Elucidating the Binding Properties of β-Cyclodextrin with Human Serum Albumin by Multispectroscopic and Molecular Docking Technique

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***Abstract*— Biomolecular interaction of an inclusion compound β-cyclodextrin (β-CD) is studied with human serum albumin (HSA) using a set of multi spectroscopic tools viz., fluorescence, UV-visible spectroscopy, Circular dichroism spectroscopy (CD), synchronous fluorescence spectroscopy (SFS), three dimensional fluorescence (3D) and molecular docking method. Fluorescence data revealed the involvement of dynamic quenching mechanism in the HSA-β-CD binding. Stern–Volmer equation is used to calculate the binding constant and number of binding sites. The average binding distance (r) between HSA and β-CD is evaluated as per Forster's non-radiation energy transfer theory. Hydrophobic forces played a major role during the binding as per thermodynamic parameters data. Three dimensional (3D) and CD spectra showed the change in conformation and micro-environment of HSA upon binding with β-cyclodextrin**

***Index Terms*— Fluorescence quenching, β-cylodextrin, human serum albumin, subdomain IIB, molecular docking**

I. INTRODUCTION

Cyclodextrins belongs to giant family of cyclic oligosaccharides consisting of a macrocyclic ring of glucose sub units joined by α-1,4 gyosidic bonds. These are produced from starch by enzymatic conversion. They are used in food pharmaceutical drug delivery and chemical industries as well as agriculture and environment engineering.[1]

Human serum albumin is a major circulatory protein of well-known structure. The crystal structrure analyses have revealed that the drug binding sites are located in subdomains IIA and IIIA.[2] A large hydrophobic cavity is present in IIA subdomain. The geometry of the pocket in IIA is quite different from that found for IIIA. HSA has one tryptophan (Trp-214) residue in subdomain IIA**.**

Studies on drug binding to human serum albumin may provide information about structural features that determine the therapeutic effectiveness of drugs and thus become an important research field in the life science, chemistry and clinical medicine. Furthermore, the pharmaceutical companies need information about drug–protein interaction for the first step of new drug design. The degree of binding determines the availability of drug to the organism. Interaction of cyclodextrins with human and bovine serum albumins are studied using spectroscopic and computational studies.[3] In the present work, we have demonstrated the binding behavior of β-cyclodextrin with human serum albumin (HSA) by fluorescence spectroscopy, UV-vis absorption studies, three dimensional spectroscopy (3D), circular dichroism (CD) and synchronous fluorescence spectral (SFS) studies and molecular docking studies.

II. EXPERIMENTAL

2.1. Materials and sample preparation

Human serum albumin (HSA, lyophilized powder, 96%) is purchased from Sigma Aldrich Chemical Company, St. Louis USA. β-cyclodextrin hydrate is obtained from Alfa aesar Ward Hill, Massachuetts. Analytical grade chemicals and double-distilled water are used throughout the work. Solutions of β-cyclodextrin and human serum albumin (molecular weight of HSA is 66,000) are prepared in 0.1 M phosphate buffer at pH 7.4 containing 0.15 M sodium chloride.

2.2. Multi spectroscopic studies and measurements

Fluorescence spectra are recorded using a RF-5301 PC spectrofluorimeter Model FT-2000 (Hitachi, Japan) with a150-W xenon lamp, a 1-cm quartz cell and thermostatic cuvette holder. The temperature of the

sample is maintained by recycling water throughout the experiment. Steady state fluorescence observations are completed at the temperatures of 288, 298, 309 K, in the range 300-500nm upon excitation at 280nm keeping HSA concentration constant (5μM), and varying the β-CD solution concentration (5-45μM). Similarly, synchronous fluorescence assays are carried out with the same procedure only at 298 K, with the $\Delta\lambda$ ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$) parameter set to 15 and 60 nm which characterizes the changes in the microenvironment around tyrosine and tryptophan residues, respectively. Three dimensional (3D) fluorescence measurements are evaluated with the same procedure in the presence and absence of β-CD. The 3D spectra are recorded using defined ranges of excitation and emission of 200–350 nm and 200–600 nm respectively. The effect of some common ions (Zn^{+2}, Ca^{+2}, Ba^{+2}, Fe^{+2} and K^{+}) on the binding is studied with the same procedure in the range 200-550nm upon excitation at 280nm. To further elucidate the binding site of β-CD on HSA, monitoring and recording of the emission spectra are repeated in the presence of site probes (warfarin, ibuprofen and digitoxin for sites I, II and III respectively). In site probes study, the concentration of HSA and probe concentration is kept constant (5μM each) while concentration of β-CD is varied.

The absorption spectra are recorded on a double-beam CARY 50-BIO UV-visible spectrophotometer (Varian, Australia) equipped with a 150-W xenon lamp and slit width of 5nm. The UV measurements of HSA in the presence and absence of β-CD are made in the range 200-800nm. The concentration of HSA is fixed at 5μM while the β-CD concentration is varied (5-45μM). Jasco J-715 spectropolarimeter (Tokyo, Japan) is used for circular dichroism (CD) spectral analysis. The circular dichroism spectral studies for HSA (5μM) are recorded in the presence and absence of β-CD in the range of 200–260 nm. The HSA to β-CD concentration is varied in the ratio 1:0, 1:4 and 1:6 at room temperature.

Based on Förster's theory of energy transfer, the overlap of the UV–visible absorption spectrum of β-CD with the fluorescence emission spectrum of HSA is used to calculate the energy transfer between β-CD and HSA. The absorption spectrum of the drug in the range of 200–500 nm and emission spectrum of protein are recorded in the range of 200–550 nm.

The crystal structuresof the *β*-CD and HSA are obtained from the RCSB Protein Data Bank having PDB IDs: 2Y4S & 1AO6. Auto-Dock program (Version AutoDock 4.2.3) is used to identify the ligand binding sites to generate the 20 docked conformers of the protein ligand complexes by using lamarckiangenetic algorithm. The water molecules are removed; Kollmannunited partial atom charges and polar hydrogen atoms are added to the PDB structure of HSA before analysis. Grid size of 114 × 114 × 114 along X, Y and Z axis with 0.75 Å grid spacing is generated to carry out blind docking of *β*-CD with HSA.The docking parameters used are: maximum number of energy evaluations: 250,000; GA population size: 150; and the number of GA runs; 20. For visualization of the docked conformations, PyMOL v 1.5 is used.[4]

III. RESULTS AND DISCUSSION

3.1. Binding study between HSA and β-CD

The intrinsic fluorescence intensity of HSA is recorded (in the presence and absence of β-CD) inorder to findout the conformational changes of protein. A decrease in the fluorescence intensity of HSA (5μM) is observed with varying concentration of β-CD (5-45μM) (Figure. 1). Furthermore, the maximum wavelength of HSA shifted from 344nm to 340nm after adding β-CD, so a slight blue shift in the maximum emission wavelength implies that the fluorophore of protein is placed in a more hydrophobic environment. It is probably owing to the loss of the compact structure of hydrophobic sub domain IIA where tryptophan is placed.[5] Fluorescence quenching can be elucidated by two types of quenching mechanisms viz., dynamic and static quenching mechanisms which results from the collision between the fluorophore and quencher and non fluorescent complex formation in the ground state of the fluorophore respectively. Quenching mechanisms can be determined by analyzing the data using stern-volmer equation (1)

$$\frac{F_o}{F} = 1 + K_{sv}[Q] = k_q \tau_o [Q] \quad \text{——} \quad (1)$$

Where F_o and F are the fluorescence intensities in the absence and presence of the quencher, respectively. k_q is the quenching rate constant of the

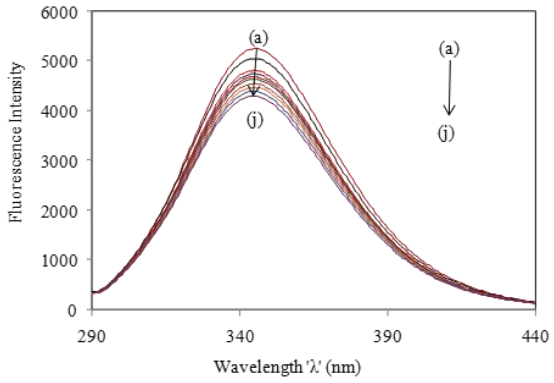

Figure 1. Fluorescence spectra of HSA (5µM) (a) in the presence and absence of β–CD (5-45µM) (b - j)

biomolecule, K_{sv} is the Stern-Volmer quenching constant and $k_q = K_{sv}\backslash\tau_o$. τ_o is the average life time of the biomolecule without quencher (10^{-8}/sec) and [Q] is the quencher concentration.[6] K_{sv} and k_q values are evaluated from slope of the plot F_o/F versus [Q] (Figure. 2) (Table 1). The k_q of various quenchers with the biopolymer[7] is reported to be 2 x 10^{10} $LM^{-1}S^{-1}$. The order of magnitude of k_q is calculated to be 10^{11} in the present study. So, the rate constant of the protein quenching procedure initiated by β–CD is greater than the value of k_q for the scatter mechanism. These results conclude the involvement of dynamic quenching mechanism in the interaction of β–CD with HSA. Further the decreased K_{sv} values with increasing temperature, supports the results.[8]

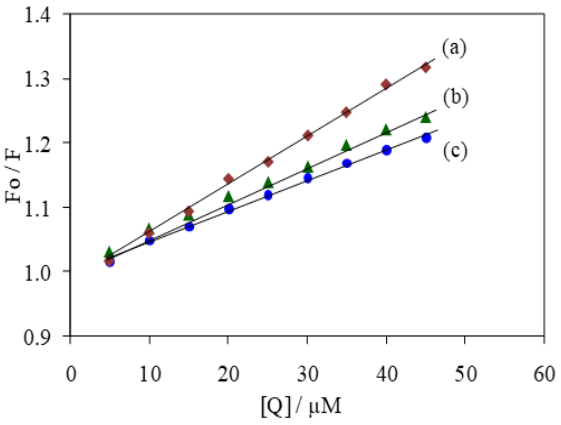

Figure 2. Stern-Volmer curves at 288K (c), 298K (b) and 308K (a); λ_{ex} = 280 nm; λ_{em}= 340 nm; [HSA] = 5µM, [β-CD] = 5 - 45µM

Temp in (K)	K_{sv} x 10^{-3} (L M^{-1})	k_q x 10^{-11} (L M^{-1} s^{-1})	,K_A x 10^{-2} (L M^{-1})	n
288	3.17	3.17	1.17	0.960
298	4.02	4.02	2.52	1.017
308	5.30	5.30	4.42	0.991

Table 1: Stern - Volmer quenching constant (K_{sv}), quenching rate constant (k_q) and binding constant (K_A) for β-CD-HSA system

3.2. Binding constant (K_A) and binding sites (n):

The obtained fluorescence data are used to evaluate the binding constant (K_A) and number of binding sites (n) using the equation (2);

$$\log \frac{(F_0-F)}{F} = \log K_A + n \log [Q] \quad \text{—— (2)}$$

The slope and intercept of the plot log(F_o - F/F) versus log[Q] (Figure. 3) gives 'n' and 'K_A' values respectively. The corresponding values of K_A and 'n' at different temperatures are given in Table 1. The values of 'n' are close to unity indicates the presence of single binding site on HSA for β–CD.

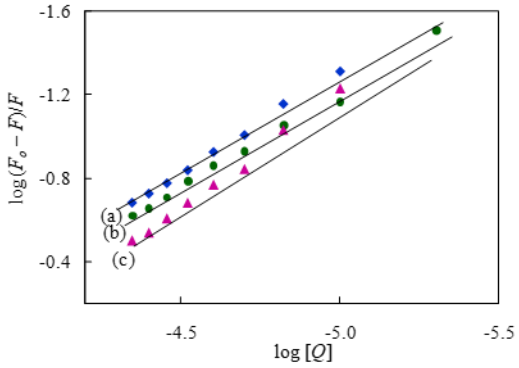

Figure 3. The plot at 288K (a), 298K (b), 308K (c); λ_{ex} = 280 nm ; λ_{em}= 340 nm ; [HSA] = 5µM, [β-CD] = 5 - 45µM

3.3. Thermodynamic analysis and binding modes

The binding forces that contribute to stabilize the binding properties mostly come from hydrogen bonds, Van der Waal's attractions, electrostatic interactions and hydrophobic interactions. The binding modes can be determined by the analysis of thermodynamic

parameters viz., change in Gibb's free energy (ΔG^o), change in enthalpy (ΔH^o) and change in entropy (ΔS^o). The following equations are employed to evaluate the thermodynamic parameters;

$$\log K_A = -\frac{\Delta H^o}{2.303\,RT} + \frac{\Delta S^o}{2.303R} \quad \text{—— (3)}$$

$$\Delta G^o = \Delta H^o - T\Delta S^o \quad \text{—— (4)}$$

Where, K_A and R are the binding constant and gas constant respectively. From log K_A versus 1/T plot ΔH^o and ΔS^o can be obtained. The ΔG^o can be calculated using the equation (4) and results are given in Table 2.

Thus the following conclusion can be drawn from the obtained data (Table 2). Both ΔH^o and ΔS^o are positive i.e., 76.52 kjmol^{-1} and 304.7 JK^{-1} mol^{-1} respectively, which indicates hydrophobic association between β–CD and HSA.[9] The major contribution to ΔG^o arises from the ΔH^o term rather than from ΔS^o. The values of ΔH^o and ΔS^o indicate that the binding process is mainly driven by the change in entropy (ΔS^o). Negative values of ΔG^o (–83.15 kJ mol^{-1}) revealed that the binding process is spontaneous.

Temperature (K)	ΔH^o (kJ M^{-1})	ΔS^o (JK^{-1} M^{-1})	ΔG^o (kJ M^{-1})
288			–80.10
298	76.52	304.7	–83.15
308			–84.20

Table 2: Thermodynamic parameters for β-CD-HSA

3.4. UV-visible absorption spectral study

UV-vis absorption studies are carried out in order to know the change in hydrophobicity[10] and complex formation between drug and protein. The UV absorption intensity of HSA increased with increasing β-CD concentration (5μM to 25μM) (Figure 4). The spectrum shows no shift which indicates there are no complex formation and it further supports dynamic quenching mechanism.

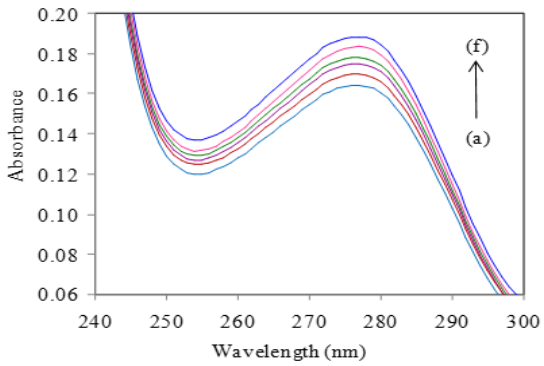

Figure 4. UV-vis absorption spectra of HSA- β–CD system

3.6. Effect of some metal ions on binding

Some metal ions present in the blood plasma can have ability to affect the binding of drugs with serum albumins. Especially the bivalent metal ions play an important structural role in biological systems and are involved in cellular and subcellular functions. The important micro and macro-elements are zinc, chromium and calcium ions.[11] Hence, the effect of Zn^{+2}, Ca^{+2}, Fe^{+2}, Ba^{2+} and K^+ on the binding are studied. Solutions of cat ions are prepared from the chlorides of their respective cat ions and no cat-ion gave a precipitate under the experimental conditions. The binding constants in the presence of above ions are evaluated and the corresponding results are shown in Table 3. A decrease in binding constant values in presence of Fe^{2+}, Ba^{2+}, Zn^{2+} and K^+ indicates shorter storage time of the drug in blood plasma and availability of more free drug which leads to the requirement of higher dose of the drug to achieve the desired therapeutic effect.[12] In case of Ca^{+2}, binding constant value is increased thereby indicating strong binding between the drug and protein and availability of more drug content for the action. This led to the need for less dose of drug for desired therapeutic effect.

System	K_A x10^{-2} (LM^{-1})
HSA + β-CD	2.52
HSA + β-CD + K^+	1.40
HSA + β-CD + Fe^{+2}	0.23
HSA + β-CD + Zn^{+2}	1.20
HSA + β-CD + Ca^{+2}	7.30
HSA + β-CD + Ba^{+2}	0.78

Table 3. Effect of some metal ions on HSA – β-CD

3.7. Energy transfer between HSA to β-CD

Using fluorescence resonance energy transfer (FRET) and Forster's theory the distance 'r' between the Trp-214 in native HSA (donor) and β-CD (acceptor) can be determined. According to Förster, the energy transfer is effective when (i) the donor produces fluorescence light (ii) the emission spectrum of the donor overlaps with the UV–vis absorbance spectrum of the acceptor (iii) the distance between the donor and the acceptor lies between 2-8nm. The energy transfer effect is related not only to the distance between the acceptor and the donor, but also on the critical energy transfer distance (R_o). The energy transfer efficiency (E) is calculated using the equation:

$$E = 1 - \frac{F}{F_o} = \frac{R^6_o}{R^6_o + r^6} \quad \text{——— (5)}$$

$$R^6_o = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \quad \text{——— (6)}$$

Where k^2 is spatial orientation factor of the dipole, N is refractive index of the medium, Φ is the fluorescence quantum yield of the donor and '*J*' is the overlap integral of the fluorescence emission spectrum of donor and absorption spectrum of the acceptor. Where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. In the present case,[13] $k^2 = 2/3$, $N = 1.336$ and $\Phi = 0.15$. From eqn (5)–(7) we are able to calculate the value of overlap integral (J) i.e., $J = 1.15 \times 10^{-14}$ cm^3 L mol^{-1}. The values of R_o, E and r are found to be 3.02 nm, 0.047 and 2.80 nm respectively. An essential criterion for energy transfer to take place is that the distance between donor and acceptor must be within 2–8 nm. This criterion is satisfied in the present study and hence quenching of Try fluorescence of HSA in the presence of the probe is attributed to energy transfer. The shorter distance between the bound β-CD and Try residues in the proposed study suggested the significant interaction between human serum albumin and β-cyclodextrin.

3.8. Conformational studies

3.8.1. Synchronous fluorescence spectral (SFS) studies

The changes in the microenvironment near fluorophores (Tyr/Trp) can also studied by the synchronous fluorescence measurement. The difference in the wavelength among the excitation and emission wavelengths ($\Delta\lambda = \lambda_{emission} - \lambda_{excitation}$) at 15 and 60 nm, respectively for the deviations of the microenvironment around tyrosine and tryptophan residues are studied.[14] When $\Delta\lambda = 15$ nm, the emission wavelength represents a negligible shift for tyrosine residue indicates that the HSA conformation is unchanged around tyrosine residue. Whereas a red shift is observed when $\Delta\lambda = 60$ nm, indicates the change in conformation of HSA upon adding β-CD near tryptophan residue (Figure 5).

3.8.2. 3D fluorescence spectroscopy

The outstanding advantage of three-dimensional fluorescence spectra is that information regarding the

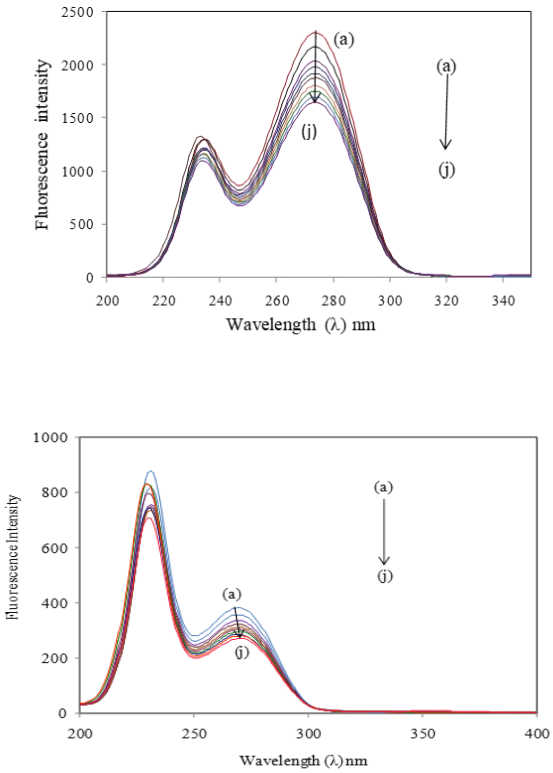

Figure 5. Synchronous fluorescence spectra of HSA–β-CD system (A) $\Delta\lambda = 15$ nm, (B) $\Delta\lambda = 60$ nm. (a) [HSA] = 5 μM; [β-CD] = 5 μM–45 μM, (b) –(j)

fluorescence characteristics can be entirely acquired by simultaneously changing the excitation and emission wavelengths. The maximum emission wavelength and the fluorescence intensity of the residues have a close relation to the polarity of their micro – environment.[15] The conformational and microenvironmental changes of HSA are investigated

by comparing their spectral changes in the presence and absence of β-CD (Figure 6A and 6B). Peak 'a' denotes the Rayleigh scattering peak ($\lambda_{ex}= \lambda_{em}$), where as strong peak 'b' mainly reveals the spectral characteristics of Trp and Try residues on proteins.

In the present study, the fluorescence intensity of peak 'a' increased with the addition of β-CD due to the formation of β-CD-HSA complex. So that diameter of the micro molecule increased and resulted in the enhancement of scattering effect.[16] The fluorescence intensity of peak 'b' which shows the spectral behavior of Trp and Tyr residues, decreased markedly with addition of β-CD. Analyzing the intensity changes of peak 'a' and peak 'b' revealed that the binding of β-CD to HSA induced some conformational and micro environmental changes in HSA.

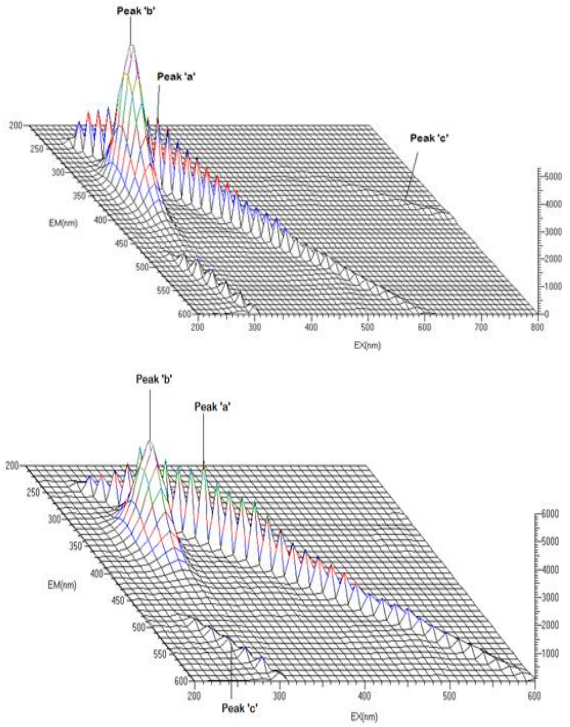

Figure 6. Three dimensional (3D) fluorescence spectra of HSA (A) and HSA+β-CD (B) system

3.8.3. CD spectroscopy studies

Circular dichroism is a convenient technique for detecting and monitoring the extent of conformational changes that is associated with the activity or regulation of a protein. The CD spectra of the HSA exhibited two negative bands in UV region at 208 and 222 nm. The addition of β-CD decreases the ellipticity at these wavelengths without any significant shift in the peaks (Figure 7). This indicates that β-CD changed the secondary structures of HSA upon interaction. The CD results are expressed in terms of mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$.

$$\text{MRE} = \frac{\text{Observed CD (mdeg)}}{C_p n l \times 10} \quad (7)$$

Where C_p is the molar concentration of the protein, n is the number of amino acid residues, and l is the path length.

$$\alpha\text{-helix (\%)} = \frac{[-\text{MRE}_{208} - 4000\,]}{[33000 - 4000]} \times 100 \quad (8)$$

Where MRE_{208} is the observed MRE value at 208 nm; 4,000 is the MRE of the α-form and random coil conformation cross at 208 nm, and 33,000 is the MRE value of a pure α-form at 208 nm. Using eqn (9) the percentage of α-helicity in the secondary structure of the HSA is calculated. The α - helical content of protein decreased from 56.08% in free HSA to 48.08% in bound HSA (at 1:4 ratio of HSA to β-CD). The CD spectra of HSA in the presence and absence of β-CD are observed to be similar in shape, indicating thereby that the structure of HSA is predominantly α-helical even after binding with drug.

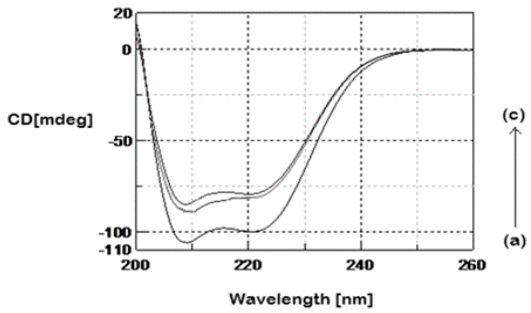

Figure 7. CD spectra of (a) HSA (10μM) in presence of (b) 40μM and (c) 60 μM of β-cyclodextrin

3.8.4. Molecular docking studies

Molecular docking gives the full picture of the binding site of ligands, its site and locations of the protein. This is a key as it explains the relationship between molecular properties of complexes. Autodock 4.2.3 software is used further to confirm the precise binding site and residues on HSA upon binding of *β*-CD. Since the binding location of the protein is of main importance to study the biological activity of the drug and it also plays a major role in pharmacokinetics and pharmacodynamics of the drug.

The stereo view of docked conformation of *β*-CD with the protein, HSA is presented in figure 8A. The cartoon model of figure 8B suggests that the *β*-CD bind to subdomain IIB of HSA by hydrogen bond formation.

This binding of CDs in the sub-domain IIB or IIIA affects the spatial conformation of the tryptophan unit located in the sub-domain IIA as domains II and III share a common interface.[3] The binding of *β*-CD with the HSA depends on the cavity sizes of the *β*-CD. Based on the docking results the hydrophobic interaction plays a major role in the HSA–*β*-CD complexation process.

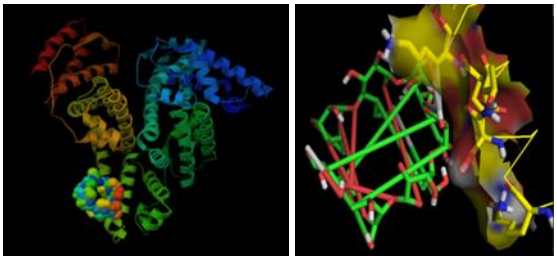

Figure 8. An overview picture of HSA and *β*-CD bound to the IIB sub domain on HSA (A). Cartoon model of HSA showing *β*-CD docked in the binding pocket (B)

IV. CONCLUSION

Interaction of β-cyclodextrin (*β*-CD) with human serum albumin (HSA) has been studied by multi spectroscopic techniques such as fluorescence, UV-visible spectroscopy, Circular dichroism spectroscopy (CD), synchronous fluorescence (SFS) and three dimensional fluorescence (3D) spectral studies. The intrinsic fluorescence of HSA is quenched by dynamic quenching mechanism. Thermodynamic parameters show that, mainly hydrophobic forces played major role in the binding of *β*-CD to HSA. Experimental and molecular docking results shows that *β*-CD bound to subdomains IIA of HSA. The distance r between donor and acceptor is obtained based on fluorescence resonance energy transfer. Three dimensional and CD spectra showed the change in conformation and micro-environment of HSA upon binding with β-cyclodextrin.

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