

SPECTROFLUOROMETRIC STUDIES OF THE EFFECT OF ZnO NANOPARTICLES ON BOVINE SERUM ALBUMIN

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Abstract— The study of protein nanoparticle interaction is very important for designing and fabricating nano composites which have potential medical applications. Bovine serum albumin (BSA), a major water soluble protein has wide range of physiological functions. In biomedical applications nanostructured metal oxides such as ZnO, TiO₂ have drawn great attention for further studies as they are very safe to human beings and also for animals. Therefore, the interaction between BSA and ZnO nanoparticles (nps) and the effect of ZnO nps on the conformation of BSA has been analyzed by various spectroscopic techniques. The BSA-ZnO np conjugate made significant changes in BSA fluorescence emission parameters and thus confirmed the conformational changes.

Index terms- Bovine serum albumin, ZnO nanoparticles, fluorescence, conformational changes.

I. INTRODUCTION

Nanotechnology is an enormously powerful technology, which holds a huge promise for the design and development of many types of novel products with its potential medical applications on early disease detection, treatment and prevention (Mc Bain et al. 2008). Nanoparticles with size of 1 to 100 nm have potential applications in bioimaging (Tan et al. 2004), drug delivery (Olson et al. 1996) and cancer therapeutics (Schubert 2006). There are only few nps such as ZnO, silver and gold known to us which are less toxic and biocompatible having excellent tuned luminescence property that can be used in several biomedical and pharmaceutical applications for mankind (Jiang et al. 2005). Proteins are the essential macromolecules for controlling most of the biological process inside the cell. Albumin is an abundant heart shaped globular protein found in many mammals such as humans, cattle, rats and mice (Carter et al. 1994). BSA, a major water soluble protein has wide range of physiological functions such as delivery of fatty acids, binding, transport etc. and consists of three intrinsic fluorophores: tryptophan (trp), tyrosine (tyr) and phenylalanine (phy). The major contribution of intrinsic fluorescence of albumin molecules is due to trp residues, with only a minor contribution by the numerous tyr depending on the excitation wavelength selected. BSA is an ideal protein for intrinsic fluorescence measurement due to the presence of two

intrinsic trp residues: trp- 134 in the first domain located on the surface of molecule, which can be quenched and trp- 212 in the second domain, located within a hydrophobic binding pocket (Jung et al. 2006). Since trp is highly sensitive to its local environment it can be used to observe changes in the fluorescence emission spectra due to protein conformational changes, binding to substrates and denaturation (Lakowicz 1999).

When biological molecules are integrated with nps, the natural biomolecule functions such as catalysis, recognition and electron transfer combine with the unique electronic, optical and catalytic properties of nps arising from their reduced dimension and high surface to volume ratio (Niemeyer et al. 2001). Due to their relatively large surface area nps have significant adsorption capacities therefore they are able to bind or carry other molecules such as chemical compounds, drugs, probes and proteins attached to the surface by covalent bonds or by adsorption. Hence by attaching specific chemical compounds, peptides or proteins to the surface the physicochemical properties of nps such as charge and hydrophobicity can be altered. The functionality of nps is thus enhanced or changed (De Jong et al. 2008). The native conformation and therefore the protein function can be easily changed by these interactions with the surface. Thus stabilization of the system as well as introduction of biocompatible functionalities into these nps for further biological interactions or coupling takes place by conjugation of protein with nps. It was reported that proteins undergo some structural changes at the boundary surface of nps (Roach et al. 2006). The interaction of BSA with metal/metal oxide nps such as Ag (Mariam et al. 2011), Au (Pramanik et al. 2008), Al₂O₃ (Rajeshwari et al. 2014) and TiO₂ (Togashi et al. 2007) was reported by many researchers.

Fluorescence spectroscopy is the most sensitive and one of the most versatile of the many techniques available for studying the structure and dynamics of macromolecules. This is due to high sensitivity of various fluorescence parameters such as fluorescence spectrum, fluorescence intensity and lifetime of trp residues to their microenvironment and to the peculiarities of their location in protein macromolecule. Since fluorescence measurements appear to be more sensitive to molecular environment than many other physical methods they

may even indicate small structural transitions of proteins. A shift in emission maximum, change in fluorescence intensity or change in lifetime gives ample evidence to indicate conformational changes in the protein molecule (Mariana et al. 2012; Togashi et al. 2007; Ojha et al. 2012). Therefore fluorescence spectroscopy plays a crucial role in the study of interactions between nps and the serum albumins.

In various biomedical applications particularly drug delivery or receptor targeting the conformational behavior of albumin on conjugation with nps is of great importance. However it is not very clear about the changes in the structure of protein after conjugation with nps hence some basic understanding is required before such complexes are used for biomedical applications. BSA readily undergoes conformational changes hence BSA provides a good model for investigating the effect of nanoparticles on protein conformational change. Also, the structure and property of BSA are well characterized and due to its major physiological significance, unusual ligand binding properties, high purification, stability in biochemical reactions and its soluble nature in water BSA has been taken as a model protein in this study (Ravindran et al. 2010). ZnO is a multifunctional wide and direct band gap semiconductor with excellent size dependent tunable optical property which is of great interest in the np based drug delivery, bioimaging and biomedical research (Dutta et al. 2012). Therefore, in the present work the interaction between BSA protein and ZnO nps was studied by using various spectroscopic techniques.

II. MATERIALS AND METHODS

A. Chemicals

BSA and ZnO (<50 nm) nps were purchased from Sigma-Aldrich, USA. The samples were used as received without any further purification. Double distilled water was used for the interaction between BSA and ZnO nps.

Stock preparation of BSA and ZnO nps

BSA stock solution was prepared by dissolving 100 mg of BSA in 10 ml double distilled water and it was used for further studies. Similarly, stock solution of ZnO np was prepared by dissolving 0.016 g of ZnO in 20 ml double distilled water. This dispersion was used further for the interaction studies.

Interaction of BSA with ZnO nps

BSA was interacted with various concentrations of ZnO nps. This mixture was homogenized and kept for 30 min for incubation. All measurements were recorded after 30 min of incubation required for the interaction between ZnO nps and BSA. 3 ml of the sample was subjected to analysis at the excitation wavelength of 290 nm and emission spectra in the range of 310-450 nm. All measurements were performed at room temperature.

B. Characterization

Steady state fluorescence measurements

The fluorescence measurements were carried out with JASCO FP-8600 spectrofluorometer. The excitation wavelength of BSA was 290 nm. The excitation slit width was 2.5 nm, emission slit width 2 nm and scan rate 500 nm/min

were maintained constant for all measurements. The quartz cuvette (4 cm x 1 cm x 1 cm) with path length of 1 cm was used.

C. Time resolved fluorescence measurements

Fluorescence lifetime measurements were carried out in a picosecond time correlated single photon counting (TCSPC) spectrometer. The excitation source is the tunable Ti-sapphire laser (Tsunami, Spectra Physics, USA).

III. RESULTS AND DISCUSSION

Nowadays there is considerable increase in the usage of nps and other nanomaterials in biomedical applications. Before development of any therapeutic and diagnostic applications it is important to understand the properties of biomolecule bound nanoparticle (Tokonami et al. 2008). Fluorescence spectroscopy is sensitive to protein dynamics because the excited fluorescent state persists for nanoseconds, which is exactly the timescale of many important biological processes such as the rotational motion of protein side chains, molecular binding and protein conformational changes (Shang et al. 2007). Np-protein binding can be monitored by steady-state or time resolved fluorescence spectroscopy.

Steady- state fluorescence analysis

In order to evaluate the conformational changes around trp residues the intrinsic fluorescence spectra for native BSA and BSA-ZnO np complex were analyzed at the excitation wavelength of 290 nm (Fig.1). When excited at 290-295 nm the emission of proteins is generally dominated by the trp fluorescence. Similar excitation wavelength 290 nm was reported earlier specifically on the interaction of BSA with metal/metal oxide nps (Zhao et al. 2012, Bhunia et al. 2013). The emission maximum of BSA is at 345 nm when excited with 290 nm wavelength. Several previous works also have reported emission band at the same wavelength for BSA (Togashi et al. 2007). Fluorescence spectra of ZnO nps attached with BSA were found to be different than in the native BSA (Fig.1).

After the addition of ZnO nps fluorescence intensity of BSA increased for all the three concentrations of ZnO nps. The maximum increase in fluorescence intensity was observed for the lowest concentration of ZnO nps. But when the concentration of ZnO nps increased a gradual decrease in fluorescence intensity was observed compared to that of BSA-ZnO complex at lowest concentration of ZnO nps. This clearly indicates that the fluorescence intensity increase of BSA is due to lower concentrations of ZnO nps and at higher concentrations of ZnO nps fluorescence of native BSA can be quenched. The fluorescence intensity increase at low concentrations of ZnO nps may be due to conformational changes leading to a more folded protein aroused from BSA-ZnO nps interaction. The change in fluorescence intensity is due to change in local environment around the trp residues (Lakowicz 1983). This suggests that ZnO nps change the native state of BSA and altering the microenvironment around the trp residues. Similar results were obtained when BSA was interacted with Au nps. They emphasized that the increase in fluorescence intensity may be due to lower concentrations of Au nps in BSA (Ojha et al. 2012). An increase in fluorescence

intensity occurred at lower concentrations of Al_2O_3 nps when interacted with BSA (Rajeshwari et al. 2014).

A 3 nm blue shift in emission maximum was observed by the addition of increasing concentrations of ZnO nps indicates a change of the microenvironment polarity around the trp residues and they were exposed to a hydrophobic environment. The blue shift in the spectral maximum suggests a decrease in the polarity or an increase in the hydrophobicity of the microenvironment surrounding the fluorophore site (Kragh-Hansen et al. 2001). The significant blue shift in emission maximum indicates that polarity near trp residues is decreased or hydrophobicity is strengthened as a result of binding of ZnO nps to BSA.

This result is quite contradictory to the earlier studies in which it was shown that the fluorescence intensity of BSA was quenched with a shift in maximum of the emission peak (red/blue/no shift) with increasing concentrations of

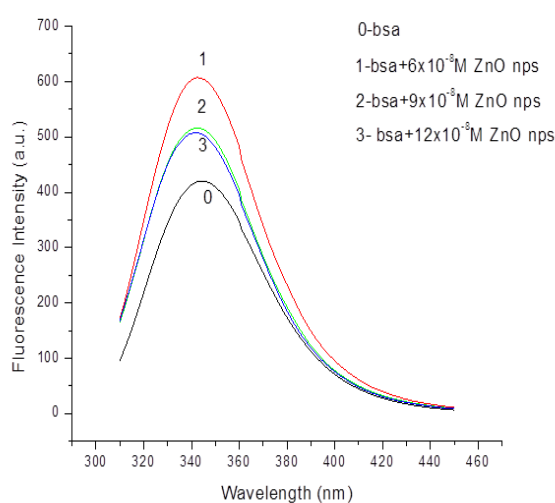


Fig. 1 Fluorescence spectra of BSA at different concentrations of ZnO nps (0, 6, 9 and 12×10^{-8} M)

ZnO nps (Bhunia et al. 2013), colloidal ZnO nps (Kathiravan et al. 2009 a), colloidal AgTiO_2 nps (Kathiravan et al. 2009 b) and colloidal capped CdS nps (Jhonsi et al. 2009). When carefully examined it was observed that in all these works either the np concentration was more (Kathiravan et al. 2009 b) or BSA concentration was less (Bhunia et al. 2013) compared to that of the present study. In some cases lower BSA concentration with higher np concentration was used for the interaction studies (Kathiravan et al. 2009 a, Jhonsi et al. 2009) compared to that of the present study. Thus it is concluded that in the present study fluorescence quenching of native BSA was not observed because of the use of either lower concentrations of ZnO nps or higher concentrations of BSA. No change in fluorescence was observed when Al_2O_3 nps interacted with human plasma proteins (Rosana et al. 2014). Increased gold np concentration in BSA resulted in decrease in fluorescence intensity due to quenching arising from energy transfer to the nps (Shang et al. 2007). Quenching of the intrinsic fluorescence was reported in case of *Bacillus amyloliquefaciens* α -amylase by tin oxide nps (Khan et al. 2011).

Time resolved fluorescence analysis

The fluorescence lifetimes of BSA in the absence and presence of ZnO nps were measured. The exponential decay curves of BSA and BSA with different concentrations of ZnO nps are shown in fig.2. The fluorescence decays of BSA were fitted by two exponential with $T_1=7.0$ ns and $T_2=3.56$ ns. The two lifetimes indicated that BSA contained two trp residues that fluoresced in two different environments (Johansson et al. 1997). This result is consistent with the studies in which it was shown that the lifetimes of trp fluorescence are rather short and often multi exponential (Sarkar et al. 2011). The significant changes between the two lifetimes indicated that one of the trp residues in the protein may be relatively exposed whereas the other trp residue appears to be deeply buried inside the protein (Ghiron et al. 1988).

The time resolved fluorescence studies showed that the lifetime of both trp residues in BSA decreased with the lowest ZnO np concentration. The fluorescence lifetime of BSA decreased for lifetime T_1 from 7.00 ns to 6.56 ns and for lifetime T_2 from 3.56 ns to 3.06 ns upon interaction with the lowest concentration of ZnO nps. But on further increasing the ZnO np concentrations in BSA the lifetimes of both trp residues does not undergo any significant change with respect to that of BSA with lowest concentration of ZnO nps (Fig.2 & Table 1). Also, from steady state fluorescence analysis in the present study a decrease in fluorescence intensity was observed at higher concentrations of ZnO nps compared to that of BSA with lowest concentration of ZnO nps. As reported by Lakowicz (1999) for a static quenching the complex formation will not disturb the fluorescence lifetime of BSA. However, the fluorescence lifetime will be cut down due to the collision between the excited protein fluorophore and nps in a dynamic quenching procedure. Therefore, in this case, the consistent fluorescence lifetime of both trp residues of BSA after binding with ZnO nps indicated static quenching process. Similar results were obtained by time resolved fluorescence studies in which it was shown that when BSA interacted with starch capped CdS (SCdS) nps the decay curve changed from mono to biexponential with two lifetimes 5.27 and 2.02 ns respectively from a lifetime of 6 ns in the absence of SCdS nps. But further increasing the concentration of SCdS nps the fluorescence lifetime of BSA remains unaltered (Jhonsi et al. 2009). When BSA was interacted with SnO_2 nps the fluorescence lifetimes of BSA ($T_1=6.8$ ns and $T_2=3.5$ ns) did not get affected by time resolved fluorescence analysis (Togashi et al. 2007).

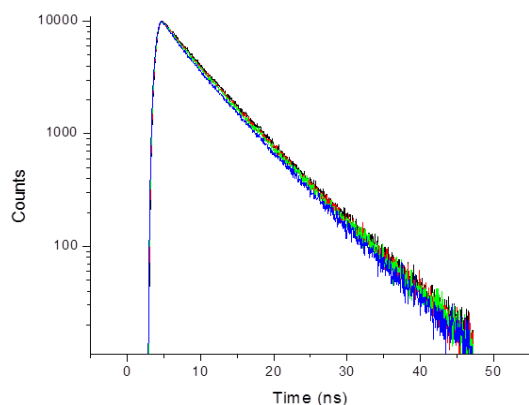


Fig. 2 Time resolved fluorescence decay of BSA at different concentrations of ZnO nps (0,6,9 and 12 x 10⁻⁸ M)

By time resolved measurements static quenching mechanism was confirmed when BSA interacted with colloidal ZnO nps (Kathiravan et al. 2009 a), TiO₂ nps (Kathiravan et al. 2008), SnO₂ nps (Togashi et al. 2007), colloidal AgTiO₂ nps (Kathiravan et al. 2009 b) and colloidal capped CdS nps (Jhonsi et al.2009). Time resolved studies showed that the average lifetime of trp in the BSA-gold np conjugate does not undergo any significant change with respect to that of native BSA (Mandal et al. 2010; Ojha et al. 2012). Time resolved fluorescence measurements showed that consistent dynamic quenching occurred when BSA interacted with TiO₂ nps (Togashi et al. 2007) and silver nps (Mariana et al. 2012).

Monitoring conformational changes of ZnO np-bound BSA

The study of protein np conjugation will provide us with the information of the phenomena occurring at the protein np-interface at the molecular level as well as the information about conformational changes of protein occurring at the protein-np interface. Proteins undergo varying degree of conformational changes in the presence of nps. Conformational changes in proteins may disturb the microenvironment around the trp residues and thus influence the fluorescence emission. Therefore the trp fluorescence is widely used to monitor conformational changes in proteins (Ojha et al. 2010). Depending on the hydrophobicity of the surrounding of a trp residue, the center of its emission band can vary over several tens of nanometers as well as its quantum yield and lifetime changes. Therefore steady state spectroscopic studies even with proteins containing several trp residues can provide insight into conformational changes upon substrate binding or other catalytic processes.

The interaction of BSA with different concentrations of ZnO nps resulted in an increase in fluorescence intensity for all the three concentrations. The maximum fluorescence intensity increase was observed for the lowest concentration of ZnO nps. On further increasing the concentrations of ZnO nps a quenching in fluorescence of BSA was observed. Also a blue shift in the fluorescence emission maximum was observed. When the proteins bind to other substances or the conformation of protein is changed, the corresponding fluorescence intensity and/ or the wavelength of the maximum peak of the protein will generate some alterations (Soares et al. 2007). Therefore this behavior means that the conformation around the trp

residues of the BSA- ZnO np complex changes as compared to that of the native BSA. The lifetime of both the trp residues in BSA reduced for the lowest concentration of ZnO nps but on further increasing the concentrations of ZnO nps in BSA there was no change in both the lifetimes of the trp residues in BSA. In the present study the significant blue shifts in the fluorescence emission maximum and the significant decrease in fluorescence intensity (fig. 1, fig.2 and table 1) implies the probable conformational changes induced by ZnO nps on BSA. Spectroscopic investigations showed conformational changes when BSA interacted with silver nps (Mariam et al. 2011), gold nps (Ojha et al.2012) TiO₂ nps (Kathiravan et al. 2008), ZnO nps (46 Bhogale et al. 2013), colloidal Ag TiO₂ nps (Kathiravan et al. 2009 b), and C60 fullerene nps (47 Liu et al. 2012).

Table 1. The wavelength of emission maximum, corresponding fluorescence intensity and lifetimes of BSA and BSA with different concentrations of ZnO nps

Sample	Emission	Fluorescence	Lifetime	
	Maximum(nm)	Intensity (A.U.)	T ₁ (ns)	T ₂ (ns)
BSA	345	420.32	7.00	3.56
BSA+ 6x10 ⁻⁸ M ZnO nps	342	607.18	6.53	3.06
BSA+9x10 ⁻⁸ M ZnO nps	343	515.90	6.54	3.04
BSA+12x10 ⁻⁸ M ZnO nps	342	507.56	6.44	2.90

IV. CONCLUSION

In the present study the interaction between ZnO nps and BSA has been studied by various spectroscopic techniques. The increase in fluorescence intensity and blue shift of fluorescence emission peak of ZnO nps- BSA conjugated system confirms that the microenvironment close to trp residues of BSA is perturbed. In addition the hydrophobicity of both trp residues increased in the presence of ZnO nps. The analysis of lifetime measurements indicated that lifetime of both trp residues in BSA decreased at lowest concentration and then remains constant while increasing ZnO np concentrations in BSA. The decrease in fluorescence intensity with constant lifetimes indicated that static quenching process occurred at higher concentrations of ZnO nps in BSA. The two lifetimes indicated that BSA contained two trp residues that fluoresced in two different environments. The significant changes between the two lifetimes indicated that one of the trp residues may be relatively exposed whereas the other trp residue appears to be deeply buried inside the protein. The results presented clearly showed that ZnO nps induced conformational changes in the structure of BSA.

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