Chapter V

FRET Biosensor: Interaction between QD with BSA

V. 1 Introduction

Nanotechnology is an extremely powerful emerging technology, which is expected to have a substantial impact on the field of pharmaceutical and medical diagnostics; there is an intensive interest in understanding the interaction between nanomaterials and biomolecules like proteins [1-7]. Nanomaterials can show a strong effect on the structural and functional properties of proteins and thereby have led to steadily increasing interest to understand the effects of various nanomaterials for interaction with biomolecules.

Bovine serum albumin (BSA) is the most studied protein having a high percentage of the total plasma proteins. Serum albumin is the most abundant proteins in blood plasma whose major physiological role is to carry various ligands to their respective target organs [8, 9]. These proteins manage transportation of drug and nutrition through human body [10]. They maintain the pH of blood and greatly contribute in maintaining colloidal osmotic pressure [11]. Therefore, binding of ligands to serum albumin is an important determinant of their distribution and fate in the body [12].
Bovine serum albumin (BSA) has been considered as a model protein due to its water-soluble nature which is important for interaction studies. Albumins also possess catalytic activity for broad range of organic molecules such as ester, amides, phosphate, etc.

BSA contains 582 amino-acid residues with a molecular weight of 66 KDa. It has two tryptophan moieties at positions 134 and 212 as well as tyrosine and phenylalanine and the intrinsic fluorescence of BSA occurs due to aromatic amino-acid residues [13, 14]. Nanoparticle probes act as biosensors in chemical and biochemical fields and their applications are becoming more extensive. Three types of nanoparticles in biochemical analysis are used: gold nanoparticles (NPs) [15], silica nanoparticles [17] and luminescent quantum dots (QDs) [17, 18]. Depending on the size and shape of NP’s/QDs, one can broadly assign three distinct kinds of molecular interactions between NPs/QDs surfaces and proteins namely electrostatic, hydrophobic and hydrogen bond interaction. These probes have been applied to ultrasensitive detection of proteins, DNA sequencing, clinical diagnostics, etc. Absorption and fluorescence spectroscopy are among the most powerful techniques to investigate the interaction between nanoparticles and biological molecules.

Semiconductor quantum dots have emerged as far better candidates for optoelectronic and biomedical applications than organic dyes [19-21]. QDs have unique spectral properties such as size-dependent tunable photoluminescence (PL) with broad excitation spectra and narrow emission bandwidths, which allow simultaneous excitation of particles of different sizes at a single wavelength. In addition, their high photobleaching threshold renders continuous or long term monitoring of slow biological processes possible. As a consequence of these advantages, QDs find enormous
applications in biological investigation. Among these water soluble CdSeS/ZnS alloyed quantum dots have high chemical stability, higher brightness and high PL quantum yield. Alloyed quantum dots have recently attracted considerable interests due to their promising optoelectronic properties and applications. Blends of conjugated polymers and colloidal semiconductor quantum dots have been advantageously used for light-emitting diodes [22, 23], ultrasensitive radiation detection [24], and solar energy conversion [25]. In addition, the alloyed QDs are promising candidates for luminescent bifunctional applications [26-28]. Therefore, it is of prime importance to understand the mechanisms of interaction of biosynthesized CdSeS/ZnS QDs with BSA.

This study has been actively pursued for decades because of its importance in the wide range of biomedical applications, such as artificial tissue and organ [29], drug delivery system [30], biosensor [31], solid-phase immunoassay [32], immunomagnetic cell separation [33] and immobilized enzyme or catalyst [34] and so on.

Fluorescence quenching measurement of albumin is an important method to investigate the interactions of drugs with serum albumins. It can reveal the accessibility of quenchers to albumin’s fluorophore groups, help to understand the binding mechanisms of albumins with drugs and provide clues to the essentials of binding phenomenon [35, 36]. In general, the mechanism of fluorescence quenching can be followed by either dynamic or static but in some cases both. The dynamic quenching mechanism results from diffusive encounters between fluorophore and quencher during the lifetime of the excited state. This type of fluorescence quenching is described by Stern–Volmer equation [36].
The present investigation emphasizes on understanding the biophysical mechanism of interaction between CdSeS/ZnS QDs and BSA. Here we investigate the interaction between BSA and CdSeS/ZnS QD in order to monitor the effect of CdSeS/ZnS QDs on the fluorescence of BSA employing various spectroscopic techniques. The fluorescence of BSA was found to be strongly quenched by alloyed CdSeS/ZnS QDs. The efficiency of energy transfer between QDs and BSA, binding interaction and thermodynamic parameters have been investigated by fluorescence methods and underlying mechanism of interaction are discussed in the light of these.

V. 2 Experimental

V. 2.1 Materials

The COOH functionalized CdSeS/ZnS alloyed core-shell QDs (6 nm dia), dispersed in water and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemicals Co., USA.

V. 2.2 UV-vis and fluorescence quenching measurements

Absorption spectra of BSA and BSA in presence of varying concentration of QDs were recorded in a quartz cuvette (10x10 mm) using Uv-vis spectrophotometer (Hitachi U-2800) in the wavelength range of 250-570 nm. The sample solutions for the measurements were prepared in double distilled water.

The fluorescence spectra were recorded using spectrofluorometer (Fluoromax 4, Horiba Jabin Yvon). The excitation wavelength was set 290 nm and emission was recorded in the wavelength range of 310-570 nm, with excitation and emission slit widths fixed at 2.0 nm. The spectra were recorded at three different temperatures of 298, 310 and 320 K for different concentration of QDs.
V. 2.3  **Resonance Light Scattering (RLS)**

Resonance light scattering, RLS, an elastic scattering, occurs when an incident beam is close to an absorption band. RLS is a sensitive and selective technique for monitoring molecular assemblies. RLS has attracted great interest among researchers and was used to investigate proteins, drugs and metal ions [37]. For RLS spectrum the excitation and emission monochromators were scanned simultaneously (Synchronous mode) in the wave length range 200-600 nm with \( \Delta \lambda = 0 \).

V. 2.4  **Synchronous fluorescence scan**

In case of synchronous fluorescence measurement, the initial excitation wavelength was set at 200 nm and scanned up to 500 nm, with the difference between excitation and emission wavelength \( \Delta \lambda \) set at 15 nm (for tyrosine residues) and 60 nm (for tryptophan residues). The spectral data were collected using Fluorescence software and OriginPro 8.0 software was used for further data analysis.

V. 2.5  **Energy transfer between BSA and QDs**

The absorption spectrum of alloyed CdSeS/ZnS QDs and emission spectrum of BSA at \( \text{Ex} = 290 \) nm (specific for tryptophan excitation) were recorded in the range of 300–550 nm. The overlap of the absorption spectrum of QDs with the fluorescence emission spectrum of protein (BSA) was used to calculate the energy transfer employing the Förster’s theory [38].

V. 2.6  **Fluorescence lifetime measurements**

Fluorescence lifetime measurements were performed with time correlated single photon counting (TCSPC) using a Chronos-BH spectrometer system. Excitation light was
derived from pulsed laser diodes (375 nm) maintaining the excitation polarizer at 0° and the emission polarizer at the magic angle (54.7°). The standard light detector is the fast PMT (model- H5773) by Hamamatsu PMC-100. The instrument response function was measured using starch in water as a scatterer, and multi-exponential curve fitting was done with the Vinci Analysis software. The instrument’s response function of this instrument is about 40 ps.

V. 3 Results and discussion

V. 3.1 Absorption characteristic of BSA-QD

Uv-Vis spectroscopy was carried out of BSA in absence and presence of CdSeS/ZnS QDs of increasing concentration and the spectra are shown in Fig. 5.1. It may be seen from this figure as quencher concentration increases, the absorption intensity at the wavelength of 278 nm increases significantly along with a blue shift of 2 nm. This indicates that absorption spectra of BSA were changed due to formation of ground-state complex of BSA-QD. Similar type of interaction has been reported for the interaction of fluorescence quenching measurements.

V. 3.2 Fluorescence quenching measurements

There are number of techniques to study the interaction between QDs/NPs and BSA, but the most convenient method is to study the fluorescence quenching of BSA. Fluorescence spectroscopy is useful to obtain local information about the conformational and/or dynamic changes of protein. Typically, from the interpretation of fluorescence parameters, one can obtain information such as the degree of exposure of the fluorophore to the solvent and the extent of its local mobility [39]. BSA constitutes three amino acids
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residues: tryptophan (Trp), tyrosine (Tyr) and Phenylalanine (Phe) which emit intense fluorescence when excited with photon of certain wavelength. For proteins with intrinsic fluorescence, more specific local information can be obtained by selectively exciting the tryptophan (Trp) residues. When these protein were excited at 290 nm, among these three residues light was absorbed by Trp group only. 3D contour spectrum of BSA is shown in Fig. 5.2A from which one can see the fluorescence emission of BSA at different excitation wavelength range from 270 -300 nm. It is noticed that BSA has maximum emission intensity at excitation wavelength 290 nm which is due to major contribution of Trp residues. The choice of 290 nm as the excitation wavelength was to avoid the contribution from tyrosine residues. Any change in the local environment of tryptophan leads to the variation in the fluorescence intensity of BSA. Thus, on observing the fluorescence emission of Trp in the bioconjugates, information about the protein conformational behavior around the Trp residues can be obtained. Hence, the most convenient method to study the interaction between QDs/NPs and BSA protein is to measure the fluorescence quenching of BSA.

The steady-state emission spectra of BSA with QD are shown in Fig 5.2 B. Here it can clearly be noticed that BSA has a strong emission band at 345 nm when excited at 290 nm. However, significant changes have been observed for Trp emission in the presence of QDs. Thus the figure clearly displays the effect of increasing concentration of QDs on fluorescence of BSA. With gradual addition of QDs the fluorescence of Trp gets quenched and maximum emission peak is also blue shifted by 4 nm for maximum concentration of QDs. Furthermore, the blue shift of the emission maximum observed in the bioconjugates indicated the occurrence of conformational changes for BSA at tertiary
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structure levels since the shift in the position of emission maximum reflected the changes of the polarity around the Trp residues. Commonly, the red shift indicates that Trp residues are, on an average, more exposed to the solvent, whereas the blue shift is a consequence of transferring Trp residues into a more hydrophobic environment [40]. Thus, the blue shift here indicated that Trp residues were in a more hydrophobic environment due to the tertiary structural change of the albumin when conjugated at the boundary surface of QDs.

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of different processes may be responsible for the quenching of fluorescence that include, excited state reactions, molecular rearrangements, energy transfer, ground state complex formation (static quenching) and collisional or dynamic quenching. Dynamic and static quenching can be distinguished by their differing dependence on temperature. Higher temperature results in faster diffusion and larger amounts of collisional quenching and will typically lead to the dissociation of weakly bound complexes and smaller amounts of static quenching. The mechanism of observed fluorescence quenching could be due to either static or dynamic or a combination of both these processes. To reveal the mechanism and the extent of quenching, we have used the Stern-Volmer (SV) equation which can be expressed as

$$\frac{F_0}{F} = 1 + K_{sv} [Q] = 1 + K_{sv} [Q] = \frac{\tau_0}{\tau}$$

(5.01)

where $F_0$ and $F$ are the steady-state fluorescence intensities of BSA in the absence and presence of quencher QD, respectively. $K_{sv}$, the Stern-Volmer quenching constant and $[Q]$ is the concentration of quencher. $K_q$ is bimolecular quenching constant and $\tau_0$ and $\tau$
are the fluorescence lifetimes in the absence and presence of quencher, respectively. The SV constant and quenching constant can be calculated from a plot of $F_0/F$ against $[Q]$ (Fig. 5.3). The figure shows the steady-state SV of BSA-QDs conjugate at 298K.

A plot of $F_0/F$ versus $[Q]$ yields a slope equal to SV quenching constant. A linear SV plot is generally indicative of a single class of fluorophores, all equally accessible to the quencher. In many cases, the fluorophore can be quenched by both collision and complex formation with the same quencher. When this is the case, the SV plot exhibits upward curvature, concave toward the y-axis at high $[Q]$ [41].

Hence, the present investigation indicates that either the static or dynamic type of quenching mechanism is responsible for the observed fluorescence quenching.

V. 3.3 Characteristics of Resonance Light Scattering

Resonance Light Scattering (RLS) studies are usually performed at wavelengths away from absorption band. RLS is shown to be sensitive and selective method for studying electronically coupled chromophore arrays. This technique helps in detecting the aggregates in the solution. The RLS spectra of BSA, QDs and their mixture are shown in Fig. 5.4. QD and BSA show very weak RLS signals in the entire wavelength range of 200-600 nm. When QD is added to BSA, the RLS intensity of (BSA+ QD) complex is enhanced. RLS spectra with the varying concentration of QD in the wavelength range 300-550 nm mainly resulted from the aggregation of QDs on the molecular surface of BSA. It is clearly seen from the figure that at lower concentration of QD to BSA the RLS signal increases to some extent at the same time higher concentration of QD to BSA RLS significantly increases. The characteristic dimension of
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the resultant complex could be larger than that of BSA and may be much lower than that of incident wavelength and thus enhanced light scattering occurred under the given condition [37]. Therefore, this could again further highlight the both static and dynamic quenching plays a key role BSA-QD interaction.

The extent to which a particle absorbs and scatters light depends on its size, shape and refractive index relative to surrounding medium and scattering due to each sphere is proportional to the square of the volume, so the amount of scattering is directly proportional to the volume of each sphere.

**V. 3.4 Effect of temperature and thermodynamic of binding**

Fig. 5.5A displays the SV plots for the fluorescence quenching of BSA by alloyed CdSeS/ZnS QDs at three different temperatures. Linear Stern-Volmer plots are in general indicative of dynamic quenching process. The values of $K_{SV}$ were found from slopes of this graph and the values of $K_q$ were calculated from equation

$$K_{SV} = K_q \tau_0$$  \hspace{1cm} (5.02)

By linear fitting of data, the slopes of plots, $K_{SV}$ and estimated bimolecular quenching constant $K_q$ at different temperatures ranging from 298 K to 320 K are listed in Table 5.1. The observed bimolecular quenching constants $K_q$ were much greater than the limiting diffusion rate constant of the biomolecule ($2.0 \times 10^{10}$ L mol$^{-1}$ S$^{-1}$), which indicated that the probable quenching mechanism of BSA–QD interactions was initiated by complex formation rather than by dynamic collision. While the results also showed that the values of SV quenching constants $K_{SV}$ and $K_q$ increased with increasing
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temperature, it indicated that the probable quenching mechanism of BSA–QD interactions was initiated by dynamic collision [42].

The binding constant \((K_b)\) and the number of binding sites \((n)\) between QDs and BSA were determined using following eqs.

\[
\log[(F_0 - F)/F] = \log K_b + n \log[Q] \tag{5.03}
\]

Fig. 5.5B shows the double-log plot of \(\log (F_0 - F)/F\) versus \(\log [Q]\) gives \(K_b\) from intercept and \(n\) (number of binding sites of QDs on BSA) from the slope of the curve at different temperatures. The values of \(K_b\) and \(n\) are presented in Table 5.1.

There are four non-covalent binding types between a small molecule and biomolecule which may involve various molecular interactions namely, electrostatic, hydrophobic, hydrogen bond or van-der Waal’s interactions. The magnitude and polarity of the thermodynamic parameter enthalpy change \(\Delta H^0\), entropy change \(\Delta S^0\) and Gibbs free energy \(\Delta G^0\) can be calculated from the van’t Hoff equation given by

\[
\ln K_b = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R} \tag{5.04}
\]

where \(K_b\) is binding constant determined by eq. (5.03) at corresponding temperature, and gas constant, \(R\) (8.3145 \(J\) mol\(^{-1}\) K\(^{-1}\)).

The changes in enthalpy \((\Delta H^0)\) and entropy \((\Delta S^0)\) of the association process between BSA and QDs have been calculated by plotting \(\ln K_b\) vs \(1/T\) . (Fig. 5.6) was fitted linearly to obtain the values of \(\Delta S^0\) and \(\Delta H^0\) from the slope and intercept, respectively. The Gibbs free energy \(\Delta G^0\) is estimated from the following equation.
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\[ \Delta G^0 = \Delta H^0 - T \Delta S \]  \hspace{1cm} (5.05)

The calculated thermodynamic parameters for interaction BSA with QDs are listed in Table 5.2. According to enthalpy and entropy changes, the model of interaction between QD and biomolecules can be summarized as follows: In general, if \( \Delta H^0 > 0 \) and \( \Delta S^0 > 0 \), association processes are triggered by hydrophobic interactions between proteins and ligands, if \( \Delta H^0 < 0 \) and \( \Delta S^0 < 0 \), processes are triggered by hydrogen and van-der Waal’s force, and \( \Delta H^0 < 0 \) and \( \Delta S^0 > 0 \) indicate that electrostatic interaction [43].

The eq. (5.04) yields the values of \( \Delta H^0 \) and \( \Delta S^0 \) to be 476 KJ/mol and 1.77 J/mol/K, respectively. The observed binding site (n) values lie near to unity suggesting that number of QD per BSA is found to be one. The calculated values of thermodynamic parameter \( G^0 \) show negative sign which indicates the spontaneity of the binding of QDs to BSA. Here, we propose that the positive values of \( \Delta H^0 \) and \( \Delta S^0 \) show that the binding process is an entropy-driven and endothermic process. Such endothermic and entropy-driven binding events are associated with the hydrophobic effect [43]. Interaction between BSA and NP are associated with a hydrophobic force of interaction are also been reported by Ravindran et al. [44]. Thus it can be concluded that hydrophobic forces may play a major role in the binding between BSA and QDs.

V. 3.5 Synchronous fluorescence scan

Synchronous fluorescence spectroscopy is a method that is widely used to explore the microenvironment of amino acid residues via measurement of the emission
wavelength shift [45]. The process is beneficial because of its characteristic sensitivity, spectral simplification, spectral bandwidth reduction, and avoidance of perturbing effects [46].

To explore the structural changes of BSA by the addition of aqueous alloyed CdSeS/ZnS quantum dots, we recorded the synchronous fluorescence spectra (SFS) of BSA with the concentration of CdSeS/ZnS QDs used for fluorescence quenching study. SFS provides the information on the microenvironment in the vicinity of the fluorophore functional group. The fluorescence of BSA is due to presence of tryptophan, tyrosine and phenylalanine residues. Hence spectroscopic methods are usually applied to study the conformation of serum protein.

In SFS the choice of appropriate scanning interval (Δλ) is mainly dictated by the different spectra requirements of resolution and sensitivity. For selecting the appropriate Δλ, the spectra have been recorded for BSA at Δλ = 15 nm, 25 nm, 45 nm, 60 nm, 85 nm. The best λ_{SFS}^{max} have been obtained for BSA at Δλ = 60 nm shown in Fig. 5.7.

As is well known, the synchronous fluorescence spectrum of BSA provides the characteristic information for the Tyr and Trp residues [43]. According to Miller et al. [46] when Δλ =15 nm, synchronous fluorescence spectra indicates the changes in the microenvironment of tyrosine residues and when Δλ = 60 nm, it provides information on the microenvironment of tryptophan residues. It could be seen form Fig. 5.8 (A and B) that when Δλ = 60 nm there is a slight blue shift of approximately 2 nm of the emission wavelength of tryptophan residues and when Δλ = 15 nm tyrosine fluorescence emission slightly decreases but no significant change in wavelength has been observed. This
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indicates that microenvironment of tyrosine remains unaffected in presence of QDs. The blue shift in the emission maxima of tryptophan is likely due to the fact that the hydrophobic amino acid structure surrounding tryptophan residues in BSA tends to collapse slightly and thus tryptophan residues are exposed more to the aqueous phase. Similar observation has been reported [45]. In a word, the synchronous fluorescence spectra indicated that the conformation of BSA has been changed.

V. 3.6 Energy Transfer between BSA and CdSeS/ZnS QDs

Fluorescence resonance energy transfer (FRET), is a process whereby the electronic excitation energy of a donor chromophore is nonradiatively transferred to a nearby acceptor molecule via dipole-dipole interaction between a donor-acceptor pair [38, 41]. It occurs when there is appreciable overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, with the centre to centre distance typically being 1-10 nm [41, 48, 49]. The efficiency of this energy transfer can be used to estimate the distance (r) between QDs and fluorophore in bio molecule.

This energy transfer depends on the extent of overlap of emission spectrum of the donor with the absorption spectrum of the acceptor. Fig. 5.9A clearly shows that the absorption spectrum of CdSeS/ZnS overlaps with emission spectrum of BSA, indicating possibility of energy transfer from excited state of BSA fluorophore to ground state CdSeS/ZnS QDs.

According to Förster theory the energy transfer efficiency can be calculated using the equation
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\[ E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \]  

(5.06)

where \( F \) and \( F_0 \) are the fluorescence intensities of BSA (as donor) in presence and absence of CdSeS/ZnS QDs (as acceptor), \( r \) is the distance between donor and acceptor and \( R_0 \) is the Förster distance when the transfer efficiency is 50\%. The value of \( R_0 \) is given by,

\[ R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \Phi_D J(\lambda) \]  

(5.07)

where \( \Phi_D \) is the quantum yield of the donor in the absence of acceptor, \( n \) is the refractive index of the medium and the term \( k^2 \) is a parameter given by the mutual orientation of the two dipoles of the donor and acceptor usually assumed to be equal to 2/3 [50].

The overlap integral \( J(\lambda) \) is expressed as

\[ J(\lambda) = \int_0^{\infty} F_D(\lambda) \varepsilon_\lambda(\lambda) \lambda^4 d\lambda \]  

(5.08)

where \( F_D(\lambda) \) is the normalized emission spectrum of the donor and \( \varepsilon_\lambda \) is the absorption coefficient of the acceptor at wavelength \( \lambda \) (in nm). \( F_D(\lambda) \) and \( \varepsilon_\lambda \) were calculated by analysis of integrating the spectrum in Fig. 5.9A using the OriginPro 8.0 software. The overlap integral, \( J(\lambda) \) was calculated to be \( 1.426 \times 10^{12} \text{M}^{-1}\text{cm}^{-1}\text{nm}^4 \). The Förster distance, \( R_0 \), corresponding to 50\% energy transfer from BSA to alloyed QD was calculated to be 5.78 nm from eq. (5.07) when \( K^2 = 2/3 \), \( N = 1.36 \), and \( \Phi_D = 0.118 \) [51]. Using eq. (5.06) we found \( r = 5.91 \) nm. The observed intermolecular distance is in the range of 2-8 nm, which suggests that non-radiative energy transfer occurs between BSA and alloyed QDs.
V. 3.7 **Fluorescence lifetime measurements**

Fig. 9B shows the time resolved fluorescence decay curves of BSA in the absence and presence of alloyed CdSeS/ZnS 490 QDs. BSA exhibits a bi-exponential lifetime component.

It was found that BSA having life time value 5.68 ns and upon addition of acceptor to the donor BSA a shorting of lifetime is observed 5.01 ns. This indicates that fraction of energy is transferring from BSA protein to CdSeS/ZnS QDs. This again confirms the steady state estimation. Hence, further confirms the dynamic quenching mechanism involves in BSA-QD interaction process.

V. 4 **Conclusion**

The binding interaction study of BSA with QDs is of great importance in understanding chemico-biological interactions for drug design, pharmacy, pharmacology and biochemistry. In this Chapter, we have studied the binding interactions of alloyed QDs with BSA using various fluorescence spectroscopic techniques. The experimental results indicate that CdSeS/ZnS alloyed QDs quench the fluorescence emission of BSA (Trp) through dynamic as well as static mechanism. The presence of a BSA-QD complex was confirmed by resonance light scattering and absorption spectra of BSA. The extent of fluorescence quenching of BSA increases with increasing temperature which further, highlights the dynamic quenching mechanism. The binding constant and binding sites for BSA-QD have been calculated. The thermodynamic parameters ($\Delta H^0, \Delta S^0$ and $\Delta G^0$), evaluated from Vant’t Hoff plots. The positive values of enthalpy and entropy change indicted that the interaction of BSA and QD was driven mainly by hydrophobic forces.
The process of binding is spontaneous as Gibb’s energy change was found to be negative. Synchronous fluorescence spectra indicate a small change in the microenvironment of tryptophan residues. This work can be one of the most significant advancements in protein NPs/QDs technology and their use in drug delivery application.
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