Synthesis, Characterization and the Application of Zno Nanoparticles in Biotechnology

Subhankar Paul and Deependra Kumar Ban

***Abstract*--**In our present investigation, we have observed the effect of chemically synthesized Zinc oxide (ZnO) nanoparticles in biological system. Chemically synthesized nanoparticles was characterized using UV-Vis spectrophotometer, SEM (Scanning Electron Microscope) image analysis, XRD (X-ray diffraction) analysis, DLS (Dynamic Light Scattering) particle size analysis and TGA (Thermogravimetricanalysis) analysis. From the characterization techniques like SEM analysis and DLS particle size analysis, the average size of the ZnO NP was found to be 91 nm. When ZnO NPs were applied at various concentrations in *Bacillus subtilis*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa* and *Escherichia. coli* cultures, it was observed that 15 mM NPs was found to inhibit the growth of all four microorganisms with a maximum effect on *Streptococcus pneumonia.* When *E. coli* growth was monitored in the presence of NPs in liquid medium, NPs with 10 and 15 mM concentration showed significant bacterial growth inhibition. When ZnO NP was administered in the *E. coli* cells to see the expression level of β-glucosidase in terms of enzymatic activity, after 24 h of culture growth, it was found that a sharp increase in enzymatic activity with maxima at a ZnO NPs concentration of 0.8 mM.

***Keywords*--** ZnO nanoparticles, antibacterial activity, β-glucosidase, Nanoparticle characterization.

I. Introduction

ZINC oxide nanoparticles are useful as antibacterial and antifungal agents when incorporated into materials, such as surface coatings (paints), textiles, and plastics [1, 2]. Due to bacteriostatic and fungistatic behavior of Zinc Oxide, it was well studied and utilized in personal care products[3]. When ZnO is reduced to nanoscale, it shows unique properties in comparison to its bulk counterpart. These unique properties of ZnO NPs are due to enhanced surface area, which allows for increased interaction of NPs with bacteria [4]. This permits using a smaller amount of Zinc Oxide NPs for the same or improved biostatic behavior.

Due to its non-toxicity and compatibility with skin, makes it a suitable additive for textiles and surfaces that come in contact with human body [5].

Dr. Subhankar Paul is with the Department of Biotechnology and Medical Engineering, National Institute of Technology Rourkela, Rourkela-769008, Odisha, India. (corresponding author's phone: +91-6612462284; e-mail: spaul@nitrkl.ac.in)

Deependra Kumar Ban is with the Department of Biotechnology and Medical Engineering, National Institute of Technology, Rourkela, Odisha 769008, India. (e-mail: 510bm112@nitrkl.ac.in)

Zinc Oxide's UV attenuation properties also make it an effective additive for packaging plastics to prevent UV mediated damage. Zinc Oxide is also used as a catalyst for methanol synthesis[6].

The increase in surface area of nanoscale Zinc Oxide compared to larger powders has the potential to improve the efficiency of both aqueous and organic solvents, allowing for incorporation into most material processes.

In our present investigation, we have observed the various effects of chemically synthesized ZnO nanoparticles in biological system. Chemically synthesized nanoparticles were characterized using UV-Vis spectrophotometer, SEM (Scanning Electron Microscope) image analysis, XRD (X-ray diffraction) analysis, DLS (Dynamic Light Scattering) particle size analysis and TGA (Thermogravimetric analysis) analysis. Following characterization, the average size of the ZnO NP was found to be 91 nm. When ZnO NPs was applied with varying concentrations in *B. subtilis*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa* and *E.coli* cultures, it was observed that 15 mM NP was found to inhibit the growth of all four microorganisms with a maximum effect on *Streptococcus pneumonia.* E.coli growth when monitored in the presence of, 10 and 15 mM concentration of NP revealed significant inhibition of growth.Administration of ZnO NPs in the *E.coli* cells followed by 24 h of growth, revealed a sharp increase of the enzymatic activity of β-glucosidase. Themaxima were found at a NP concentration of 0.6 mM.

II. Materials And Methods

A. Synthesis of Zno Nanoparticles

The zinc oxide nanoparticles ((ZnO NPs) were prepared by wet chemical method using zinc nitrate and sodium hydroxides precursors and starch as a stabilizing agent[5]. Different concentrations of starch (0.1%) were dissolved in 500 ml of luke warm distilled water. Zinc nitrate, 14.874 g (0.1 mol), was added in the above solution, followed by constant stirring for 1 hour using magnetic stirrer to completely dissolve the zinc nitrate. After complete dissolution of zinc nitrate, 0.2 mol of sodium hydroxide solution was added drop by drop under constant stirring. The reaction was allowed to proceed for 2 h. After the completion of reaction, the solution was kept overnight and the supernatant solution was discarded carefully. Rest of the solution was centrifuged at 10,000 g for 10 min and the supernatant was discarded. Thus, the nanoparticles obtained were washed thrice using distilled water. Washing was carried

out to remove the by-products and the excessive starch bound with the nanoparticles. After washing, the nanoparticles were dried at 80°C overnight. During drying, complete conversion of $Zn(OH)_2$ into ZnO takes place

B. Uv-Vis Spectroscopy

The ZnO NPs were characterized in a Perkin-Elmer UV-VIS spectrophotometer, Lambda-19.The scanning range for the samples was 200-800 nm at a scan speed of 480 nm/min. The spectrophotometer was equipped with "UVWinlab" software to record and analyze data. Base correction of the spectrophotometer was carried out by using a blank reference. The UV-Vis absorption spectra of the samples were recorded and numerical data were plotted.

C. X-Ray Diffraction (Xrd) Measurement

The ZnO NPs solution thus obtained was purified by repeated centrifugation at 8000 rpm for 15 min followed by re-dispersion of the pellet of ZnO NPs into 10 ml of deionized water. After freeze drying of the purified NPs, the structure and composition were analyzed by XRD. The dried mixture of ZnO NPs was characterized by an X'Pert Pro X-ray diffractometer (PANalytical BV, The Netherlands) operated at a voltage of 40 kV and current of 30 mA with Cu Kα radiation in θ- 2θ configurations.

D. Growth inhibition in liquid medium

The antibacterial effect of both ZnO NPs, both in liquid nutrient growth medium andon agar plates werestudied against four microorganisms *B. subtilis* (B) *Streptococcus pneumonia* (C) *Pseudomonas aeruginosa*, *E.coli*. *E.coli* culture in nutrient media (1gyeast extract,1 g beef extract,0.5 g NaCl, dissolved in100 ml distilled water),were used for both studies. Frozen *E.coli*cells were grown overnight in the nutrient medium to prepare inoculum. The bacteria cultures were allowed to grow in a shaking incubator at 310K (37 ^{0}C), and 200 rpm. ZnO NPs was dispersed in autoclaved deionized water by ultrasonication. Aqueous dispersion of silver nanoparticles of desired concentration was made.For this experiment, freshly grown bacterial inoculums (10^4 cells/ml)of *E. coli* was incubated in the presence of arrange of ZnO NPs with concentration of 0,5mM, 10mM, and15mM that added in each flask to observe the bacterial cell growth pattern at 310 K (37 ^{0}C)and 150 rpm. Total solution volume used in each flask was50ml. In liquid medium, growth of *E. coli* was indexed by measuring optical density (OD). Optical density measurements on the samples collected from the solution were carried out at λ_{max}=600 nm against growth media control by UV-Vis spectroscopy after every 2 h time interval and up to 24h. Control flask obtained 50ml of all the initial reaction components except the nanoparticles. The specific growth rate of the bacterial culture was calculated using the following eqn (1):

$$\mu = \ln (m_2/m_1)/t_2-t_1 \quad (1)$$

Where μ is specific growth rate**,** m_1 and m_2 is the biomass produced at time t_1 and t_2 of the bacterial culture.

E. Disc Diffusion Method

The antibacterial assays were performed by standard disc diffusion method. Nutrient broth/agar (1g beef extract, 1 g peptone, and 0.5g NaCl dissolved in100 ml of double distilled water) was used to cultivate bacteria. The media was autoclaved and cooled. The media was poured into the Petridis and kept for 30 minutes for solidification. After 30 minutes the fresh overnight cultures of inoculum (100 μl) of four different cultures were spread on to the solidified nutrient agar plates. Sterilepaper discs made of Whatmanfilter paper, 5mm diameter that was earlier dipped in 50 mg/liter ZnO NPs along with four standard antibiotic containing discs were placed in each plate. The cultured agar plate was incubated at 37 °C for 24h. After 24h of incubation the zone of inhibition was investigated.

F. Cfu Measurement

E. coli was used for colony forming unit (CFU) measurements on the solid medium plate. Samples treated with different concentrations of ZnO NPs (50,100,150 μg/ml) were spread on nutrient agar plates. These samples were diluted at 10^9 folds to get the better colonies. After incubation at 37 ^{0}C for 24 h, the numbers of CFU were counted.

G. Effect of Zno Nps on the Level of B-Glucosidase Production in E.Coli

250 ml of *E. coli* culture was grown at 25 ^{0}C for 24 h in the absence and presence of ZnO NPs (0.1-0.8 mM) and centrifuged at 4500 rpm for10 minutes at 4 °C. The pellet was washed with chilled phosphate buffer (pH 7.0) of 10 ml and the sample was sonicated. The sonicated sample was centrifuged at 10,000 rpm and 4°C and the supernatant was separated.100 μl of the supernatant was added in each flask along with 100 μl of citrate phosphate buffer (0.1M, pH 5).This was followed by addition of 100 μl of 0.55×10^{-2}M of *p*NPG and incubated at 37°Cfor 30min.Afterincubation, 1M sodium carbonate was added to stop the reaction allowing the yellow color of the *p*-nitrophenolateion to develop and 2.0 ml of ethanol was added. This was followed by centrifugation at10,000 rpmfor15 minutes. The supernatant was taken and the optical density of each sample was measured to know the relative β-glucosidase activity. Here we assumed that the genetically expressed β-glucosidase molecules all are folded and active.

III. Results

A. Uv–Vis Spectral Analysis

ZnO NPs was synthesized according to the protocol described in the 'Material and Methods' section. The UV-Vis spectra of ZnO NP prepared with 0.5% concentration of soluble starch was shownin Fig.1. The peak was found at 370 nm which was the characteristics of ZnO NPs formation.

B. X-Ray Diffraction Pattern For Zno Nps

The XRD pattern of ZnO NPs was presented in Fig.2.All the peaks were hexagonal and approximately close to the reported information (jcpds-79-0206). Due to the crystal symmetry and related face velocities, the common crystal habit of ZnO is hexagonal in shape. Also the ZnO NPs is the thermodynamically stable crystallographic phase. The width of the peaks in case of ZnO NPs has increased due to the quantum size effect. The average particle size was estimated to be 42 nm using Scherer equation (discussed in 'Material and Methods' section 3.6.2).

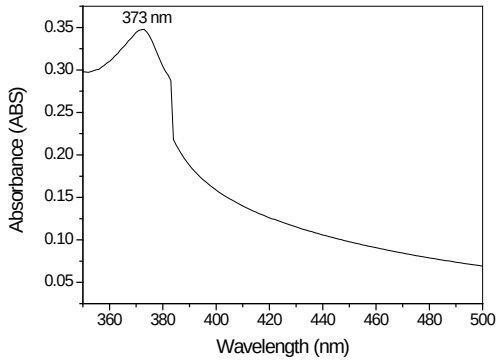

Fig. 1 UV-Vis absorption spectrum of ZnO NPs synthesized by using starch (0.5%). maxima at 373 nm.

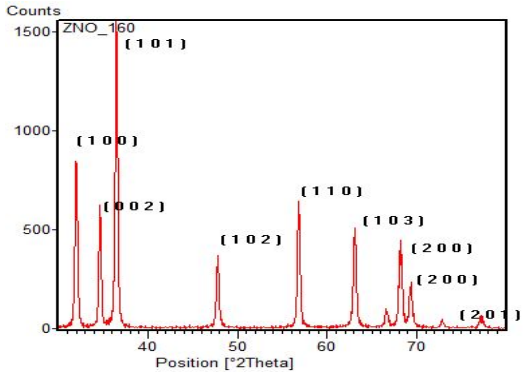

Fig. 2 XRD analysis of ZnO NPs showed wurtzite structure.

C. Scanning Electron Microscope Image Analysis

The SEM images of Zinc oxide are shown in figure 3.The figure shows that ZnO NPs are spherical with some aggregation.

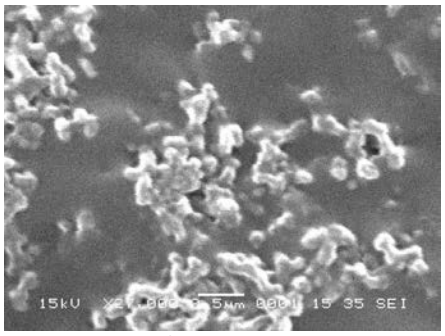

Fig. 3 SEM analysis of ZnO nanoparticle synthesized by chemical reduction method

D. Dynamic light scattering particle size analyzer

The Fig. 4 shows the particle size of the ZnO NP samples. After analyzing data, it was found that ZnO NPs size were in the range of 80-120nm. However, beyond 100 nm range the percentage of nanoparticles present is less. The highest fraction of ZnO NP present in the solution was of 90 nm. From the Fig. 4, it was evident that the solution was consisting of NPs having various sizes which are indeed in agreement of the result obtained by SEM analysis.

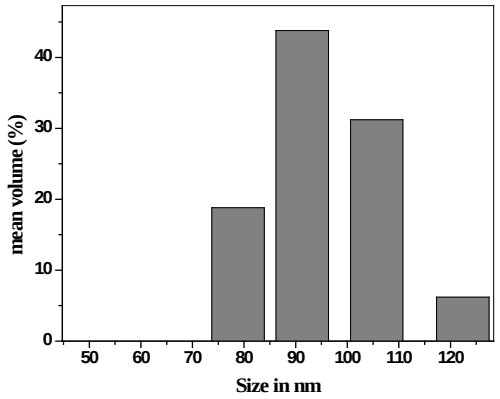

Fig. 4 Particle size distribution of ZnO NPs synthesized by chemical reduction method. The average sizes of nanoparticles were found of 91 nm

E. Discs Diffusion Assay

The effect of different concentration of ZnO NPs like-5mM, 10 mM, 15 mM and 0.2 mM, 0.3 mM and 0.6mM of ZnO NPs on bacteria was performed. As shown in Fig. 5, as we increased the concentration of ZnO NPs the antibacterial activity of ZnO NPs increased. Fig. 5 shows a clear inhibition zone treated with ZnO NPs whereas the standard antibiotics like vencomycin, erythromycin, and tobramycin shows smaller zone of inhibition as compared to the nanoparticles treated discs.

TABLE 1
ZONE OF INHIBITION OF ANTIBACTERIAL TEST OF ZNO NP.

Bioactive agent		Zone of inhibition (Diameter, cm)			
		E.coli	*B. subtilis*	*P. aeruginosa*	*S. pneumonia*
ZnO NPs	5 mM	3.2	2.2	nil	0.2
	10 mM	4.7	4.1	nil	3.1
	15 mM	4.8	4.8	3.1	4.9
Erythromycin (10mcg/disc)		0.8	0.7	0.6	4.8
Vancomycin (10mcg/disc)		nil	nil	nil	0.6
Tobramycin (10mcg/disc)		nil	3.1	nil	3.2

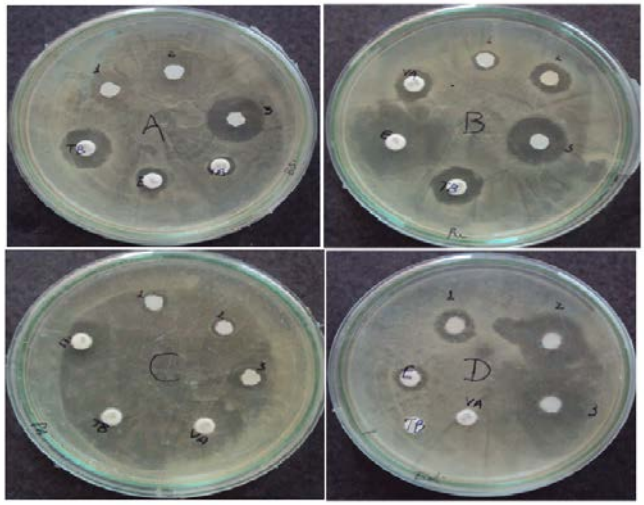

Fig. 5 Images of antibacterial activities of discs of different concentration of ZnO nanoparticles 1) 5mM, 2) 10mM, 3. 15mM and other antibiotics on (A) *B. subtilis* (B) *Streptococcus pneumonia* (C) *Pseudomonas aeruginosa* (D) *E.coli* (N=nanoparticles, VA= Vancomycin, E= Erythromycin, TB = Tobramycin).

F. Antimicrobial test by the estimation of Colony Forming Units (CFU)

Fig. 6 shows the plot of number of bacterial colonies grown on nutrient agar plates as a function of concentration of ZnO NPs. The numbers of CFU have been observed to reduce significantly with the increasing ZnO NPs loading.

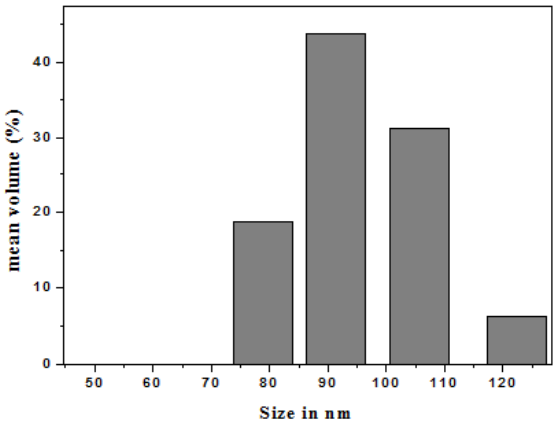

Fig. 6 Antibacterial Characterization by CFU as a function of ZnO NPs concentration on Agar plates.

G. The effect of ZnO NPs on the growth of E. coli

The synthesized ZnO NPs were administered to *E. coli* with various concentrations to investigate the growth behavior. From the Fig. 7, it was noticed that when the concentration of ZnO NPs was increased the growth was reduced. This clearly indicates that NP produced toxicity to *E. coli* and therefore the growth was inhibited.15 mM ZnO. Also specific growth rate, μ was calculated in each case and presented in the tabular form in Table 2.

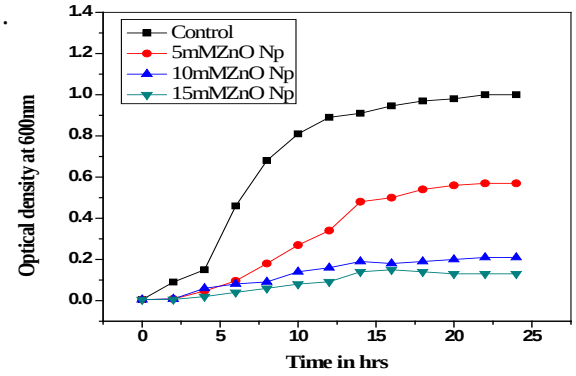

Fig. 7 The effect of ZnO NPs on the growth of *E.Coli.*

H. Effect Of Zno Nps In The Level Of β*–Glucosidase Expression In Vivo*

β–glucosidase was expressed in *E. coli* and its biological activity was monitored using the protocol mentioned in 'Material and Methods' section. The culture was incubated with various concentrations of ZnO NPs and the biological activity of the expressed β–glucosidase was measured.

TABLE II
SPECIFIC GROWTH RATE VALUES OF *E.COLI* IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF ZNO NPS.

ZnO NPs concentration (mM)	**Specific Growth Rate (h^{-1})**
0	0.21
5	0.14
10	0.05
15	0.02

The activity unit presented absorbance (A) was measured at 400 nm and expressed as the relative activity of β–glucosidase and this was plotted against ZnO NPs concentration which shows in Fig. 8**.** It was observed from the plot that with the increase of NP concentrations, the biological activity of β–glucosidase was enhanced substantially *in vivo* and below 0.1 mM and beyond 0.8 mM concentration of ZnO NPs, there was no further change of activity.

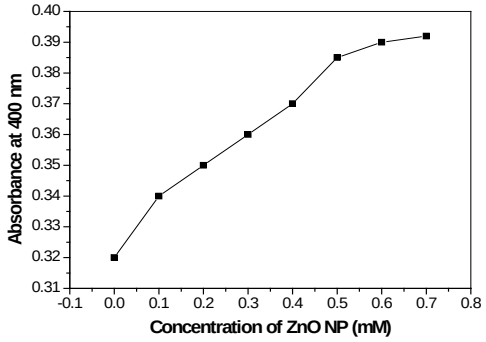

Fig. 8 Activity of β-glucosidase in *E.coli* when treated with ZnO NPs.

IV. DISCUSSION

ZnO NPs has been synthesized using chemical reduction process and has been tested in various biological systems and

evaluated their effect on them. The average ZnO NPs size was found to be 91 nm. The antibacterial potential of ZnO NPs were evaluated against four different pathogenic bacteria like *B. subtilis, S. pneumonia, P. aeruginosa* and *E.coli*. When the ZnO NPs applied at three different concentrations like 5, 10 and 15 mM, it was observed that 15 mM ZnO NPs inhibited the growth of all cells with a maximum effect on *E. coli* and *B. subtilis*. Three antibiotics (erythromycin, vancomycin, tobramycin) was also applied simultaneously as a control with 100 µg/ml final concentration, however, ZnO NPs was observed to be a better choice as an inhibitor than antibiotics (Fig. 5, Table 1). The reason was perhaps the multi-drug resistance that was developed by these four pathogens. The NPs were new killing agents to them and hence ZnO NPs could inhibit the growth of these four pathogens.

When colony forming units (CFUs) were monitored in agar plates in the presence of 5,10 and 15 mM ZnO NPs, it was found that at 15 mM ZnO NPs, only 2% CFUs of the control was counted, which indicated that 15 mM ZnO NPs can almost completely stop the growth of *E .coli* culture in agar plate (Fig. 7). When ZnO NPs was applied in *E. coli* liquid culture (broth) and growth profile was studied for 24 h, the growth inhibitory effect of ZnO NPs was also observed. All three concentrations of NPs brought inhibitory effect; however, highest effect was seen with 15 mM ZnO NPs (Fig. 7, Table 2).The result was in the agreement with the antimicrobial properties of NPs that we found in the agar plate experiment. While the NPs can have growth inhibitory effect, it might effect on the genetic expression level of various genes. To check this, we have tested β-glucosidase expression in *E .coli*. The β-glucosidase expression level in *E. coli* was also monitored in the presence of different concentrations NPs at 37 °C. When 0.1-0.8 mM ZnO NPs was applied after the inoculating the seed culture and culture was grown for 24 h. The level of the β-glucosidase expression was increased with in increasing the NPs concentration reached optimum for 0.8 mM NPs. Here our assumption was that the all protein molecules that are expressed must be in soluble and native and hence, the enzymatic activity was correlated with the level of expression.

These results showed that ZnO NPs at low concentrations enhanced the growth and β-glucosidase expression level, but when we applied higher concentration of NPs, it showed antimicrobial property with different bacteria. Many research groups also investigated the antimicrobial properties of ZnO NPs. Xie *et al.* showed antibacterial properties of ZnO NPs with *Campylobacter jejuni* [1]. Premanathan *et al* showed ZnO NPs selective toxicity to gram-positive bacteria and cancer cells by apoptosis by lipid peroxidation [7]. Joshi *et al.* investigated the effect of ZnO NPs (4-5 nm) surface ion on antibacterial property with *E. coli* (K-12). They proposed that enhanced antibacterial property of ZnO NPs was due to release of reactive oxygen species [8]. From this research, it is confirmed that ZnO NPs effect as antimicrobial agent is dose dependent. Further verification of mechanism of ZnO NPs action on different bacteria is required.

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Subhankar Paul: Dr. Subhankar Paul completed his undergraduate and postgraduate degree in Chemical Engineering and Ph.D in protein folding in cell. He is the Chief Investigator of Structural Biology and Nanomedicine group in the Department of Biotechnology and Medical Engineering, National Institute of Technology, Rourkela, India. His current research mainly focuses on understanding the nanoparticle-protein interactione and development of nanoparticle- biomolecule conjugates for the treatment of various diseases like cancer and neurodegenerative disease.

Deependra K. Ban: Mr. Ban completed his undergraduate degree in biotechnology and graduate degree in Food and Biochemical Engineering. After that he had joined as a Ph. D. student in structure biology laboratory under the supervision of Dr. S. Paul. His current research focuses on understanding the nanoparticle protein interaction and role of nanoparticles in protein folding, misfolding and aggregation.