Photophysical study of the interaction between ZnO nanoparticles and globular protein bovine serum albumin in solution and in a layer-by-layer self-assembled film

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A B S T R A C T

In this study, we investigated the spectroscopic properties of the water-soluble globular protein bovine serum albumin (BSA) while interacting with zinc oxide (ZnO) semiconductor nanoparticles (NPs) in aqueous medium and in a ZnO/BSA layer-by-layer (LbL) self-assembled film fabricated on poly (acrylic acid) (PAA)-coated quartz or a Si substrate via electrostatic interactions. BSA formed a ground state complex due to its interaction with ZnO NPs, which was confirmed by ultraviolet–visible absorption, and steady state and time-resolved fluorescence emission spectroscopic techniques. However, due to its interaction with ZnO, the photophysical properties of BSA depend significantly on the concentration of ZnO NPs in the mixed solution. The quenching of the fluorescence intensity of BSA in the presence of ZnO NPs was due to the interaction between ZnO and BSA, and the formation of their stable ground state complex, as well as energy transfer from the excited BSA to ZnO NPs in the complex nano-bioconjugated species. Multilayer growth of the ZnO/BSA LbL self-assembled film on the quartz substrate was confirmed by monitoring the characteristic absorption band of BSA (280 nm), where the nature of the film growth depends on the number of bilayers deposited on the quartz substrate. BSA formed a well-ordered molecular network-type morphology due to its adsorption onto the surface of the ZnO nanostructure in the backbone of the PAA-coated Si substrate in the LbL film according to atomic force microscopic study. The as-synthesized ZnO NPs were characterized by field emission scanning electron microscopy, X-ray powder diffraction, and dynamic light scattering techniques.

1. Introduction

Recently, nanotechnology research has attracted interest among scientists and in industry due to its possible applications in various fields [1–4]. Nanomaterials exhibit excellent novel properties because of their high specific surface area to volume ratio, distribution, and unique surface morphology compared with the bulk crystalline phases [5,6]. These unique characteristics of nanomaterials enable them for their significant role in the development of modern biosensors, biomedicine and bio-nano-technology [7–9]. In particular, nanoparticles (NPs) can be readily accumulated or adsorbed at biomolecular interfaces via various selective and specific interactions [10]. However, our understanding of the bio-compatibility and potential risks of NP exposure is still in the early stage. Organisms may be exposed to nanomaterials via various routes, e.g., accidentally, intentionally, or in consumer products such as cosmetics and foods etc. [11–13]. Thus, it is essential to understand the mechanisms that allow some nanomaterials to interact with biomolecular interfaces in order to elucidate their potential as bio-hazards. However, it is also important to study the beneficial effects of the interactions between NPs and biological materials in order to assess their possible applications in drug therapy, bio-sensing, bioelectronics, and other uses [14–16].

In this study, we investigated the interaction between the important globular protein bovine serum albumin (BSA) and the semiconductor nanomaterial zinc oxide (ZnO), as well as that in an alternate layer-by-layer (LbL) self-assembled film fabricated on a solid substrate via

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electrostatic interactions. Albumin is present in the blood plasma as the most abundant protein and it plays vital roles in the mammalian physiological system as well as in overall immunity. Albumin also participates in the transport of various drug molecules, where it may be required for their adsorption and efficacy [17]. The widely studied water-soluble protein BSA maintains the plasma pH, controls the colloidal osmotic pressure, and participates in other functions [18]. BSA is a protein of 583 amino acid residues (molecular weight = 66.5 kDa). The structure of BSA contains two tryptophan (Trp) residues, where Trp-212 is located in the hydrophobic pocket of the molecule and Trp-135 is bound to the surface which is exposed to the hydrophilic plasma environment [19]. Trp exhibits strong fluorescence compared with tyrosine (Tyr). BSA can interact with a variety of materials such as fatty acids, steroids, and inorganic ions, including Fe, Ni, and Zn etc. [20–22].

Since the advent of nanotechnology, the interactions and assembly of nanomaterials such as semiconductor NPs, e.g., ZnO, have attracted much interest [23]. ZnO NPs have unique optical properties, and thus they have possible application in drug delivery, bio-imaging, and consumer products [24,25]. Hahn et al. [26] demonstrated that various ZnO nano-platforms can serve as excellent signal enhancing media for a wide variety of proteins.

In biological environments, nanomaterials may interact with macromolecules such as proteins, nucleic acids (DNA and RNA), lipids and many biological metabolic residues due to the unique morphology and physicochemical properties of NPs. Therefore, controlling or manipulating their possible interactions with biomolecules is of both for fundamental and technological importance. Among the various biomaterials, proteins adsorb significantly onto the surfaces of NPs to form protein–NP complexes (commonly referred to as protein corona) [27]. These complex structures modify the aggregation properties of NPs as well as other interfacial properties, thereby giving the NPs biological characteristics. The interactions between proteins and NPs can be reversible or irreversible in some cases [28,29]. In addition, NPs may have some toxic effects when they interact with proteins with specific functionalities in cells [30].

The electrostatic LbL self-assembly method originally developed by Decher et al. [31] has attracted much attention in recent years because it allows the fabrication of nanodimensional organized molecular films on solid substrates [32]. A wide diversity of materials, such as biomolecules, polymer NPs, organic dye lasers and semiconductors with interesting physical and physicochemical properties have been successfully assembled using the LbL self-assembly technique [33–35]. In particular, proteins are promising candidates for encapsulation with nanostructured host geometries prepared using the LbL self-assembly method and they have been utilized in various applications, i.e., drug delivery, bio-sensing, direct electrochemistry and bioelectronic devices [36–38]. The electrostatic interactions between proteins and the host nano-matrix greatly influence the formation of ultrathin assemblies on solid substrates. LbL assemblies of encapsulated proteins can be employed for the sustained release of protein drugs due to the controlled degradation of the host polymer matrix while interacting with the cell membrane [39]. However, retaining the bio-functionality of adsorbed proteins is important for their actual and potential biomedical applications [40]. Various water-soluble proteins can be adsorbed onto the surfaces of NPs and they have interesting properties in aqueous media. However, assembling proteins onto the surfaces of NPs in LbL self-assembled films via the electrostatic adsorption technique can produce much more confined structures due to the restricted solid state geometry of LbL films.

In the present study, we investigated the complexation of ZnO NPs with BSA as well as their LbL self-assembled film on solid substrates by electronic spectroscopy, particularly ultraviolet-visible (UV–Vis) absorption and fluorescence emission spectroscopy. Fluorescence emission spectroscopic analysis of BSA in the presence of ZnO NPs was conducted to obtain details of the local microenvironment for the protein fluorophores Trp and Tyr, and to understand their structural stability while adsorbed onto the surfaces of NPs. Our results provide important fundamental insights into nano-bio-complex molecular systems, thereby making a significant contribution to the field of biomedical research. We used atomic force microscopy (AFM) to investigate the surface morphology of LbL films deposited onto a Si substrate. Field emission scanning electron microscopy (FESEM) and X-ray diffraction (XRD) were employed to characterize the as-synthesized ZnO NPs. Dynamic light scattering (DLS) analysis was also used to determine the size distribution of ZnO NPs and BSA in aqueous media.

2. Materials and characterization methods

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Anionic polyelectrolyte poly (acrylic acid) (PAA) (MW_{avg} = 1,30,000) and BSA (lyophilized powder, MW = 66.5 kDa) were purchased from Sigma Aldrich Chemical Company (USA) and used without further purification. Zinc nitrate [Zn(NO_{3})_{2}] was procured from Merck (Germany) and used as received. All of the sample solutions were prepared using triple distilled deionized Milli-Q water (resistivity = 18.2 MΩ cm; Millipore Inc., USA). BSA aqueous solutions with different molar concentrations were prepared from a stock solution and the pH values of these solutions were adjusted to 7.2 using phosphate buffer. Fluorescence grade quartz substrates and smooth Si substrates were used to deposit LbL self-assembled films. Before fabricating the films, the substrates were thoroughly cleaned in an oxidative bath for 30 min with piranha solution (i.e., 3:1 vol/vol mixture of 96% H_{2}SO_{4} and 30% H_{2}O_{2}) [41]. The reaction mechanism for piranha solution is as follows.

\[ \text{H}_{2}\text{SO}_{4} + \text{H}_{2}\text{O}_{2} = \text{H}_{2}\text{SO}_{5} + \text{H}_{2}\text{O} \]

H_{2}SO_{4} (peroxysulfuric acid) is a strongly oxidative product and thus piranha solution is a strong oxidizer that could hydroxylate the surface of glass or silicon by increasing the abundances of silanol groups and Si-O species. In addition, H_{2}SO_{4} boosts H_{2}O_{2} into a more aggressive form that could dissolve any elemental carbon present in the substrate. The substrates were rinsed with deionized water several times and then dried under an N_{2} flow for 10 min. The substrates were then preserved by vacuum incubation before being used for film fabrication. The cleaning and rinsing procedures were important for removing any organic impurities attached to the substrates in order to ensure their high hydrophilicity by generating -OH groups [41].

2.1.1. Synthesis of ZnO NPs

Zinc nitrate [Zn(NO_{3})_{2}] was used as the NP precursor. A typical soft chemical route was employed to prepare the nanocrystalline ZnO samples. At first, a sol of Zn(NO_{3})_{2} was prepared by adding small amounts of H_{2}O and alcohol. This solution was stirred for 1 h after adding a few drops of nitric acid so that the pH of the solutions remained in the acidic range. The sol was then subjected to gelation for 3 days at 60°C and then evaporated to dryness in order to obtain the sample in powdered form. The powder was crushed and heated at 400°C for 1 h to obtain ZnO NPs. The reaction scheme for the preparation of ZnO NPs is shown as a block diagram in Fig. 1. The ZnO NPs were then used to analyze their interaction with BSA.

2.1.2. Fabrication of LbL self-assembled films

To deposit LbL films onto the substrate, the thoroughly cleaned (as described above) quartz substrate was dipped into an aqueous solution of PAA (0.5 mg mL^{-1}) for 15 min and then dried in an N_{2} flow for 30 min. Then PAA-coated substrate was immersed in an aqueous colloidal suspension of ZnO (concentration of 10^{-5} M) for 15 min, followed by subsequent rinsing and same drying procedure as described above, thereby obtaining the PAA/ZnO LbL film. Finally, the PAA/ZnO
film was dipped into BSA solution for 15 min, before rinsing with deionized water and drying for 30 min. Thus, a bilayer ZnO/BSA LbL film was prepared on the quartz substrate. The same dipping cycle with rinsing and drying was repeated to fabricate the multilayered LbL self-assembled film on the solid substrate. A microcontroller-based commercial dip-coating instrument (model: Xdip-SV1; Apex Instrument Co, India) was employed for deposition onto the solid substrate.

2.2. Characterization methods

UV–Vis absorption and steady state fluorescence emission measurements of the samples were obtained using a dual beam UV–Vis absorption spectrophotometer (model: UV1800; Shimadzu, Japan) and fluorescence spectrophotometer (model: LS 55; Perkin Elmer, USA). The absorption spectra were recorded in the wavelength range of 200–800 nm. The excitation wavelength used to obtain fluorescence emission measurements from the samples was selected as 280 nm because the maximum excitation of BSA molecules occurs at this wavelength. FESEM images of the as-prepared ZnO were obtained using a field emission scanning electron microscope (model: Inspect F50; FEI, Czech Republic). XRD analysis of the ZnO NPs was performed using an X-ray powder diffractometer (model: D8 Advanced; Bruker, Germany).

Fig. 1. Reaction scheme employed for the preparation of ZnO NPs.

Fig. 2. (A) FESEM image of pure ZnO nanopowder. (b) XRD results obtained for the pure as-prepared ZnO NPs. (c) DLS results showing the size distribution of the as-prepared ZnO NPs in aqueous colloidal dispersion.
with CuKα radiation (wavelength = 1.54 Å) as the source, which was operated at an accelerating voltage of 40 kV and a current of 120 mA. Time-resolved fluorescence decay measurements were acquired using a time-correlated single photon counting (TCSPC) system (model: FluoroHub; Horiba Scientific, UK). The samples were excited at 280 nm using a picosecond pulsed diode laser in an IBH Fluorocube apparatus. The fluorescence decay data for the samples were collected over 200 channels, which were calibrated on a nonlinear time scale with increasing time according to an arithmetic progression with a Hamamatsu MCP photomultiplier tube (R3809). The raw decay data were analyzed using IBH DAS6 software. The particle size distributions were estimated in the ZnO NPs and BSA in aqueous solution by DLS analysis using a Zetasizer Nano ZS from Malvern Instruments (UK), where a 2 mW He-Ne laser at a wavelength of 633 nm was used to illuminate the sample. In the DLS analysis, back-scattered light at an angle of 173° was de-

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### 3. Results and discussion

#### 3.1. Characterisations of ZnO NPs

The as-prepared ZnO NPs were characterized using FESEM, XRD, and DLS techniques. Fig. 2a shows an FESEM image of the ZnO NPs obtained in this study, which indicates that the ZnO NPs had a flower-like distribution. The flower-like ZnO NPs had an average size of 49 μm and the morphology was possibly due to the aggregation of individual NPs in the medium. Thus, the exact size of the individual NPs could not be estimated because of the variable sizes of the aggregates throughout the sample. However, Fig. 2a shows that the ZnO NPs had hexagonal edges, which is consistent with the nanocrystalline phase of ZnO, as reported in a previous study [42].

We performed XRD analysis to obtain the details of the crystalline phase of the ZnO sample. Fig. 2b shows the XRD results for the pure ZnO powder sample, where all of the diffraction peaks originating from various lattice planes corresponded to the wurtzite hexagonal lattice structure of ZnO, and they were consistent with international standard data file for JCPDS Card No. 36–1451 [43,44]. The highly intense peak observed at 2θ = 36.2° corresponds to the (101) plane of the hexagonal crystal geometry of ZnO and the estimated d-spacing for the plane was 2.47 Å. No peaks were observed due to impurities, thereby suggesting that the synthesized ZnO NPs had high purity. The crystalline grain size of the ZnO NPs was also calculated using the Debye–Scherrer equation [45]:

\[ d = \frac{0.89\lambda}{\beta \cos \theta} \]  

where 0.89 is Scherrer’s constant, \( \theta \) is the Bragg diffraction angle, \( \lambda \) is the wavelength of the X-rays used, and \( \beta \) is the full width at half-maximum (FWHM) of the diffraction peak corresponding to any diffraction plane, as shown in Fig. S1 (Supplementary information). Thus, the average crystalline grain size of the ZnO NPs was estimated at 54.8 nm by considering the diffraction peaks originating from the (100), (101), and (002) planes of the crystalline lattice of the ZnO NPs. It should be noted that the grain size of the crystalline NPs as obtained by XRD was lower than that determined by FESEM, where the latter only indicated the size and morphology of the aggregated flower-like structures of the ZnO NPs [46].

DLS was performed to further characterize the ZnO NPs in colloidal dispersion. Fig. 2c shows the size distribution of the ZnO NPs determined by DLS, where the average hydrodynamic ZnO particle size was 38 nm. The results also demonstrated that the ZnO was dispersed well in the aqueous medium.

#### 3.2. UV–Vis absorption spectroscopy and steady state fluorescence emission spectroscopy

Fig. 3a shows the UV–Vis absorption spectra obtained for BSA in the absence and presence of ZnO NPs at different concentrations (10⁻⁶ to 10⁻² M) in aqueous solution. The molar concentrations of the ZnO NPs were calculated based on the UV–Vis absorption spectrum (results not shown) using the equation for the absorption coefficient and extinction coefficient of the ZnO colloidal suspension and according to the Beer–Lambert law.

The absorption coefficient “\( \alpha \)” was calculated using the relationship:

\[ \alpha = \frac{2.023 A}{t} \]  

where \( A \) is the optical absorbance at any particular wavelength (\( \lambda \)) and \( t \) is the thickness or path length of the sample medium.

The NP extinction coefficient (\( K \)) was calculated using the following relationship [47].
\[ \alpha = 4 \times 3.14 \times \frac{K}{\lambda} \] (3)

According to the Beer–Lambert law, the concentration \( c \) was calculated using the following equation:

\[ c = \frac{A}{(\text{extinction coefficient})} \times 1 \]

where \( A \) is the experimental optical absorbance at the peak wavelength of the absorption band (368 nm) for the ZnO nanocolloids and \( \lambda \) is the path length of light through the quartz cuvette.

Thus, the concentration of the stock dispersion of the ZnO NPs was obtained based on the UV–Vis absorption spectrum as described above and different concentrations were obtained by serial dilution with deionized water. The BSA sample was almost dissolved in water at a pH close to physiological pH and the size distribution in aqueous media was determined by DLS analysis (Fig. S2 in electronic supplementary information) in order to elucidate its solubility. Less than 5% of the protein was aggregated in solution according to the DLS results.

Fig. 3a shows that BSA produced a strong absorbance band at around 280 nm [48] due to the phenyl ring of Trp and Tyr amino acid residues and the intensity of this band was increased with increase in ZnO concentration in ZnO/BSA mixed solution because ZnO does not produce an absorbance band at 280 nm. This absorbance band was originated from the \( \pi-\pi^* \) transition between the electronic states. However, at higher ZnO concentrations, the absorbance band obtained for the mixed solution was broadened, possibly due to the change in the microenvironment for the Trp and Tyr amino acid residues present in BSA molecules [49]. At relatively lower ZnO concentrations, i.e. \( 10^{-6} \) to \( 10^{-5} \) M, some of the BSA molecules may have been adsorbed onto the surfaces of the colloidal ZnO NPs, thereby allowing the BSA to form a ground state surface complex with ZnO NPs, and thus the shape and peak position of the absorbance band in this concentration range were similar to those for the pure BSA. The formation of this complex was basically due to spontaneous molecular interactions on the surfaces. The isoelectric point of ZnO is 9–10 [50]. It is well known that ZnO has an overall positive surface charge in an aqueous medium with a pH close to physiological pH due to the transfer of a proton from the aqueous environment to the hydroxylated ZnO surface [50]. Therefore, the charge distribution on the surface of ZnO NPs in aqueous colloidal dispersion should be cationic in nature at pH close to physiological pH (7.4) [50]. On the other hand, the isoelectric point of BSA is 4.7 at 25 °C [51]. Therefore, near the physiological pH (7.2), the negatively charged BSA molecules can be electrostatically adsorbed onto the surfaces of the NPs. As the ZnO concentration increased, the number of complex molecular sites increased, as demonstrated by the increased absorbance of BSA at 280 nm (Fig. 3a). Thus, the ZnO/BSA nano-bioconjugated complex was formed in their ground electronic states [52]. The increased absorbance of BSA after the addition of the colloidal ZnO NPs is also consistent with the results obtained in a previous study [53]. At higher ZnO concentrations, the broadening of the absorbance band was probably due to the penetration of ZnO NPs in close proximity to the Tyr residue of BSA causing a conformational transformation by the protein, which was further confirmed by fluorescence spectroscopy.

We also prepared alternate LbL self-assembled films of BSA and ZnO on solid substrates by exploiting their electrostatic interactions. Initially, the cleaned quartz substrate was immersed in aqueous PAA solution for a specific time. The surface of quartz has Bronsted acid sites, which have a high capacity to donate protons. The aqueous PAA solution was electronegative and its carboxylic group conferred its functionality. Therefore, there was a high probability of hydrogen bonding interactions between the Bronsted acid adsorption sites on the quartz surface and the carboxylic group of PAA facilitating their adsorption. The proton donor is also more important for hydrogen bond formation [54]. The PAA chains reached an entropically favorable configuration on the quartz surface where the adsorption energy was minimized. As mentioned earlier that the ZnO NPs in aqueous colloidal dispersion (concentration of \( 10^{-5} \) M) had positive surface charge so they were adsorbed onto the surface of the PAA deposited on the quartz substrate via electrostatic interactions. The PAA coating was only added to support ZnO NPs on the substrate via electrostatic interactions. BSA is negatively charged at neutral or physiological pH, so it was also adsorbed electrostatically onto the positively charged ZnO surface on the LbL film. Fig. 3b shows the normalized UV–Vis absorption spectrum obtained for the one bilayer ZnO/BSA LbL self-assembled film deposited on the PAA-coated quartz substrate as well as those for the pure BSA solution and the BSA/ZnO mixed solution. The BSA in the LbL film produced the same absorbance band but it was broadened with a peak centered at around 280 nm compared with the absorption spectrum for the solution. This change in the absorbance profile of BSA in the LbL film may have been due to the different microenvironment for the light-absorbing chromophores in BSA while interacting with the ZnO NPs as well as the formation of the self-associated BSA molecular structure on the surfaces of the NPs in the LbL film because the protein had a different adsorption energy in the restricted geometry of the solid state LbL films. The self-association of the BSA structure in the LbL film was also investigated by AFM. The alternate LbL assembly of ZnO and BSA on the PAA-coated solid substrate is illustrated schematically in Fig. 4 in order to explain the proposed film formation mechanism.

3.2.1. Multilayer film growth

The multilayered ZnO/BSA LbL self-assembled film was fabricated on the PAA-coated quartz substrate by dipping it alternately in ZnO and BSA aqueous dispersions and solutions, respectively. After deposition and rinsing each layer in deionized water, and then drying, nucleation and film growth were monitored by observing the changes in absorbance by BSA at 280 nm using UV–Vis absorption spectroscopy. No significant losses of materials (results not shown) were observed due to rinsing with deionized water, thereby confirming the successful alternate deposition of BSA and ZnO on the complex LbL film. Fig. 5 shows the UV–Vis absorption spectra obtained for different number of layers (1–12 bilayers) of the alternate ZnO/BSA LbL self-assembled film deposited on the PAA-coated quartz substrate. The absorption spectra obtained for all of the bilayers on the LbL films were very similar but the absorbance increased with a broadened absorption peak at around 280 nm, which is the characteristic absorption peak of BSA. Therefore, ZnO and BSA were successfully and alternately adsorbed onto the LbL film via electrostatic interactions, and the BSA was probably immobilized on the surfaces of the NP in a LbL manner. The increased amount of protein in the multilayer build-up may enhance the catalytic activity and improve the performance in artificial bio-recognition.
The concentrations of the BSA and ZnO solutions and the dispersion were multilayer Fig. 5. However, after deposition of seven bilayers, the decrease in the slope might have formed a stable attachment with the quartz substrate. This type of quenching has also been observed for BSA or hemoglobin in the presence of various metal or metal-based nanomaterials such as silver, gold, or magnetic iron oxide [60–62]. As mentioned earlier, BSA contains three key amino acid residues, i.e., Trp, Tyr, and phenylalanine (Phe) [63]. The two Trp residues and one Tyr residue exhibit intrinsic fluorescence, where Trp-212 is located within a hydrophobic pocket in the protein and Trp-134 is located on the surface of the molecule, and thus it is hydrophilic [64]. When the concentration of ZnO NPs was relatively low (10−6 or 10−5 M) in the mixed solution, the hydrophilic Trp-134 amino acid residue formed a ground state surface complex by interacting with ZnO NPs, thereby decreasing the overall number of excited free unbound BSA molecular species in the mixed solution. As a consequence, there was a decrease in the steady state fluorescence intensity in the BSA/ZnO mixed solution. This type of quenching can be considered as static quenching due to the interaction between BSA and the ZnO NPs at very low concentrations, this complexation mostly occurred in the ground electronic states [65]. In addition, there was considerable spectral overlap between the absorption spectrum of ZnO and the emission spectrum of BSA, as discussed later in the Section 3.4. Thus, BSA probably transferred its excited state energy to ZnO NPs due to their interaction and they finally returned to their ground states. At higher ZnO concentrations, the Trp residue was buried within the protein structure and some ZnO NPs penetrated into the hydrophobic pocket of BSA to facilitate covalent binding between the aromatic Tyr amino acid residue and the ZnO NPs [49] because of the close association between the ZnO and BSA molecules.

BSA is a well characterized protein with a distinct hydrophobic region in its molecular architecture. Previous studies indicate that the structure of BSA comprises homologous domains with three subdomains (I, II, and III). It has been suggested that the hydrophobic pocket in subdomain II is dynamic in nature and it possesses two sites (I and II) with Trp, Tyr, and Phe amino acids in specific positions. BSA usually binds to different ionic and molecular species at site I (cavity size = 2.53 Å) or site II (cavity size = 2.6 Å) depending on the specific combination of hydrophobic, hydrogen bonding, and electrostatic interactions [66–68].

The types of binding interactions between BSA and ZnO NPs were determined according to the blue shift observed in the emission band of BSA. The binding interactions could also influence the local microenvironment for the Tyr residues and decrease their hydrophobicity, thereby reducing the excitation of BSA molecules at higher ZnO concentrations [69]. Thus, the reduced fluorescence emission intensity and the blue shift of the emission band (340 nm) for BSA at higher ZnO concentrations were probably due to conformational changes in the protein structure because of the increased polarity around the Trp residue [69]. The interaction between the Trp residue and ZnO NPs in these conditions may have occurred via the OH-group bound to the Trp residue. At lower ZnO concentrations, there was less possibility of processes [55]. To illustrate the adsorption behavior and nature of multilayer film growth, the absorbance of the 280 nm band in the LbL film is plotted as a function of the bilayer number in the inset of Fig. 5, which shows that the adsorption of BSA onto the LbL film was almost linear for the first few layers (1–7 bilayers) but the slope then decreased suddenly after seven bilayers. Thus, the multilayer build-up was approximately homogeneous, but after every layer of deposition with either ZnO or BSA, charge overcompensation occurred in the LbL self-assembly process. UV–Vis absorption spectroscopy analysis of sequential multilayer growth by the LbL film also suggested that the PAA chain might have formed a stable attachment with the quartz substrate. However, after deposition of seven bilayers, the decrease in the slope showed that there was an imbalance in the overall charges in the multilayer molecular assembly, which reduced further BSA adsorption on the LbL films at higher layer numbers [56]. Furthermore, with more layers, the imbalance in the overall surface charges on the ZnO assembled on the LbL film hindered the electrostatic interactions between ZnO and BSA compared with those in the first few multilayers [41], probably because the roughness of the film surface might have increased with the layer number. The imbalanced surface charge on the ZnO assembled on the LbL films at higher layer numbers was probably due to the aggregations of ZnO NPs and BSA in the LbL films. These aggregations were also investigated by AFM later.

Various analytical methods have been employed to understand the interactions and bio-conjugation of inorganic NPs with biological macromolecules, e.g., proteins, but the steady state fluorescence emission spectroscopic technique is one of the most convenient and reliable methods. Fig. 6a shows the steady state fluorescence emission spectra obtained for the pure BSA aqueous solution (concentration of 10−5 M) and the BSA/ZnO mixed solution with various concentrations of ZnO (10−6 to 10−2 M). The BSA aqueous solution produced a strong emission band with a peak centered at around 340 nm when excited with light at a wavelength of 280 nm. This was due mainly to the excitation of Trp amino acid residues, which are very sensitive to their local microenvironment. It has been reported that ZnO NPs luminescence due to oxygen vacancies on their surfaces [57,58] but we detected no fluorescence emission in the concentration range tested in this study during photoexcitation, possibly because the low concentration was beyond the detection limit of the instrument employed. Interestingly, the intensity of the fluorescence band (340 nm) produced by the BSA/ZnO mixed solution decreased gradually as the ZnO concentration increased, and it was accompanied by a slight blue shift only at higher ZnO concentrations of 10−3 to 10−2 M. Furthermore, it was previously reported [59] that the luminescence of ZnO may be quenched in the presence of biomolecules. A reduction in the fluorescence emission band intensity of BSA occurred in the presence of ZnO NPs due to the possible interaction between BSA and the ZnO NPs. This type of fluorescence quenching has also been observed for BSA or hemoglobin in the presence of various metal or metal-based nanomaterials such as silver, gold, or magnetic iron oxide [60–62]. As mentioned earlier, BSA contains three key amino acid residues, i.e., Trp, Tyr, and phenylalanine (Phe) [63]. The two Trp residues and one Tyr residue exhibit intrinsic fluorescence, where Trp-212 is located within a hydrophobic pocket in the protein and Trp-134 is located on the surface of the molecule, and thus it is hydrophilic [64]. When the concentration of ZnO NPs was relatively low (10−6 or 10−5 M) in the mixed solution, the hydrophilic Trp-134 amino acid residue formed a ground state surface complex by interacting with ZnO NPs, thereby decreasing the overall number of excited free unbound BSA molecular species in the mixed solution. As a consequence, there was a decrease in the steady state fluorescence intensity in the BSA/ZnO mixed solution. This type of quenching can be considered as static quenching due to the interaction between BSA and the ZnO NPs at very low concentrations, this complexation mostly occurred in the ground electronic states [65]. In addition, there was considerable spectral overlap between the absorption spectrum of ZnO and the emission spectrum of BSA, as discussed later in the Section 3.4. Thus, BSA probably transferred its excited state energy to ZnO NPs due to their interaction and they finally returned to their ground states. At higher ZnO concentrations, the Trp residue was buried within the protein structure and some ZnO NPs penetrated into the hydrophobic pocket of BSA to facilitate covalent binding between the aromatic Tyr amino acid residue and the ZnO NPs [49] because of the close association between the ZnO and BSA molecules.

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accessing the Trp residue by covalent bonding, so the protein structure may have been unaffected, as shown by the unaltered emission peak position for BSA at low ZnO concentrations in the mixed solution. In particular, the incorporation of ZnO NPs close to the hydrophobic zone of BSA probably induced a slight reduction in the size of the protein structure as well as the redistribution of the electronic states i.e. highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) due to the hydrophilic interaction between Trp residues in the presence of the ZnO NPs. The proposed interactions between BSA and ZnO at low and high concentrations are shown schematically in Fig. 6b.

Therefore, the ZnO NPs clearly influenced the gradual fluorescence quenching of BSA in an aqueous medium at a pH close to physiological pH. In general, the quenching of organic fluorophores can be described by the Stern–Volmer equation [70]:

\[
\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_q[Q]
\]

where \( F_0 \) and \( F \) represent the steady state fluorescence intensity in the absence and presence of quencher molecules (ZnO in our case),
respectively, $K_{SV}$ is the Stern–Volmer quenching constant, $k_q$ is the quenching rate constant of the biological macromolecule, $[Q]$ is the concentration of quencher molecules (ZnO), and $\tau_0$ is the average lifetime ($\sim 10^{-8}$ s) of BSA in the excited state \cite{71}. The inset in Fig. 6a shows the variations in $F_o/F$ as a function of the ZnO concentration in the mixed solution. At lower ZnO concentrations, i.e., $10^{-6}$ M and $10^{-5}$ M, the variation in $F_o/F$ was approximately linear, but it then started to deviate toward the X-axis and the slope decreased as the ZnO concentration increased. Considering only the linear portion of the curve and based on the slope, the quenching constant was calculated as $1.38 \times 10^4$ M$^{-1}$ at room temperature (25°C). Thus, we can conclude that at lower ZnO concentrations, the fluorescence quenching of BSA by ZnO NPs was static and it did not affect the intrinsic fluorescence of BSA. However, at higher concentrations of ZnO, the NPs could not access the Trp residues because they were buried within the protein molecules and the amino acid microenvironment was greatly affected. Therefore, the plot of $F_o/F$ versus $Q$ does not conform to the linear Stern–Volmer quenching mechanism, and the quenching of BSA at higher ZnO concentrations was accompanied by a blue shift of the emission band due to a conformational transformation by the protein.

In addition, at a sufficiently low quencher concentration, some energy transfer might have occurred between BSA (donor) and the ZnO NPs (acceptor) during the photo-excitation of the complex molecular species because there was considerable overlap between the ZnO absorption spectra and the BSA emission spectra, as mentioned earlier. However, it is likely that some free BSA molecules were still present in the mixed solution, which did not participate in complex formation at low ZnO concentrations. Therefore, the steady state fluorescence intensity as we obtained was still the intrinsic fluorescence emission from BSA in presence of ZnO. As the quencher concentration increased, the probability of energy transfer decreased due to the reduced excitation of BSA as a consequence of its conformational changes.

Fig. 7 shows the normalized steady state fluorescence emission spectra for the one bilayered ZnO/BSA LbL self-assembled film deposited on the PAA-coated quartz substrate as well as those for the BSA solution and ZnO/BSA mixed solution. The ZnO/BSA LbL self-assembled film exhibited an overall spectral shift of about 18 nm compared with the emission spectra for the pure BSA solution. The spectral shift of the BSA emission band in the LbL film was due to the complex formation with ZnO NPs as well as the aggregation of protein molecules on the ZnO matrix assembled in the LbL film. The BSA/ZnO mixed solution had a similar spectral profile, except for some quenching of the fluorescence intensity. The spectral shift of the emission band for BSA in the LbL film was possibly due to the self-association between the BSA molecular domains adsorbed onto the ZnO surfaces in the restricted solid state geometry of the LbL films, which is consistent with the broadened spectral profile obtained for BSA by UV–Vis absorption spectroscopy. This change in the association of BSA molecules in the LbL films compared with that in solution can be explained by the protein molecules adsorbing onto the LbL films with an entropically favorable different adsorption energy in the solid state. By contrast, they had more flexibility and structural freedom in aqueous solution.

### 3.3. Time resolved fluorescence emission spectroscopy

Time-resolved fluorescence emission spectroscopy is a powerful method for measuring the excited state lifetime of fluorophores, thereby helping to understand various molecular interactions when excited with light at a suitable wavelength. In this study, we observed quenching of the steady state fluorescence intensity for BSA after adding colloidal ZnO NPs, which indicated the formation of a ground state complex of the ZnO NPs and BSA in aqueous solution at very low concentrations. However, the steady state measurements alone cannot explain the nature of quenching as a function of the quencher concentration. Fig. 8 show the fluorescence decay curve for pure BSA and the BSA/ZnO mixed aqueous solution. The fluorescence lifetimes of the pure BSA and BSA/ZnO mixture were measured based on the fluorescence of the Trp amino acid residues in the protein structure when excited at 280 nm and the corresponding emission was monitored at a wavelength of 340 nm. The fluorescence decay of the pure BSA was fitted by two exponential functions with life times of $\tau_1 = 2.75$ ns and $\tau_2 = 6.23$ ns. The first component was responsible for around 27% of the decay in the intensity. The biexponential decay of BSA in these cases clearly indicates that two different species contributed to the overall fluorescence emission. The average lifetime was calculated using the following equation:

$$\tau = \frac{\sum_{i=1}^{n} \tau_i \cdot [A_i]}{\sum_{i=1}^{n} [A_i]}$$

where $\tau_i$ is the lifetime of species $i$, and $[A_i]$ is the concentration of species $i$.


\[ \tau_{av} = \frac{\sum a_i \tau_i^2}{\sum a_i} \]  

(5)

where \( a_i \) is the normalized pre-exponential factor for the component with lifetime \( \tau_i \). Using Eq. (5), the average lifetime of pure BSA was calculated as 5.26 ns. In the presence of ZnO NPs (concentration of \( 10^{-5} \) M and \( 10^{-4} \) M), BSA had a similar decay profile with almost no change in the average fluorescence lifetime compared with that determined for the pure BSA. This implies that the BSA fluorescence quenching as observed based on steady state measurements was static in nature due to the formation of the ZnO/BSA ground state complex in aqueous solution. Also, the intrinsic fluorescence lifetime of BSA remained almost unchanged because of some free excited BSA molecules, which produced normal fluorescence emissions. However, as mentioned earlier, during the photoexcitation of the ZnO/BSA complex, energy could be transferred from BSA to ZnO [64], but the presence of some free BSA molecules in the mixture means that the lifetime was not changed. Moreover, as the ZnO concentration increased, especially in the range of \( 10^{-4} \) to \( 10^{-3} \) M, the average lifetime of the excited state for BSA decreased to 3.12 ns. This decrease in the excited state lifetime for BSA in the presence of ZnO NPs was attributed to changes occurred in the local microenvironment of the Trp and Tyr residues in the BSA molecules [72]. In fact, at high quencher concentrations, it is possible that the ZnO NPs could penetrate into the BSA molecule and expose the Trp residue to the polar environment. The reduced fluorescence lifetime of BSA in the presence of ZnO NPs corresponded to conformational changes in the protein structure [73], as also shown for the milk protein \( \alpha\)-lactalbumin in the presence of ZnO NPs according to Chakraborti et al. [74]. In particular, at high ZnO concentrations, the Trp residue could enter the hydrophobic pocket of BSA [72] and then decrease the overall lifetime of BSA molecules in the mixed solution. Therefore, according to these results, it is clear that the average fluorescence lifetime and the components of the BSA fluorophore were sensitive to their microenvironment.

3.4. Energy transfer from excited BSA molecules to ZnO NPs

Our steady state and time-resolved fluorescence analyses indicated that the ZnO NPs could form a stable ground state complex with BSA in aqueous solution because the fluorescence lifetime of BSA was not altered in the presence of ZnO at low concentrations. The quenching of the steady state fluorescence intensity is shown in Fig. 6a. The BSA molecules that still remained free in the mixed solution returned to their ground electronic states after photoexcitation and subsequent vibrational relaxation, and thus there was no change in the fluorescence lifetime. However, during the photoexcitation of the ZnO/BSA complex, the excited BSA chromophores could transfer their excited energy to the ZnO NPs non-radiatively while returning to their ground states. This excited state energy transfer depended on the relative orientation, distance, local microenvironment, and long range dipole interactions. Recently, Bhunia et al. [75] reported that the rate of this energy transfer is very fast at ambient temperature. The energy migration from BSA to ZnO NPs occurred because the excited state energy of BSA (3.646 eV when calculated based on the emission peak) was considerably higher than the optical band gap energy (3.3 eV) of the ZnO NPs [76], and there was a considerable overlap between the ZnO (acceptor) absorption spectrum and the BSA (donor) fluorescence emission spectrum, as shown in Fig. 9. However, the luminescence of ZnO (although small) might have been quenched due to changes in the defect states associated with the surfaces of the ZnO NPs [59]. Therefore, the excited state energy of BSA was eventually transferred to ZnO via a non-radiative mechanism and the adsorbed energy from the excited ZnO NPs could have been lost as heat without any further emission of light while returning to the ground electronic state. Thus, we can conclude that the quenching of the fluorescence intensity was due to the interaction between the BSA and ZnO NPs, as well as the formation of their ground state complex at low ZnO concentrations in aqueous media. However, at higher ZnO concentrations, the ZnO NPs could access both the Trp and Tyr amino acid residues according to the observed blue shift of the emission band (Fig. 6a) for BSA, and thus no appreciable energy transfer occurred because of some conformational changes in BSA at higher ZnO concentrations. In addition, the fluorescence lifetime of the ZnO/BSA complex was reduced compared with that of the pure BSA solution because of changes in the Trp or Tyr microenvironment.

In general, the energy transfer efficiency between molecules can be written as:

\[ E = 1 - \frac{F}{F_0} \]  

(6)

where \( F \) and \( F_0 \) are the steady state fluorescence intensities of the donor (BSA) in the presence and absence of the acceptor (ZnO), respectively. Therefore, according to our analysis, we can conclude that the steady state fluorescence intensity decreased due to the interaction between BSA and ZnO NPs, and the formation of the ground state complex (ZnO/BSA), as well as the migration of energy from excited BSA to ZnO in aqueous media, but without any spectral shift of the band position for BSA at low ZnO concentrations.

3.5. AFM analysis of the ZnO/BSA LbL self-assembled film

We employed AFM in the tapping mode to analyze the surface topography and roughness of the as-prepared ZnO/BSA LbL self-assembled film deposited onto the PAA-coated Si-substrate. Fig. 10a and b shows AFM topographic images of the one bilayer PAA/ZnO and PAA/ ZnO/BSA LbL self-assembled film deposited on the Si substrate, respectively. The ZnO NPs were successfully deposited on the PAA layer on the substrate, where the root mean square (RMS) and average roughness were 3.076 nm and 2.391 nm, respectively, as summarized in Table 1. The ZnO/BSA LbL film and imperfections in the bilayer film, thereby leading to a much lower average height on the LbL film compared with...
the sizes of the ZnO NPs as determined by DLS. Similar observations were obtained in previous studies [77, 78]. Moreover, the ZnO NPs were not very tightly or closely packed with each other, thereby creating porous structures in the LbL film, which was confirmed by the height profile analysis. However, the dimensions of the porous structures were not uniform throughout the film. Eita et al. also found similar porosity in a PAA/ZnO LbL film deposited on a solid substrate [78]. They also showed that the PAA/ZnO LbL film exhibited superhydrophilicity due to its porosity and surface roughness. This type of film is highly optically transparent, even to the naked eye. The AFM image of the ZnO/BSA LbL film deposited on the PAA-coated Si substrate shown in Fig. 10b provided some interesting insights into the morphological parameters for the ZnO/BSA LbL self-assembled film.

**Table 1**

<table>
<thead>
<tr>
<th>Systems</th>
<th>RMS roughness</th>
<th>Average roughness</th>
<th>Average height</th>
<th>Surface skewness</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAA/ZnO</td>
<td>3.076 nm</td>
<td>2.391 nm</td>
<td>7.99 nm</td>
<td>3.450 nm</td>
</tr>
<tr>
<td>ZnO/BSA</td>
<td>6.433 nm</td>
<td>5.510 nm</td>
<td>12.742 nm</td>
<td>0.655 nm</td>
</tr>
</tbody>
</table>

4. Conclusion

In this study, we successfully determined the photophysical behavior of BSA when combined with semiconductor nanoparticle ZnO in aqueous solution and in a LbL self-assembled film. DLS showed that the average hydrodynamic size of the as-synthesized ZnO NPs was 38 nm. UV–Vis absorption spectroscopy confirmed the alternate multilayer growth of the ZnO/BSA LbL film deposited on a quartz substrate via the electrostatic interactions between them. The multilayer growth was almost linear up to seven bilayers, but the rate of deposition then decreased because the increased roughness of the films disrupted the electrostatic interactions. In aqueous solution, BSA formed a ground state complex with ZnO NPs. In addition, the excited BSA could transfer energy to the ZnO NPs during the photoexcitation of the conjugated or complex molecular species at lower ZnO concentrations. The quenching of the fluorescence intensity of BSA confirmed the possible interaction between BSA and ZnO, and this fluorescence quenching was static in nature because the emission lifetime of the free unbound BSA was not altered. However, at higher ZnO concentrations, the fluorescence quenching was also accompanied by a gradual blue shift of the emission band for BSA due to changes in the Trp amino acid residue micro-environment and some ZnO NPs could penetrate the hydrophobic pocket in BSA to affect the Tyr residue. The combined effects led to conformational changes in BSA in the presence of high ZnO concentrations in aqueous solution. FESEM confirmed the morphology of the as-synthesized ZnO with clear hexagonal edges and XRD analysis confirmed the nanocrystalline structure of ZnO with an average crystallite grain size of 54.8 nm. AFM demonstrate the formation of the ZnO/BSA LbL self-assembled film on the PAA-coated Si substrate. AFM images also confirmed that the PAA/ZnO LbL film had a porous structure. In the PAA/ZnO/BSA LbL film, BSA molecular domains formed a well-ordered network that connected the ZnO NPs and the surface roughness was increased compared with that of the PAA/ZnO LbL film.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpcs.2018.05.029.

**References**