticles. This clearly indicated a concentration dependent effect in serum protein. The intrinsic fluorescence of BSA is mainly contributed by its two Tryptophan (Trp) moieties: Trp 212 and Trp 134 which is very sensitive to the local environment [30]. Therefore, the intrinsic fluorescence spectra of the BSA–NPs complex were analyzed at the excitation wavelength of 280 nm, to evaluate the conformational changes around the Trp residues**.** The reduction in fluorescence intensity of BSA in presence nanoparticles indicated that the nanoparticles might have interact with the amino acid residues present in the BSA polypeptide chain and alter the conformation of protein. The quenching rate constant (Kq) for the complexation could be calculated from the slope and the result was **6.1 x M^{-1} $S^{-1}10^9$ M^{-1} S^{-1}**, which was higher than the maximum diffusion collision rate constant for various biopolymers (2.0 x10^{10} M^{-1} s^{-1}) which indicates a static quenching mechanism [28]. From the slope and intercept, the binding constant (K_b) and the number of binding sites (n) were obtained as **5.4 x 10^9 M^{-1}** and **0.81**. The value of 'n' for the interaction of BSA with *Catharanthus roseus*–ZnO NPs was close to 1, indicating that there was only one binding site available in BSA for *Catharanthus roseus*–ZnO nanoparticles. The larger size of the ZnO nanoparticles prepared with *Catharanthus roseus* may be due to agglomerations observed in Fig.14(a)and (b), which was probably attributable to the high surface energy of ZnO nanoparticles and to densification of the nanoparticles.

Furthermore CD spectral analysis of BSA protein in presence of nanoparticles confirms the conformation change in protein. The α-helix contents decreased when compared with that of the indigenous BSA [29]. The reduction in α-helicity of BSA showed that the nanoparticles might have interacted with the amino acid residues there in the BSA polypeptide chain and demolished the hydrogen bonding networks [31].

CHAPTER 4

Conclusion

It is well acknowledged that the surface of nanoparticles will be roofed by protein corona upon the interaction. In the present work, we used different nanoparticles and BSA as a means to recognize the method of protein interaction with the nanoparticles. The Stern-Volmer plot showed the static quenching in the fluorescence of BSA with increasing concentration of nanoparticles, which proved that the binding of one or two molecules of protein with nanoparticles. With Circular Dichorism the loosening of the native structure of the protein with the exposed hydrophobic amino acids was observed. Hence to understand the dynamics of the complex nanoparticle-protein interaction gives useful understanding for development or for designing the safer and value added nanoparticles for treatment of many diseases related to protein misfolding, it can be used as diagnostic kit or tool and also can be used for delivering the controlled amount of drug [32].

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