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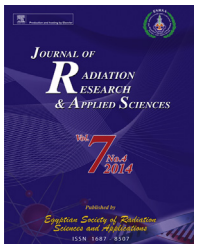

UV radiation sensitivity of bovine serum albumin bound to silver nanoparticles

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ABSTRACT

We report here the UV radiation sensitivity of Bovine Serum Albumin (BSA) bound silver nanoparticles (62 nm diameter) to various power density between 468 mJ/cm^2 to 1872 mJ/cm^2 under physiological conditions. The functional properties associated with BSA such as esterase activity, free thiols and copper ion binding have been studied. Decrease in free thiols, with increase in copper ion binding and P- nitrophenyl acetate (PNPA) turnover were observed in BSA bound silver nanoparticles (SNP) in the presence of UV radiation. Intrinsic fluorescence intensity of BSA bound SNP was decreased with UV radiation. Circular Dichroism results indicated a decrease in alpha helical content of BSA bound SNP. The overall results suggest modifications in structure–function properties of BSA bound to SNP in the presence of UV radiation. The possible mechanisms of interaction between BSA and SNP have been explained in presence of UV radiation.

1. Introduction

Understanding the mechanism of radiation induced damages either X-ray or UV radiation, in biological systems is of prime importance in radiation biology (Durchshlag, Hefferle, & Zipper, 2003). Radiation induced damage especially in presence of chemicals, are considered important due to the differential effects of radiation over them tumour and normal tissue. These differential effects can be achieved by effective chemical agents that specifically damage the tumour and spare normal tissue. Numerous investigators are taking efforts in this regard to develop chemical agents which could serve as efficient radiosensitizer or radioprotector. A large number of compounds have been identified as radiosensitizer or radioprotectos, and some of them have been under clinical trials. Proteins are important biomacromolecules that are sensitive to ionizing radiation as well as UV radiation (Boulton, Cleary, Papworth, & Plumb, 2001; Burke & Augenstein, 1969; Davies, 2003). Radiation induced alterations of molecular properties of proteins like breaking of hydrogen/covalent bonds, fragmentation, inactivation has been reported by several investigators (Cho & Song, 2000; Moon & Song, 2001). The hydroxyl and superoxide anion radicals generated by radiation which modify the primary structure of protein results in distortion of secondary and tertiary structure (Davis & Delsignore, 1987). Conformational changes in protein induced by UV, X-ray and γ- radiation have been reported in several studies (Durchshlag et al. 2003; Lee & Song, 2002).

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Recently nanoparticles have greatly attracted the attention of many investigators due to their unique physico-chemical properties and potential application in biology and medicine (Salata, 2004). Amongs these, silver nanoparticles (SNP) are being developed in the field of nanomedicine for treating various diseases (Couvreur & Vauthier, 2006). Expérimental studies had shown that SNP showed strong action against microbes (Ahmad, Wani, Manzoor, Ahmed, & Asiri, 2013; Dongre et al. 2010; Kim et al. 2007; Sukumaran & Poulose, 2012) viruses (Elechiguerra et al. 2005; Galdiero et al. 2011; Lara, Ayala-Nuñez, Ixtepan-Turrent, & Rodriguez-Padilla, 2010) and fungus (Jo, Kim, & Jung, 2009; Nasrollahi, Pourshamsian, Mansourkiaee, 2011; Panacek et al. 2009). SNPs are used in toothpaste, face creams, soaps and disinfectants because of their antimicrobial properties (Salata, 2004). A recent study had shown that SNP induced the antioxidants properties of some enzymes which reduced reactive oxygen species (Sharma et al. 2012). There are few reports are available on modification of radiation effect by SNP. Radiosensitizing effects of SNP have been reported on malignant glioma cells in vitro (Liu et al. 2013). Zheng et al. has used SNP alone for radiation therapy in cancer (Zheng, Yang, Wei, Tong, & Shu, 2013). The effectiveness in cancer due to shape of SNP have been studied (Boca et al. 2011). In contrast to this, radioprotecting property of SNP revealed when it is complexed with a phytoceutical Glyzyrrhizic acid (Chandrasekharan, Khanna, & Nair, 2011) and an antioxidant alpha-lipoic acid (Ramachandran and Nair 2011).

In the present study, attempts have been made to understand the UV radiation sensitivity of bovine serum albumin bound silver nanoparticles. BSA is the model of choice because it is the most abundant plasma protein (Carter, Chang, Ho, & Krishnaswami, 1994; Ziegler & Foegeding, 1990) has simple structure, small molecular weight and only one polypeptide chain. BSA is a carrier of fatty acids, amino acids, metals and drugs and has a great affinity for fatty acids, hematin and bilirubin etc. (Curry, Mandelkow, Brick, & Franks, 1998; Reed, 1997).

2. Material and methods

All the chemicals used were of an analytical grade, double distilled water used throughout the experiment. BSA and 5, 5 – Dithiobis (2- nitrobenzoic acid), silver nitrate, p-nitrophenol acetate (PNPA), EDTA, sodium ascorbate, copper sulphate and bathocuproine disulphonic acid (BCS) were used.

2.1. Synthesis and characterization of silver nanoparticles

SNP_S were synthesized by chemical reduction method. The method was slightly modified form of Lee and Meisel (Lee & Meisel, 1982). One millimole solution of silver nitrate heated on a hot plate with magnetic stirrer up to 80 °C, 1% sodium citrate solution was added drop by drop. The colourless solution turned pale yellow, which indicating the formation of SNP. SNPs were characterized by UV visible (Fig 1) and dynamic light scattering method. The average size of SNPs was approximate 62 nm.

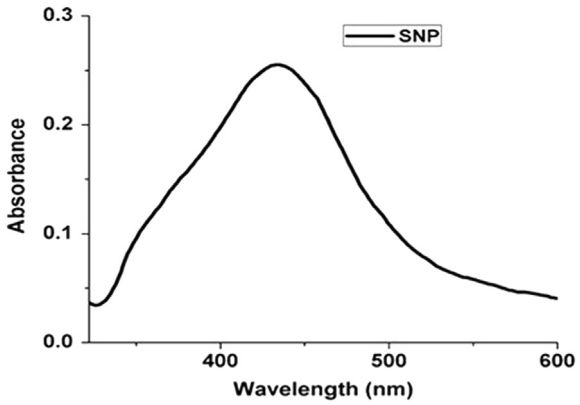

Fig. 1 – Absorption spectrum of silver nanopart.

2.2. UV radiation source

Fifteen Watts of UV radiation source was used and the energy of radiation measured by ILT 77 Germicidal radiometer (International Light Technologies, Inc.). The samples were exposed for 30 min (468 mJ/cm^2), 60 min (936 mJ/cm^2), 90 min (1404 mJ/cm^2) and 120 min (1872 mJ/cm^2).

2.3. Structural and functional assays of BSA

Fluorescence emission intensity of irradiated and non irradiated BSA (1 mg/ml) was monitored by using fluorescence spectrophotometer (VARIAN, Cary Eclipse, Netherland). The samples were excited at 280 nm. The emission spectra recorded at the range 290–500 nm in phosphate buffer (5 mM) at pH 7.4. A circular Dichroism (CD) spectrum of BSA was recorded at 190–250 nm (Jasco J-815 Spectropolarimeter). Samples were prepared in 5 mM solution of phosphate buffer at pH 7.4.

Esterase activity was measured by hydrolysis of P-nitrophenyl acetate (500 mM) catalyzed by BSA(1 mg/ml) in presence and absence of silver nanoparticles(1 mM) alone as well as after UV radiation by monitoring the formation of p-nitrophenol at 400 nm on nanophotometer.

Ellman's assay was used for the estimation of free sulfhydryl groups in bovine serum albumin. Ellman's reagent was dissolved (4 mg/ml) in sodium phosphate buffer (0.1 M) containing 1 mM EDTA at pH 8.0. Thiol groups were measured by making reaction system containing 2.5 ml of reaction buffer, 250 μl sample and 50 μl Ellman's reagent. The system was incubated at room temperature for 15 min. The absorption was measured at 412 nm. The Free thiol concentration was calculated interpolating the absorbance at 412 nm with the help of calibration curve. Results have been expressed as number of free thiol groups per mole of BSA.

Copper ion binding to BSA was measured spectrophotometrically by using bathocuproine disulphonic acid (BCS). BSA alone and with SNP was prepared in 0.15 M NaCl and incubated with 10 μM of $CuSO_4$ for 2 h. Both the samples were irradiated by UV radiation at 30, 60, 90, 120 min. After irradiation, protein was dialyzed overnight against 0.15 M NaCl. The reagent BCS was (400 μM) incubated at room temperature for

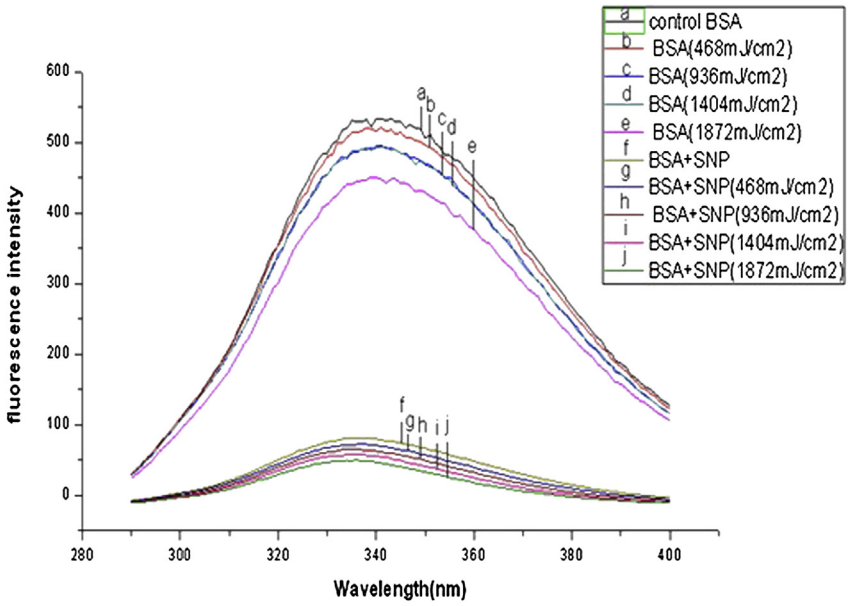

Fig. 2 – Effect of UV radiation on fluorescence intensity of BSA bound to SNP.

5 min and absorption was measured at 480 nm. The amount of copper ion bound was calculated with the help of calibration curve.

3. Results and discussion

The surface plasmon resonance band in UV visible spectra at peak 422 nm clearly indicated the formation of colloidal silver nanoparticles (Fig. 1). The average size of silver nanoparticles was found to be 62 nm which was determined by dynamic light scattering.

3.1. *Fluorescence, Circular Dichroism and UV visible study*

Fluorescence spectroscopy is one of the important biophysical techniques which reveals the ligand-drug binding/protein–-protein interactions quantitatively (Alarcón, Aspée, Abuin, & Lissi, 2012; Avis & Nilapwar, 2013; Van de Weert, Stella, 2011; Yan & Marriott, 2003). Circular dichroism (CD) is another powerful technique which throws light exclusively on alpha helicity of the proteins. Intrinsic fluorescence characteristic of BSA is due to amino acid residues such as tryptophan, phenylalanine and tyrosine which emit fluorescence light

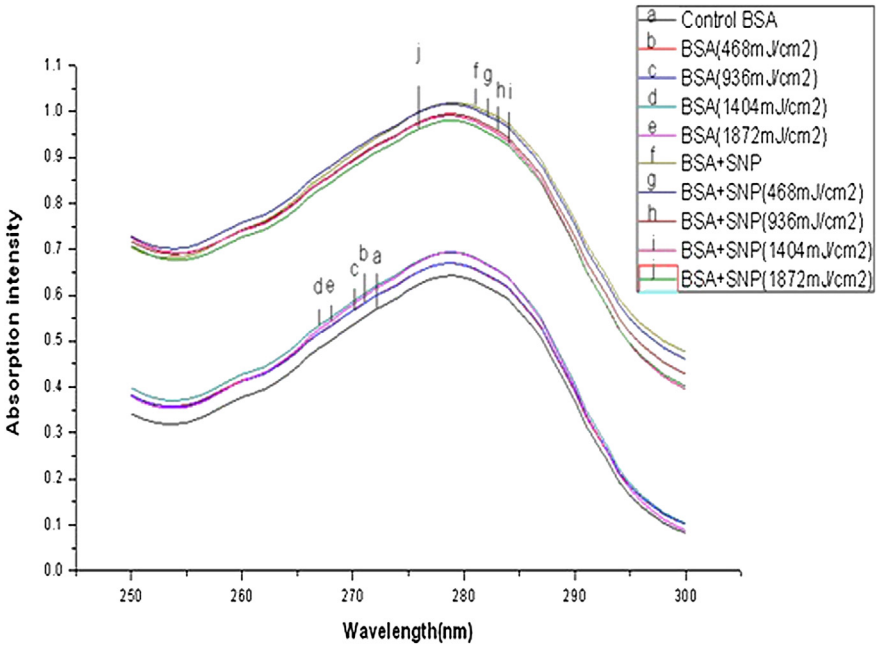

Fig. 3 – Effect of UV radiation on absorption of BSA bound to SNP.

when excited at 280 nm, however tryptophan is the most dominant amino acid (Lakowicz, 2006).

In the present study, a decrease in fluorescence intensity (Fig. 2), increase in UV light absorption (Fig. 3) and changes in CD spectra (Fig. 4) of BSA were observed in presence of SNP. These observations strongly suggest that there is an interaction between BSA and SNP. Interaction between BSA and SNP have been reported in several studies such as changes in alpha helical structure during adsorption of protein on NP surface to a certain extent (Selvakannan et al. 2004). Silver nanoparticles increases the amount of helix and decrease the beta sheet structure which lead to loosening of the protein skeleton (Rutao et al. 2009). X-ray diffraction study revealed complete coverage of SNP by BSA (Ravindran, Singh, Raichur, Chandrasekaran, & Mukherjee, 2010), Thermodynamic and fluorescence studies indicated that hydrophobic forces are prominent in SNP-BSA complex (Mariam, Dongre, & Kothari, 2011; Ravindran et al. 2010); however stabilization of secondary structure of BSA by SNP is reported by Ghosh, Jana, and Guchhait (2012). The increase in absorption intensity observed when BSA was incubated with silver nanoparticles could be attributed to the conformational changes of BSA due to binding with silver nanoparticles. It is due to the change in microenvironment around the aromatic amino acids which results in exposure of these residues. Similar results have been reported for interaction of colloidal silver nanoparticles with BSA (Lee & Song, 2002).

The CD spectrum of BSA exhibits negative bands at 208 and 222 nm (Fig. 4) which is characteristic of high α helical content (Dockal, Carter, & Ruker, 2000; Manavalan & Johnson, 1983; Price, 2000). Adsorption of BSA on SNP results in increase in negative bands at 208 and 222 nm. The far- UV CD data was taken in range of 190–250 nm. Result showed that upon interaction of BSA with SNP, α helical content of BSA decreases. Induction of more loose conformation of BSA with extended polypeptide chain may be indicated. The conformational transition may be due to exposure of hydrophobic areas to hydrophilic environment.

The SNPs and UV irradiation quenched the fluorescence of BSA with respect to UV radiation density and SNP concentration (Fig. 2). The fluorescence intensity of BSA sharply decreases with a blue shift around 4–5 nm in the presence of SNP, however fluorescence intensity decreases with UV radiaiton but no blue shift takes place. The decrease intensity is related to the quenching. The quenching could be due to energy transfer, ground state complex formation, and molecular rearrangement. This is due to interaction between BSA and SNP (Mariam et al. 2011). When protein is exposed to the wavelength of 280 nm, mainly the tryptophan and tyrosine residues of protein molecules get excited, which would reflect upon the tertiary structure of protein. UV irradiation caused a decrease in emission intensity (Fig. 2), due to change in local environment around the tryptophan and tyrosine residues. This could be attributed to change in tertiary structure of protein.

The absorption intensity of BSA decreased with increase of UV radiation density (Fig. 3). There was negligible shift in absorption maxima wavelength at different density of UV radiation. The change in absorption of BSA could be due to the minor structural and conformational changes in BSA. However there was a significant decrease in UV absorption of BSA when irradiated in presence of SNP. This shows that there could be a gross change in structural aspects.

UV irradiation of BSA affected the CD spectrum (Fig. 4). It increased the ellipticity value at 208 and 222 nm which indicates the decrease in ordered structure of protein. UV irradiation may be destabilizing α helical structure (Cho & Song, 2000). Similar results have been reported on the molecular

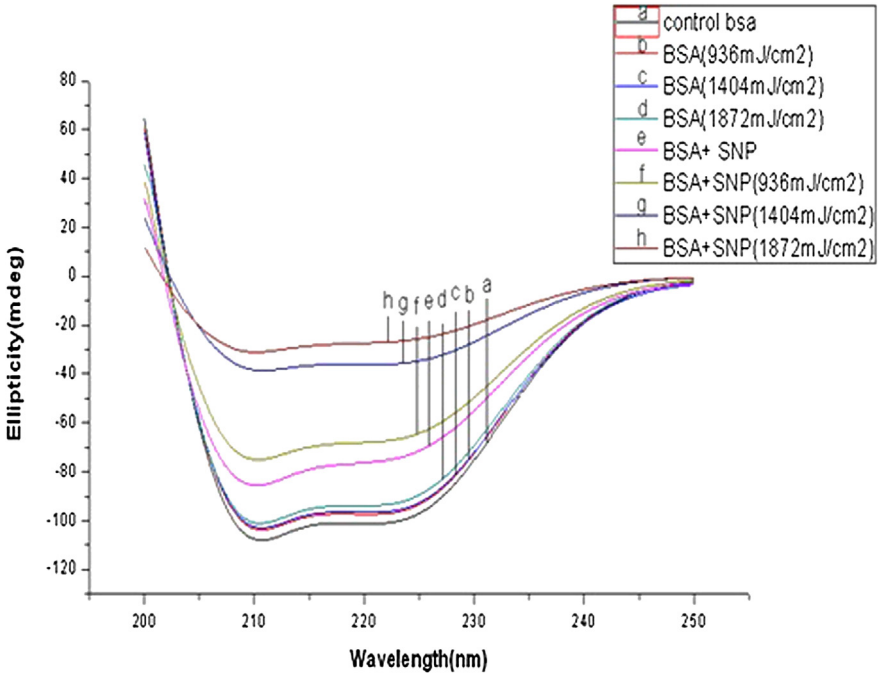

Fig. 4 – Effect of UV radiation on α- helicity of BSA bound to SNP.

properties of ovalbumin and ovomucoid when they are exposed to gamma radiation (Cho & Song, 2000). When BSA was irradiated in presence of SNP, the ellipticity value is further increased at 208 and 222 nm. It shows that both UV radiation and silver nanoparticles destabilized the α helical structure of BSA. Decrease in the ordered structure of protein is more in presence of UV radiation and SNP together than SNP alone.

3.2. *Esterase activity and free thiol assays*

Physical and chemical properties of proteins are the most important for their biological activities. Upon UV irradiation BSA exhibited increased esterase activity and free thiols with increase in UV radiation density (Figs. 5 and 6). FT-IR microscopic studies revealed the alteration in secondary structures of alpha-crystallin in aqueous solution after UV-B irradiation and more pronounced changes after prolonged UV-B exposure (Lin, Ho, & Li, 1999). Changes in secondary structure upon UV-light exposure led to decrease in alpha-helical contents in prion protein (Thakur and Rao 2008). Earlier observations have shown that UVC-irradiation of albumin solutions caused conformational rearrangement and aggregation of the polypeptide chains (Michnik, Michalik, & Drzazga, 2008). The C-terminal fragment of BSA has been suggested to be the subject of the strongest action of UVC radiation (Michnik & Michalik, 2004), further UVC doses have been found to preserve the activity of protein causing minimal damage to the protein (Caillet-Fauquet et al. 2004; Wang et al. 2004).

The serum albumin exhibit an esterase activity which can hydrolyze p-Nitrophenyl acetate and convert it to p-nitrophenol (Awad-Elkarim & Means, 1988; Peters 1996). The reactivity of proteins with p-nitrophenol acetate is due to their nucleophilic amino acid side chain groups. This is due to acetylating of highly reactive tyrosine group at position −410 (Fig. 5). This observation related to the fact that tyrosine is negatively charged and SNP are positively charged (Dou, Jung, Cao, & Ozaki, 1999), hence there could be probability of electrostatic interaction between SNP and BSA molecules which cause the decrease in the concentration of p-nitrophenol formed. Irradiation of BSA to different density UV radiation (468 mJ/cm^2, 936 mJ/cm^2, 1404 mJ/cm^2, 1872 mJ/cm^2), increased the turnover of PNPA with increasing uv radiation density (Fig. 5). However, when SNP's are incubated to the aqueous solution of BSA and exposed to UV radiation at the

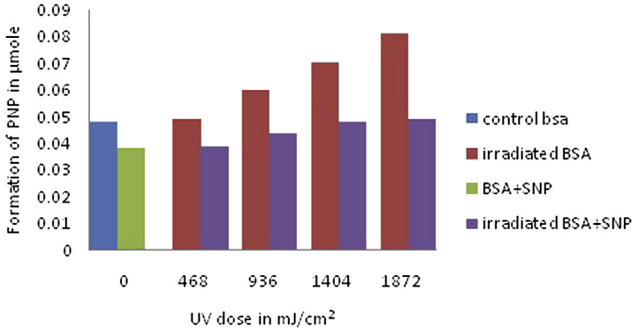

Fig. 5 – Esterase activity of BSA bound to SNP in presence of UV radiation.

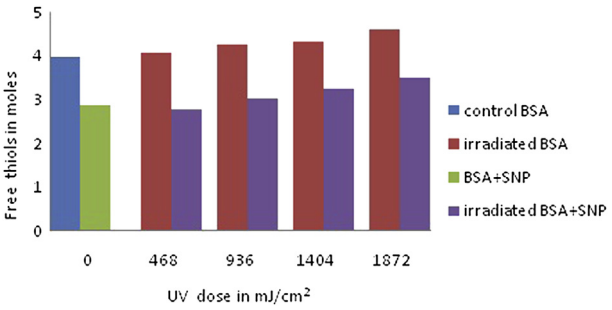

Fig. 6 – Effect of UV radiation on free thiols of BSA bound to SNP.

same density, the slight increase in formation of p-nitrophenol at higher UV radiation density in presence of SNP's but not more than naked BSA exposed to UV radiation. This could be due to tyrosine group of BSA would not be free for undergoing the nucleophilic attack, because of electrostatic interaction between BSA and SNP.

The free sulfhydryl content of native BSA was observed to be 4 mol. However this was increased with increasing UV radiation density with highest noticed at 1872 mJ/cm^2 (Fig. 6) It can be explained on the basis of reduction of large number of disulfide bonds in BSA molecules (Verhaar et al. 2009) and changes in intramolecular location of free sulfhydryl groups. The groups in hydrophobic pocket of molecules may get exposed to the surface due to changes in three dimensional structure of BSA molecule. A previous study with human serum albumin had shown that thermal treatment may change the configuration of those pockets that exposes the groups to solution (Wetzel et al. 1980). The free thiol contents decreased in presence of SNP as silver has a great tendency to bind with free -SH groups of BSA (Gordon et al. 2010). Resulting in decrease in number of free -SH groups. The free thiol contents were increased when BSA was irradiated in presence of SNP, it was UV radiation density dependent (Fig. 6). The possible explanation is that the most of cystine-34 on the surface of BSA binds with silver nanoparticles, hence the thiol content decreases.

3.3. *Copper ion assay*

The albumins have binding sites for many metal ions such as Cu^{++}, Ca^{++}, and Mg^{++} with highest affinity with copper. The four amino acids of N-terminus of human serum albumin, Asp- Ala- His- Lys, have affinity for binding with Cu^{++} ion (Laussac & Sarkar, 1984). SNP's facilitated the binding of the copper ion with serum albumin (Fig. 7). The SNP's might be altering the conformation of molecules in such a way that majority of N-termini (Asp- Ala- His- Lys sequences) will be getting exposed to solution. In presence of UV radiation, the copper binding was found to be slightly more at 1872 mJ/cm^2 radiation density than control. Copper binding is increased further when SNP were present during UV irradiation. The increase in copper binding site may be due to modification of conformation of BSA. The change in conformation by SNP is reported earlier (Banerjee & Das, 2013; Mariam et al. 2011).

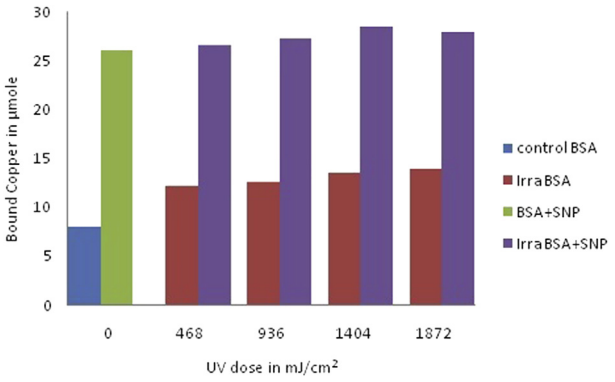

Fig. 7 – Effect of UV radiation on copper binding of BSA bound to SNP.

4. Conclusion

This study clearly indicates functional and structural modifications of BSA bound SNP in presence UV radiation. The modification of functional properties of BSA in presence of UV radiation, results changes of esterase activity, free thiol groups and copper binding ability suggesting the structural/chemical alterations. Significantly increased in esterase activity, free thiols in irradiated BSA and copper ion binding in non irradiated BSA bound SNP However, these activities did not change significantly when BSA bound SNP in presence and absence of UV radiation except of copper ion binding study. BSA bound to SNP restore antioxidant property in presence of UV radiation. This suggests that SNPs might be playing the role of some stabilization molecular structure of proteins, where a further modification by UV radiation was not possible. The fluorescence, CD and UV spectrometric study reveals the modification of structure in BSA bound to SNP's. The detailed mechanisms of interaction of silver nanoparticles in presence of UV radiation need to be studied further.

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Conflict of interest statement

I (We) certify that there is no conflict of interest regarding the material discussed in the manuscript.

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