Fluorescence and CD studies of protein denaturation in the presence of sub-picomolar gold nanoparticles

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The dependence of nanoparticle concentration on the conformation of bovine serum albumin has been studied. A very small but consistent enhancement in the tryptophan emission is observed in the presence of sub-picomolar concentration of gold nanoparticles. At this concentration, the bovine serum albumin unfolding profile in presence and absence of nanoparticles are quite similar and the protein conformation remains essentially conserved.

Keywords: Protein-nanoparticle interactions, Protein conformation, Protein denaturation, Thermal denaturation, Chemical denaturation, Nanoparticles, Gold nanoparticles, Fluorescence spectroscopy, Circular dichroism

The application of nanoparticles in different areas of research has gained much prominence of late because of the multifaceted properties that these submicron-sized particles possess by themselves or in combination with other molecules. Of special mention is the importance of conjugation of such nanoparticles to molecules of biological relevance such as proteins, enzymes and DNA. This emerging field of biotechnology that invokes the use of nanoparticles as possible biosensors, drug delivery systems and many others has already garnered considerable amount of interest among the scientific community. Central to all these functions is the nature of interaction of proteins with the nanoparticle surfaces, that is, to what extent the structures of proteins and their functions/biological activities are preserved or perturbed by surface adsorption/attachment. Indeed, knowledge of this very aspect is extremely crucial and is the primary criterion that needs to be addressed in determining the suitability of a particular protein-nanoparticle conjugate.

One of the main features of protein-nanoparticle interactions is the effect of the curvature of the nanoparticles on the biological species that remain conjugated to the former either through covalent attachment or via simple adsorption, the latter occurring reportedly through an electrostatic mechanism. Recent studies clearly delineate the effect of surface curvature/topography for proteins on silica spheres with the shape of the protein and nature of the surface (hydrophilic or hydrophobic) also playing decisive roles. Highly curved C60 fullerenes have also been found to enhance the stability of enzymes under denaturing conditions much more than flat surfaces. In a similar fashion, the stability of the protein RNase was found to be strongly influenced by the size of the silica nanoparticles. CdSe nanoparticles have also been shown to interact differently with the protein chymotrypsin by differential modification of the surface chemistry. Surface modified negatively charged gold nanoparticles have been used as synthetic chaperones thereby assisting in refolding of denatured proteins. Besides these, nanoparticles are also known to influence the aggregation kinetics of amyloid beta (Aβ) peptides. Taken together, the aforesaid reports reveal that nanoparticles can strongly affect the conformational properties and stability of adsorbed proteins and hence an in-depth understanding of such protein-nanoparticle conjugate interactions is extremely important.

In this study, we have investigated the thermal and chemical denaturation profiles, and hence, the stability to external perturbation thereby, of the protein bovine serum albumin (BSA) when conjugated to citrate capped gold nanoparticles. Bovine serum albumin, one of the main plasma proteins, is a very well-studied biological macromolecule and details of its chemical unfolding profiles in absence of nanoparticles have already been investigated through a variety of spectroscopic techniques. It is a 583 residue, predominantly helical and multidomain (domains I, II and III) protein having only two tryptophan residues of which one remains exposed while the other one is buried in the protein cleft. The interaction of BSA with different nanoparticles (TiO2, CdS, Au, SiO2 etc) including core-shell nanostructures have also been reported. pH dependent studies of BSA-GNP conjugates show substantial modification...
in the structure of the protein as evident from the change of the amide I band profile of the protein. The rate of surface energy transfer between BSA and GNP s was also shown to be modulated as a function of pH thus again revealing changes in the protein conformation when conjugated to the nanoparticles. Localized surface plasmon resonance (LSPR) and surface enhanced Raman scattering (SERS) data have provided further details about the nature of interaction between BSA and GNP s. In spite of these previous reports, a proper insight into the interaction of proteins (here BSA) with nanoparticles is yet to be obtained. In particular, the effect of the concentration of gold nanoparticles on the conformation of BSA is an important aspect that needs to be considered since the ultimate aim is to use the former for medical applications wherein it is desirable to minimize the amount of metal particles for reduced toxicity issues.

Here we have therefore mainly emphasized on the interaction of BSA with very low concentration (sub-picomolar) of gold nanoparticles. An enhancement in Trp emission is observed for BSA in presence of ‘sub-picomolar’ concentration of gold nanoparticles. This enhancement of tryptophan (Trp residue) emission in presence of gold nanoparticles is either due to (a) conformational changes in protein induced by nanoparticles or (b) coupling of surface plasmon of nanoparticles to the emission of trp residues. Moreover, thermal and chemical denaturation profiles monitored through fluorescence circular dichroism techniques reveal very similar unfolding profiles of BSA in presence and absence of gold nanoparticles in this range of nanoparticle concentration, thus signifying little perturbation (if any) of the biomolecule.

**Experimental**

Hydrogen tetrachloroaurate(III) (HAuCl₄·3H₂O, Sigma Aldrich), bovine serum albumin (BSA, fatty acid free, Sigma Aldrich), trisodium citrate (SLS, India), urea (Sigma Aldrich), disodium hydrogen phosphate and monosodium dihydrogen phosphate were used without further purification. Absorbances of the protein solutions were recorded immediately after the solutions were prepared using a UV-vis spectrophotometer (Shimadzu UV-2450). Transmission electron microscopy (TEM) images of nanoparticles were taken using a FEI-TECNAI G² electron microscope operating at 200 kV. Intrinsic fluorescence emission from tryptophan (Trp residues) of BSA (excited at 295 nm) was measured with a Cary-Eclipse spectrofluorometer using a quartz cuvette of 10 mm path length at excitation and emission slit widths of 5 nm each. Circular dichroism (CD) spectra were obtained using a temperature controlled Jasco-J700 spectropolarimeter.

Hydrogen tetrachloroaurate(III) was reduced by trisodium citrate to obtain the gold nanoparticles. Hydrogen tetrachloroauric acid solution (29 µL of 0.0173 M) was added to 18.97 mL mili-Q water. The solution was heated at 90 °C for 20 min and then 1 mL of 1 % (w/v) trisodium citrate was added to it slowly with constant stirring. The yellow colour of HAuCl₄ disappeared and the solution became reddish purple. It was stored at 4 °C and used for further experiments.

The protein stock solution was prepared with 0.0165 g of BSA in 5 mL phosphate buffer (pH 7.0, 20 mM). The concentration of the solution was determined using the absorbance at 278 nm where the extinction coefficient (ε₂₇₈) of BSA was taken as 4.4 × 10⁴ M⁻¹ cm⁻¹ (ref. 15). For all the experiments, 139 µL of the protein stock solution was taken and the final volume of each sample was maintained exactly at 5 mL. Final concentration of the protein in each sample was 4.3 µM.

BSA stock solution (139 µL) was mixed with varying volumes of colloidal solution of Au nanoparticles (as mentioned above). Phosphate buffer (pH = 7.0) was added to each solution to make the final volume to 5 mL. All the solutions were stirred for 5 min and left overnight. No separation techniques were employed for removing excess protein to avoid any change in the original conformation of the protein. Various optical measurements were then carried out on the above solutions.

For studying the denaturation of the protein by urea, protein solutions were prepared with varying concentrations (0–8 M) of urea in phosphate buffer.

For circular dichroism measurements, the protein concentration was taken as 2.5 µM and 0.25 µM for thermal and chemical denaturation respectively, which takes into consideration the different path lengths (10 mm and 1 mm respectively) of the quartz cuvettes. Thermal denaturation studies were carried out by monitoring the ellipticity change at 222 nm as a function of temperature.
Results and discussion

The TEM images of Au-nps (Fig. 1a) shows the formation of uniform spherical particles with an average diameter of 15 nm. The absorption spectra of the Au-nps in absence and presence of BSA shows an SPR band with the maximum at 519 nm and 522.5 nm respectively (Fig. 1b). Thus on conjugation to BSA, the SPR band undergoes a red shift of 3.5 nm; this shift can therefore be taken as a signature of the protein interacting with the nanoparticles.

To further probe the local structural changes, the intrinsic fluorescence of the protein was also monitored with varying concentrations of Au-nps. BSA has two tryptophan residues, i.e., Trp-213 located in a primarily hydrophobic environment of the protein, and, Trp-134 which is on the protein surface and remains exposed to the surrounding solvent. As shown in Fig. 2, there is a small but definite increase in the Trp emission. The Trp emission intensity reaches a maximum at $6.4 \times 10^4\text{ nM}$ Au-np concentration. These data (reproducibly obtained thrice) are quite surprising when compared with the results observed by other groups, wherein with progressive increase in Au-np concentration, a decrease in fluorescence was observed, the latter being attributed to quenching arising from energy transfer to the nanoparticles$^{16,17}$. However, it must be emphasized that in the present studies we have used a very low concentration of Au-nps ($6.4 \times 10^{-4}\text{ nM}$), while in all earlier studies a concentration of $10^{-2}\text{ nM}$ and above have been studied. We have also studied the trp emissions of another similar protein, human serum albumin (HSA) (Supplementary Data, Fig. S1) and pure tryptophan (Supplementary Data, Fig. S2) in presence of sub-picomolar gold nanoparticles, where we did not observe any change in the trp emission. The tryptophan residue (Trp 134) which is present in BSA is absent in HSA. It may be possible that Trp 134 contributes towards the emission enhancement in BSA. The increase in fluorescence at this low concentration of Au-nps may be explained by a conformational change leading to a more folded (more sequestered Trp) protein, especially the domain-I (where the Trp 134 resides) of BSA, which leads to enhanced interaction with nanoparticles.

Time-resolved studies show that the average lifetime of Trp in the BSA-np conjugates does not undergo a significant change with respect to that of BSA in buffer (Table 1). We observed that the maximum

Fig. 1 – TEM image of gold nanoparticles. (b) Absorbance spectra of gold nanoparticles and BSA-Au conjugates. [1, Au-np; 2, Au-np-BSA].

Fig. 2 – Fluorescence spectra of BSA in presence of varying concentrations of gold nanoparticles.
variation in the decay time ($\tau$) in presence of Au-nps is $\sim 0.16$ ns (2.9 %), which can be considered to lie within the error associated with such measurements.

To get an insight into global changes in conformation of BSA when conjugated with the nanoparticles, circular dichroism studies were also carried out. CD spectra of BSA (Fig. 3) show that there is a small stabilization of the native BSA as observed from the increase in helicity of BSA at very low concentration of the gold nanoparticles. The maximum helicity of BSA was observed in the presence of $3 \times 10^{-5}$ nM gold nanoparticles, that is, the lowest concentration of nanoparticles used in this study (Fig. 3). Further increase in the concentration of nanoparticles led to a minor decrease in the helicity and the final CD signal almost superimposed with that of the protein in buffer thereby signifying very similar secondary structural content among the samples. A previous study\(^{16}\) also showed decrease in helicity of BSA (10 µM) conjugated with Au-nps (0.124 µM) which is consistent with our results involving higher concentration of Au-nps.

Chemical denaturation of BSA conjugated to GNPs was carried out in presence of different concentrations of urea and the resultant changes were monitored using fluorescence and CD spectroscopy. Urea weakens the hydrophobic interactions by perturbing the water structure\(^{18}\). This facilitates the solvation of hydrophobic residues which stabilizes the protein in its unfolded state. As protein unfolding takes place, Trp residues get exposed to the solvent (i.e. water) which results in a decrease in the quantum yield of Trp residues. Urea induced denaturation profile of BSA studied by Trp fluorescence emission (Fig. 4a) shows a similar trend as reported earlier\(^8, 19\). In presence of Au-NPs ($6.4 \times 10^{-4}$ nM) there is no significant variation in the denaturation profile. Circular dichroism and emission studies both corroborate similar behaviour (Fig. 4a & 4b) in presence and absence of gold nanoparticles. In earlier

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<th>$a_2$</th>
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Fig. 3 – Circular dichroism spectra of BSA (2.5 µM) with varying concentrations of gold nanoparticles in the wavelength range of 190 – 250 nm.

Fig. 4 – (a) Normalized fluorescence spectra, and, (b) circular dichroism spectra for the chemical denaturation of BSA by urea in presence and absence of gold nanoparticles. [1, BSA; 2, BSA-Au-np].
studies, higher concentrations of nanoparticles have been used where a significant variation in the denaturation profile was observed. At sub-picomolar concentration of gold nanoparticles, the overall trend of the conformational transition during the denaturation process, when protein was attached to the surface of gold nanoparticles, is quite similar to that of the bare protein.

We have also studied the thermal denaturation of BSA by comparing the Trp emission (at maxima) and the minima in the CD spectra at 222 nm. From the intrinsic Trp emission data (Fig. 5a) it is observed that thermal denaturation of BSA followed the same trend in presence of gold nanoparticles with respect to a solution of BSA in buffer. Here also a very small enhancement in the Trp emission is observed in presence of gold nanoparticles at each temperature up to 65 °C where BSA is in a totally unfolded state. Beyond 65 °C, the denaturation profile in presence and absence of gold nanoparticles overlap with each other. This implies that beyond 65 °C, there is no significant interaction between BSA and gold nanoparticles. Both fluorescence (Fig. 5a) and CD (Fig. 5b) data show that there is no variation in conformation and denaturation profile of BSA in presence of gold nanoparticles of sub-picomolar concentration. With increasing concentration of gold nanoparticles to $3 \times 10^{-2}$ nM, while the helicity of BSA-gold nanoparticle conjugates decreased, it follows the same denaturation profile.

In the present study, sub-nanomolar concentrations of gold nanoparticles have been found to have some visible effects on the conformation of BSA. A small but definite enhancement in the trp emission in presence of sub-picomolar gold nanoparticles has been observed. While the reason for such an enhancement is not quite clear at present, however such an enhancement shows great promise in particular for the Trp-containing proteins. Moreover, at this concentration, the overall denaturation profile is quite similar (in presence and absence of nps) and hence we can conclude that the protein conformation remains essentially conserved. This has important implications for gold nanoparticle based protein immobilization and conjugate based devices.

**Supplementary data**

Supplementary Data associated with this article, i.e., Figs S1 and S2, are available in the electronic form at http://www.niscair.res.in/jinfo/ijca/IJCA_51A(10)1561-1566_SupplData.pdf.

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**References**